AN ABSTRACT OF THE THESIS OF

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Abstract approved: _____

Daniel J. Arp

Ammonia monooxygenase (AMO) is an enzyme involved in the nitrogen biogeochemical cycle. AMO oxidizes ammonia to hydroxylamine for the utilization of ammonia as an energy source by the bacterium, *Nitrosomonas europaea*.

Thioethers are shown to be a new class of substrates for AMO. Intact cells of *N. europaea* depleted dimethylsulfide concomitant with the formation of dimethylsulfoxide. The process required O_2 and was inhibited by two specific inhibitors of AMO. Five other thioethers were also substrates for AMO. Sulfoxides were the only products identified.

Allylsulfide caused an irreversible inactivation of ammonia-dependent O_2 uptake activity in *N. europaea* . The inactivation was specific to AMO and followed first-order kinetics. Anaerobic conditions or a reversible AMO inhibitor protected AMO from inactivation. Allylsulfide prevented ^{14}C-label from $^{14}C_2H_2$ from being incorporated into the 27-kD polypeptide of AMO. Allylsulfide is suggested to be a specific, mechanism-based inactivator of AMO.

The role of bovine serum albumin (BSA) in the assay of ammonia-oxidizing activity is to absorb free fatty acids because: 1) only proteins which bind fatty acids supported activity, 2) palmitoleic acid completely inhibited activity and only proteins which bind fatty acids reversed the inhibition and 3) the palmitoleic acid concentration increased during aging of cell extracts. Assay conditions for measuring ammonia-oxidizing activity in the absence of BSA are also described. Ammonia-oxidizing activity was stabilized by agents which modify lipase activity, including BSA, $CuCl_2$, $HgCl_2$, lecithin, or phenylmethylsulfonyl fluoride.

The mechanism for the inhibition of ammonia-dependent O_2 uptake by fatty acids was investigated. Fatty acids did not inhibit activity by acting as

protonphores. The electron transfer inhibitor, n-heptylhydroxyquinoline N-oxide (HQNO) completely inhibited activity which suggested the involvement of electron transport processes. The inhibition of activity by HQNO and fatty acids was relieved by either BSA or reductant sources for AMO.

Ammonia- and hydrazine-oxidizing activities were stabilized or lost concurrently in extracts suggesting that the loss of ammonia-oxidizing activity may result from the loss of coupling integrity among enzymes involved in the ammonia oxidation pathway. This result emphasizes the need for establishing an AMO assay which is independent of hydroxylamine oxidoreductase activity.

In vivo and *in vitro* Characterization of Ammonia Monooxygenase in *Nitrosomonas europaea*

by

Lisa Yvonne Juliette

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degree of

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APPROVED:

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Major Professor, representing Department of Biochemistry and Biophysics

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CONTRIBUTION OF AUTHORS

Chapter 2 of this dissertation was published in 1993 in Applied and Environmental Microbiology Vol. 59: 3718-3727 and is entitled "Inhibition of ammonia oxidation in *Nitrosomonas europaea* by sulfur compounds: Thioethers are oxidized to sulfoxides by ammonia monooxygenase". Chapter 3 was published in 1993 in Applied and Environmental Microbiology Vol. 59: 3728-3735 and is entitled "Mechanism-based inactivation of ammonia monooxygenase in *Nitrosomonas europaea* by allylsulfide". Chapter 4 is in the process of being published in 1995 in the Journal of Bacteriology Vol. 177: 4908-4913 and is entitled "Roles of bovine serum albumin and copper in the assay and stability of ammonia monooxygenase activity *in vitro*".

The experiments were performed in the laboratory of Dr. Daniel J. Arp who contributed to all aspects of the research and especially to the writing of chapters 2 through 4. Dr. Michael R. Hyman also contributed to the design of the figures, some of the experimental work and to the writing of chapters 2 through 4. Dr. Arp and Dr. Hyman are coauthors on the three published manuscripts presented in chapters 2 though 4.

LABORATORY FOR NITROGEN FIXATION RESEARCH
Department of Botany and Plant Pathology

OREGON STATE UNIVERSITY
Cordley Hall 4100 · Corvallis, Oregon 97331-2902
Lab 503-737-4214 Office 503-737-1294 Dept. Office 503-737-3451

July 25,1995

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Chapter 1

Introduction

1.1. Nitrification.

Microbial nitrification is a two step process carried out by two groups of bacteria placed together in the family *Nitrobacteraceae*. The first step of nitrification, the oxidation of ammonia to nitrite, is performed by the ammonia-oxidizing bacteria such as *Nitrosomonas europaea* . The second reaction, the oxidation of nitrite to nitrate, is performed by the nitrite-oxidizing bacteria, like *Nitrobacter sp.* These bacteria are major contributors, via their dissimilatory reactions, to the geochemical nitrogen cycle. The nitrifying bacteria are ubiquitous in nature and are the predominant source of high amounts of nitrite and nitrate generated from ammonia. Nitrification can be considered a beneficial process as well as a detrimental process.

As natural components of the soil microbial population, the combined oxidizing activities of the ammonia- and nitrite-oxidizing bacteria lead to the loss of expensive ammonia-based fertilizers used in agriculture. It is estimated that 25% of applied ammonia-based fertilizers are lost through routes initiated by ammonia-oxidizing bacteria (Postgate, 1982). The loss of fertilizer nitrogen though nitrification has provided impetus for the designated use of nitrification inhibitors, such as nitrapyrin, in agriculture (Keeney, 1986). Additionally, the oxidation of ammonia-based fertilizers results in substantial amounts of nitrates. The leaching of nitrates into ground and surface waters leads to both the contamination and eutrophication of water reserves and to the stimulation of microbial denitrification (Keeney, 1986). Denitrification of the nitrates leads to the production of N-oxides, of which nitrous oxide has received the most notoriety. Nitrous oxide is implicated in many current environmental problems including acid rain, greenhouse effect and ozone depletion. Thus both environmental and

economic considerations have maintained the long term interest in understanding and controlling nitrification.

On the other hand, nitrifying bacteria also play a beneficial role in the removal of ammonia from sewage. During the treatment of wastewater, microbial mineralization or organic matter results in the production of high quantities of ammonia (80-120 mg/l) (Abeliovich, 1992). The ammonia must be removed from the treated water prior to its release into surface waters, due to the toxicity of ammonia to aquatic life. Therefore an aerobic stage is incorporated into the water treatment process in order to stimulate the nitrification of ammonia to nitrate. Although nitrate is considered less toxic than ammonia, a later anaerobic stage stimulates microbial denitrification for the reduction of nitrate to dinitrogen. Water treatment thus relies on the microbial processes of nitrification and denitrification to reduce the nitrogen content of the effluent wastewater with the excess nitrogen being either lost to volatilization of dinitrogen or released as nitrate.

In addition to the usefulness of ammonia-oxidizing bacteria in water treatment, these bacteria are also of potential use for the biological removal of recalcitrant pollutants, such as halogenated hydrocarbons, from contaminated soils and water. The enzyme which oxidizes ammonia in *N. europaea* has been shown to oxidatively dehalogenate various halogenated hydrocarbons (Rasche, Hicks et al., 1990; Rasche, Hyman et al., 1990; Vannelli, Logan et al., 1990). Dehalogenation results in compounds which are more susceptible to degradation by other soil microorganisms. The use of microorganisms to degrade environmental pollutants is referred to as bioremediation.

Because the ammonia-oxidizing bacteria initiate nitrification, there has been considerable research concerning their inhibition and biochemistry. Ammonia-oxidizing bacteria obtain their energy for growth solely from the oxidation of ammonia to nitrite. *N. europaea* is an autotrophic bacterium and therefore depends on CO_2 as its carbon source. It is estimated that CO_2 fixation consumes 80% of the energy budget of an autotroph (Wood, 1986).

As a result of their poor energy source, NH_3, and the necessity to fix all of their carbon from CO_2, their growth is slow. Generation times among the ammonia-oxidizing bacteria, in culture, range between 7-24 hours (Bock, Koops et al., 1991). Ammonia-oxidizing bacteria are Gram-negative, ellipsoidal or rod shaped bacteria and many strains, including *N. europaea*, are motile with polar flagella. All ammonia-oxidizing bacteria contain extensive systems of either

centrally or peripherally located internal membranes. These membranes are thought to be complex invaginations of the cytoplasmic membrane similar those of photosynthetic bacteria. *N. europaea* contain peripherally located internal membranes and it has been suggested that the function of the internal membranes may be to accommodate the high content of respiratory proteins required for energy production (Wood, 1986).

Among the ammonia-oxidizing bacteria, *N. europaea* is a rapid grower with a generation time in culture of 7 to 8 hours. For this reason, most biochemical studies of ammonia-oxidizing bacteria have been conducted with *N. europaea*. Based on these studies, an overview of the energy metabolism of *N. europaea* is presented in the following sections.

1.2. Enzymology of energy generation in *Nitrosomonas europaea*.

Although nitrogen makes up approximately 14% of the elemental composition of living organisms, the amount of ammonia incorporated into the biomass of ammonia-oxidizing bacteria is negligible when compared to the amount of ammonia oxidized to nitrite by these bacteria. The oxidation of high amounts of ammonia is required by these bacteria because ammonia provides the only source of energy for growth and because thermodynamically ammonia oxidation is a poor source of energy. The pathway for the dissimilatory oxidation of ammonia by the ammonia-oxidizing bacteria has been established.

The overall oxidation of ammonia to nitrite by *N. europaea* can be thought of as a two step process. The individual steps are shown in Scheme 1.1. In the first catalytic step, uncharged ammonia and O_2 are substrates and the resulting product is hydroxylamine. Subsequently, hydroxylamine is oxidized to nitrite with water as the source of the oxygen atom, via a second enzyme in the pathway. Evidence for the occurrence of each of these catalytic steps during the oxidation of ammonia is presented in the following sections.

$$NH_3 + O_2 + 2e^- + 2H^+ \longrightarrow NH_2OH + H_2O \quad (1)$$

$$NH_2OH + H_2O \longrightarrow NO_2^- + 5H^+ + 4e^- \quad (2)$$

Scheme 1.1. The two step oxidation of ammonia to nitrite by *Nitrosomonas europaea*.

1.2.1. Ammonia oxidation to hydroxylamine.

It was demonstrated, using both intact cells and cell-free preparations of *N. europaea*, that the apparent Km for ammonium ions fell sharply with increasing pH (Suzuki, Dular et al., 1974). When expressed in terms of free NH_3, the Km values were almost independent of pH suggesting that uncharged ammonia was the actual substrate for the ammonia-oxidizing enzyme. The apparent Km for ammonia was 20 μM for both cell-free preparations and intact cells.

Both the demonstration that exogenous hydroxylamine was oxidized by *N. europaea* and the subsequent isolation of a periplasmically located hydroxylamine-oxidizing enzyme suggested that hydroxylamine was the product of ammonia oxidation . Hydroxylamine could not initially be detected during turnover of ammonia by intact cells. However, with the addition of hydrazine, provided as an alternative substrate for the hydroxylamine-oxidizing enzyme, small amounts of hydroxylamine were detected during ammonia oxidation (Hofman and Lees, 1953). Subsequently, the oxidation of ammonia was shown to be inhibited by low concentrations of hydroxylamine (Hyman and Wood, 1984a). This result led to the suggestion that feedback inhibition may regulate the periplasmic concentration of hydroxylamine and that the inability to detect or trap hydroxylamine during ammonia oxidation probably resulted from its normally low *in vivo* concentration (Wood, 1986).

Initial evidence suggesting that ammonia oxidation involved O_2 was that ammonia was not oxidized under anaerobic conditions in the presence or absence of electron acceptors. Subsequently, the demonstration that ^{18}O-hydroxylamine accumulated when cell suspensions were incubated with ammonia, hydrazine and $^{18}O_2$ provided the first direct evidence that ammonia

oxidation to hydroxylamine proceeded by way of a direct insertion reaction involving O_2 (Hollocher, Tate et al., 1981). These results suggested that the ammonia-oxidizing enzyme was a monooxygenase. However, that ammonia oxidation to hydroxylamine concomitantly results in the formation of water, as expected for an oxidation catalyzed by a monooxygenase, has not been demonstrated.

Additional support that ammonia oxidation is catalyzed by a monooxygenase includes the requirement of reductant for ammonia oxidation. The oxidation of hydroxylamine is thought to provide reductant for ammonia oxidation *in vivo*. The coupling of the monooxygenase reaction to hydroxylamine oxidation *in vivo* became evident from several observations. In contrast to hydroxylamine oxidation, ammonia oxidation by starved cells exhibited a lag prior to the onset of ammonia oxidation. The lag was eliminated by a priming amount of hydroxylamine (10 μM) (Hooper, 1969). Hydroxylamine has also been shown to act as a priming agent for cell-free ammonia-oxidizing activity (Suzuki, Kwoks et al., 1976).

Additional evidence that reductant is required for ammonia oxidation resulted from studies with alternative substrates for the ammonia-oxidizing enzyme. Several nonpolar organic compounds were hydroxylated by *N. europaea*. These organic compounds inhibited ammonia oxidation to hydroxylamine but not the subsequent conversion of hydroxylamine to nitrite. The hydroxylation reactions had the same sensitivities to inhibitors as demonstrated for the oxidation of ammonia to hydroxylamine, which suggested that these compounds were substrates for the ammonia-oxidizing enzyme. The transformations of the organic compounds were often enhanced when either hydroxylamine or hydrazine was supplied as a substrate for the hydroxylamine-oxidizing enzyme. These results supported both the idea that the monooxygenase activity required a source of reductant and that the oxidation of either hydroxylamine or hydrazine could provide reductant for catalysis.

In summary, ammonia oxidation involves uncharged ammonia, molecular O_2 and a source of reductant, and yields hydroxylamine as the product. The enzyme responsible is referred to as ammonia monooxygenase (AMO). The supply of reductant for the monooxygenase reaction *in vivo* is regenerated via the oxidation of hydroxylamine.

Several lines of evidence have suggested that copper may be involved in ammonia oxidation (Bédard and Knowles, 1989). The first evidence to suggest

the involvement of cuprous copper was the observation that thioureas and diethyldithiocarbamate, both chelating agents with high affinity for cuprous copper, inhibited ammonia oxidation but did not affect hydroxylamine oxidation (Hooper and Terry, 1973). The idea that ammonia oxidation involved copper was perpetuated by two additional observations.

First, ammonia-oxidizing activity is inactivated by near ultraviolet light (Hooper and Terry, 1974). Photoinactivation was inhibited under either anaerobic conditions or conditions of rapid ammonia oxidation. It was proposed that absorption of light caused the dissociation of an active form of O_2 which inactivated the enzyme. It was observed using difference absorption spectrophotometry of cell suspensions of *N. europaea* that a loss of absorbancy at approximately a wavelength of 380 nm occurred when photoinactivated (Shears and Wood, 1985). It was noted that oxygenated tyrosinase, a copper-containing oxygenase with a similar inhibitor profile to AMO, also exhibited a similar loss of absorbancy when exposed to near ultraviolet light. Based on these observations the idea that AMO contained copper was strengthened. Additionally, it was proposed that AMO may have a catalytic mechanism similar to tyrosinase. However, this suggestion has yet to be supported.

The second observation which supported a role for copper in the activity of AMO involved the characterization of an enzyme of similar nature to AMO. This enzyme is referred to as particulate methane monooxygenase (pMMO) and exists in methane-oxidizing bacteria. AMO shares with pMMO a sensitivity to the same inhibitors, an ability to oxidize a variety of alternative substrates and a similar cellular location (Bédard and Knowles, 1989). Additionally, the similar natures of these enzymes is matched by the metabolic similarities that exist between the ammonia- and the methane-oxidizing bacteria. As for the role of copper, it has been reported that methane-oxidizing bacteria express pMMO only when grown in the presence of sufficient copper concentrations. This result suggested that copper may be involved in the activity and expression of pMMO. Because of both the involvement of copper in pMMO and the other similarities which exist between AMO and pMMO, it is thought that copper will also be found necessary for AMO activity. More recently, a comparison of the translated amino acid sequences of the two genes for AMO and pMMO was done (Semrau, Chistoserdov et al., 1995). The two polypeptides components of AMO had approximately 40% identity with the two polypeptides components of pMMO.

These results have further substantiated the similarities that exist between these two enzymes.

Further confirmation of early speculation that AMO may be a copper-containing enzyme came from more recent results obtained using cell extracts which demonstrated that Cu^{2+} ions were required for cell-free AMO activity (Ensign, Hyman et al., 1993). The role of Cu^{2+} ions was catalytic and it was suggested that copper reactivated a copper-depleted form of AMO which was generated during cell lysis.

The first source of information regarding a molecular component of AMO was obtained using acetylene (C_2H_2), an irreversible inactivator of AMO. The inactivation of ammonia-oxidizing activity by C_2H_2 followed first-order kinetics, was a saturable process and high concentrations of ammonia protected ammonia-oxidizing activity from inactivation by C_2H_2 (Hyman and Wood, 1985). Because of these characteristics and the fact that the oxidation of hydroxylamine was unaffected by C_2H_2 suggested that inactivation was specific for ammonia oxidation. Furthermore, the inactivation of ammonia-oxidizing activity by $^{14}C_2H_2$ coincided with the covalent attachment of ^{14}C-label almost exclusively into a single membrane-associated polypeptide of apparent molecular weight of 27-kD (Hyman and Arp, 1992). The incorporation of ^{14}C-label from $^{14}C_2H_2$ into the 27-kD polypeptide was also a saturable process. These results provided the first indication of a likely component of the active site of AMO.

The usefulness of $^{14}C_2H_2$ was employed for cloning of the gene coding for the 27-kD polypeptide (McTavish, Fuchs et al., 1993). ^{14}C-labeling of the 27-kD polypeptide with $^{14}C_2H_2$ was used as a tag to follow the isolation of the 27-kD polypeptide. Once the 27-kD polypeptide was isolated, the N-terminus was sequenced and the genomic DNA probed, the gene coding for this polypeptide was isolated. As a result of this study, it was found that *N. europaea* contained two copies of the gene coding for the 27-kD polypeptide. One copy of this gene, referred to as *amoA*, was cloned and sequenced.

A 40-kD polypeptide had copurified with the 27-kD polypeptide (McTavish, Fuchs et al., 1993). The two polypeptides were separated using SDS-PAGE and the N- terminus of the 40-kD polypeptide was also sequenced. Coincidentally, immediately downstream of the gene coding for *amoA*, in the same operon, was the gene which encoded for the copurified 40-kD polypeptide. One copy of gene coding for the 40-kD polypeptide, referred to as *amoB*, was cloned and sequenced (Bergmann and Hooper, 1994). Hydropathy plots of the translated

amino acid sequences, of *amoA* and *amoB*, were done. The amino acid sequences of both AMOA and AMOB were hydrophobic; AMOA contained potentially four membrane-spanning regions and AMOB contained potentially three membrane-spanning regions (McTavish, Fuchs et al., 1993).

AmoB and *amoB* were the only open reading frames in the putative *amo* operon (Bergmann and Hooper, 1994). Because significant sequence homology does not existed between the sequence of either *amoA* or *amoB* and available sequence data, it was suggested that AMO may be a novel type of monooxygenase (McTavish, Fuchs et al., 1993). Interestingly, despite the belief that AMO contains copper, neither the 27-kD nor the 40-kD polypeptides exhibit any local amino acid sequence homology to either of two previously sequenced copper-containing monooxygenases, tyrosinase or dopamine ß-hydroxylase or any other monooxygenase.

However, since the reported sequencing of the *amoA* and *amoB*, the sequences of two genes coding for two polypeptides thought to be components of pMMO from *Methylococcus capsulatus* Bath have been published (Semrau, Chistoserdov et al., 1995). The translated amino acid sequence of *amoA* reveals a 46% identity with one of these genes referred to as *pmoA*. The translated amino acid sequence of *amoB* showed a 43% identity with that of the open reading frame for *pmoB*. Evidence was also provided that, like *amoA* and *amoB* in *N. europaea*, *pmoA* and *pmoB* are present in duplicated gene copies in a variety of methane-oxidizing bacteria. Multiple copies of genes in procaryotes are rare. The role for duplicate copies of these genes in *N. europaea* is currently being investigated.

Additional information about other potential polypeptide components of AMO have been obtained using experiments which exploit *de novo* protein synthesis in the presence of $^{14}CO_2$. When the ammonia-oxidizing activity of cells of *N. europaea* is inactivated by exposure to either acetylene or light, they subsequently recover activity when placed in growth media. The recovery of ammonia-oxidizing activity occurs within a short time relative to cell growth and *de novo* protein synthesis is required. During recovery of activity, cells specifically resynthesize several polypeptides which includes the 27-kD polypeptide and seven other polypeptides (Hyman and Arp, 1992). However, whether any of the seven polypeptides are components associated with the ammonia-oxidizing system requires further investigation.

1.2.2. Hydroxylamine oxidation to nitrite.

Hydroxylamine is stoichiometrically oxidized to nitrite by intact cells of *N. europaea*. The source of the oxygen atom, incorporated into hydroxylamine, is water. The enzyme catalyzing this oxidation in *N. europaea* is referred to as hydroxylamine oxidoreductase (HAO). HAO is considered a periplasmic enzyme because activity is released into soluble fraction during spheroplast preparation. However, some HAO activity remains associated with membrane (Olsen and Hooper, 1983). Although the oxidation of hydroxylamine to nitrite is the energy generating step in the oxidation of ammonia to nitrite, cells can not grow using hydroxylamine as a source of energy (Bock, Koops et al., 1991).

In contrast to AMO, HAO is well characterized (Hooper, 1984). HAO is an oligomer of 2 or 3 α subunits of Mr of 63-kD (Arciero, Hooper et al., 1993). HAO has an exceptionally complex structure with each α subunit containing seven c-type hemes and a single covalently bound P-460 prosthetic group. Hydrazine, like hydroxylamine, is a substrate for HAO. Hydrazine reduces endogenous cytochromes and has a K_m similar to that of hydroxylamine (~1 μM). The P-460 prosthetic group has only been observed in the ammonia-oxidizing bacteria. Physical studies of the P-460 prosthetic group have indicated that it is an unusual form of heme. The P-460 chromophore is considered to be part of the active site of HAO because when it is destroyed using hydrogen peroxide, the c-hemes which are unaffected by hydrogen peroxide are no longer reducible by hydroxylamine (Hooper, 1984). The genome of *N. europaea* contains at least three copies of the genes coding for HAO (McTavish, LaQuier et al., 1993). The possible reasons for having multiple copies of these genes is currently being investigated.

1.2.3. Cytochromes associated with ammonia oxidation.

In addition to HAO, several heme proteins have been isolated from the periplasmic space of *N. europaea* (Wood, 1986). Two of these proteins were autooxidizable c-type cytochromes. They were designated cytochrome c-554 and cytochrome c-552 and are involved in the pathway of ammonia oxidation. The involvement of these cytochromes in the ammonia oxidation was initially

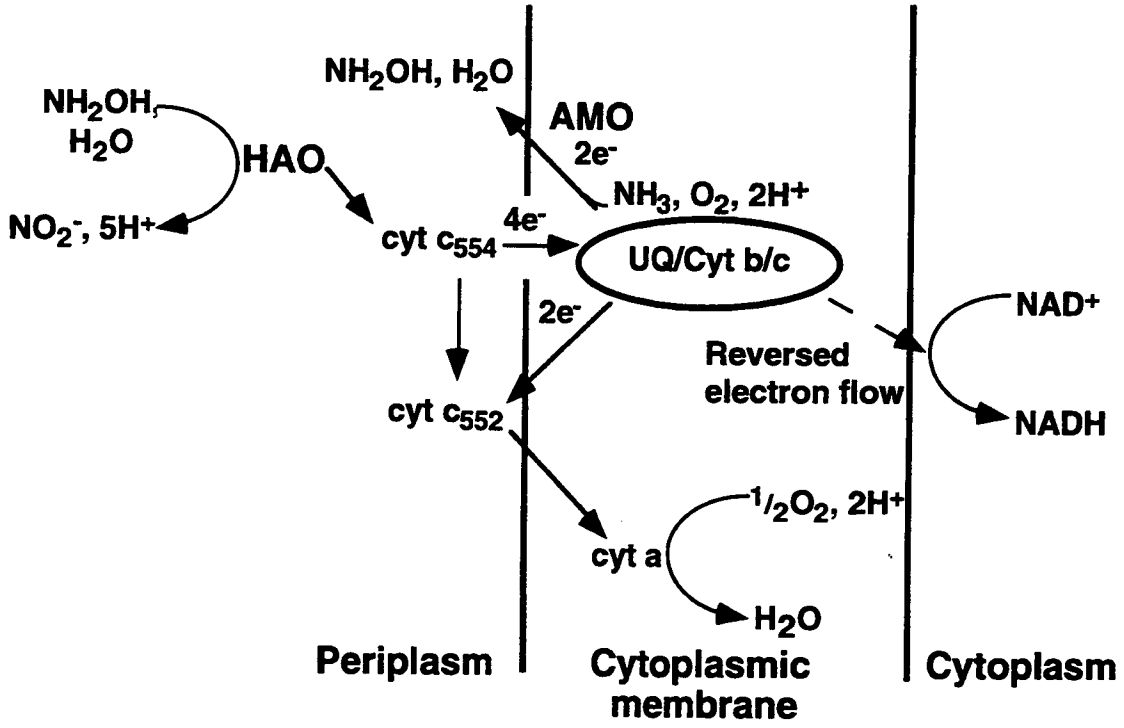
suggested by the demonstration that both cytochromes were reduced by hydroxylamine in the presence of purified HAO. Additionally, the genome of *N. europaea* contains at least three copies of the genes coding for cytochrome c-554 (McTavish, LaQuier et al., 1993). Further elaboration of the involvement of these cytochromes in electron transport and energy generation in *N. europaea* is presented in the following section.

1.3. Energy transduction in *N. europaea.*

Most chemolithotrophs, including *N. europaea*, are expected to contain all the components expected of a normal electron transport chain, namely NADH, flavoproteins, quinones, cytochromes of the b, c, c_1, and a, a_1, aa_3 or o types (Kelly, 1990). However, not all these components have been isolated and the pathway of electron flow in *N. europaea* is uncertain. In most cases, inorganic substrates, including ammonia, are thought to be oxidized to donate electrons initially at the level cytochrome c, rather than NADH, and oxidative phosphorylation is the major mechanism for ATP synthesis (Kelly, 1990).

Energy generation in *N. europaea* involves the oxidation of ammonia to nitrite coupled to the reduction of O_2 to H_2O by a type aa_3 terminal oxidase. The oxidation of hydroxylamine to nitrite is the energy producing step of the ammonia oxidation pathway. In support of this belief, ammonia oxidation to hydroxylamine requires energy and the oxidation of hydroxylamine to nitrite is closely linked to cytochrome reduction and subsequent energy generation in *N. europaea* (Bock, Koops et al., 1991).

A hypothetical scheme of electron transport in *N. europaea* is presented which is consistent with the currently existing information of the electron donor and acceptor status of various components involved in electron transfer in *N. europaea* (Scheme 1.2). Coupled proton translocations are not shown.



Scheme 1.2. Hypothetical scheme of electron transport in *N. europaea* as modified from (Wood, 1986).

The oxidation of ammonia to hydroxylamine by AMO requires a supply of reductant. When ammonia is added to resting cells, the initial source of reductant for AMO is attributed to endogenous substrates acting indirectly via NADH (Wood, 1986). AMO is located within the cytoplasmic membrane; however, it is unknown whether ammonia binding occurs on the cytoplasmic or periplasmic side of the membrane. Because of the known periplasmic location of hydroxylamine oxidoreductase, the impermeability of hydroxylamine in membranes and the mutagenic nature of hydroxylamine, it is thought that hydroxylamine is released by AMO into the periplasm.

Once hydroxylamine reaches a steady-state concentration in the periplasm, two electrons generated from hydroxylamine oxidation are used to support the continued oxidation of ammonia. The other two electrons enter the electron transfer chain and are used to support both the reduction of NAD^+ and oxidative phosphorylation. Three reactions are dependent on electrons supplied from the oxidation of hydroxylamine: the terminal oxidase, AMO and the generation of NADH (Wood, 1986). The flow of electrons from HAO to AMO and

the rest of the electron transport chain is thought to be tightly regulated *in vivo* (Wood, 1986).

Contributions to scheme 1.2 include both studies of proteins purified from *N. europaea* and existing knowledge of other bacterial electron transport chains. Purified HAO reduced cytochrome c-554; therefore, cytochrome c-554 is thought to be the immediate electron acceptor from HAO. Likewise, cytochrome c-552 is reduced by hydroxylamine but only in the presence of both cytochrome c-554 and HAO. Reduced cytochrome c-552 is oxidized by a purified cytochrome aa_3 type terminal oxidase (Yamazaki, Fukumori et al., 1985). However, between cytochrome c-554 and c-552, a hypothetical ubiquinone (UQ)-cytochrome bc complex is proposed to function as a branch point for electrons from hydroxylamine oxidation (Bock, Koops et al., 1991). Consistent with this suggestion, both ubiquinone (Q_8) and membrane-bound cytochromes (b-558, b-562, c-553) have been identified in *N. europaea*. However, the path of electron flow from HAO to AMO remains uncertain.

In general, uncouplers of oxidative phosphorylation stimulate respiration but inhibit the coupling of respiration to the formation of ATP. In *N. europaea*, uncouplers of oxidative phosphorylation, such as 2,4 dinitrophenol and carbonyl cyanide p-trifluoromethoxyphenylhydrazone, stimulate hydroxylamine oxidation but inhibit ammonia oxidation (Wood, 1988). The mechanism for the inhibition of ammonia oxidation is not known. However, the sensitivity of ammonia oxidation to uncouplers could be explained if ammonia oxidation was dependent on the membrane potential for its source of reductant. Alternatively, the stimulation of hydroxylamine oxidation by uncouplers suggests that the protonmotive force may stimulate electron flow to the terminal oxidase (Wood, 1988). Because AMO and the terminal oxidase are competing sinks for electrons, the balance between each must be regulated. The inhibition of AMO by uncouplers may thus be explained if uncouplers let electrons flow too freely to the terminal oxidase and thus starve AMO of reductant.

1.3.1. NADH generation.

The assimilation of CO_2 by *N. europaea* requires a continual supply of NADH. However, the reduction potential of either ammonia or hydroxylamine

oxidation to nitrite is higher than that of NAD^+/NADH redox couple. Therefore the reduction of NAD^+ is thermodynamically unfavorable without the addition of energy. This problem is a characteristic one for autolithotrophic bacteria, such as *N. europaea*, and it is largely responsible for the relatively low growth yields of these bacteria (Kelly, 1990). In order to overcome this problem, the reduction of NAD^+ is postulated to occur as a result of reversed electron transport (Wood, 1986).

Reversed electron transport involves the inward flow of protons down the electrochemical potential gradient so that electrons from intermediate carriers can flow to carriers of a more negative electrode potential. In support of the occurrence of reversed electron transport in *N. europaea*, cell-free preparations catalyzed ATP-dependent NAD^+ reduction using succinate, hydroxylamine or ferrocytochrome c (Aleem, 1966). The reduction of NAD^+ was stimulated by oligomycin and inhibited by uncouplers of oxidative phosphorylation. In most chemolithotrophic bacteria, the NADH dehydrogenase catalyzes an energy-dependent reversed electron transfer from quinol to NAD^+ at the expense of the proton motive force (Kelly, 1990). However, reversed proton flow has not been elucidated for *N. europaea*.

1.3.2. Energy conservation.

The generation of a proton gradient during either ammonia oxidation or hydroxylamine oxidation by *N. europaea* has been demonstrated. Endogenously respiring cells of *N. europaea* maintain a proton motive force (interior negative and alkaline) of approximately -160 mV. The electrical potential was the dominant component of the proton motive force (Frijlink, Abee et al., 1992). Proton translocation, extrapolated to the upper limit of H^+/O, was determined to be 3.4 and 4.4 for ammonia and hydroxylamine oxidation, respectively. However, interpretation of H^+/O values requires knowledge of the underlying electron transport chain in detail and uncertainties exist in the methods used to obtain H^+/O ratios for both ammonia and hydroxylamine oxidation and thus make these values uncertain (Bock, Koops et al., 1991).

In the absence of exogenous substrates, *N. europaea* has a low level of endogenous respiration. Although cells of *N. europaea* are thought to possess

a large capacity for endogenous metabolism, neither the chemical nature of the storage compounds which are oxidized nor has the metabolic pathway of endogenous metabolism have been determined. Inhibition of cytochrome aa_3 with cyanide did not influence the magnitude of proton gradient maintained by endogenous metabolism or the O_2 consumption rate, which indicated that two distinct branches exist for energy transduction. Because endogenous metabolism was insensitive to cyanide concentrations which inhibited cytochrome aa_3, it has been suggested that an alternative and cyanide resistant cytochrome o type oxidase present in *N. europaea* may be involved with endogenous metabolism (Frijlink, Abee et al., 1992b).

Storage of both polyphosphates and glycogen have been reported for the ammonia-oxidizing bacteria (Watson, 1971). Specifically, *N. europaea* has been reported to store polyphosphates (Terry and Hooper, 1970). Polyphosphate can serve as an internal reservoir of both phosphate and energy (Kornberg, 1995). However, whether polyphosphates serve as a reservoir for energy for *N. europaea* has not been determined.

1.4. Autolithotrophy.

Ammonia-oxidizing bacteria are obligate autolithotrophic bacteria which use only ammonia as an energy source and CO_2 as a carbon source. Autotrophy refers to the ability of an organism to grow using only CO_2 as a carbon source and lithotrophy refers to the use of only an inorganic energy source. The biochemical basis of obligate autotrophy has often been debated. It has been suggested that the lack of or low levels of certain enzymes involved in the citric acid cycle, such as α-oxoglutarate dehydrogenase or pyruvate carboxylase, limit the use of carbon sources other than CO_2. Additionally it has been suggested that the intracellular concentrations of carbon metabolites, such as pyruvate, is tightly regulated in autotrophic bacteria and therefore exogenous addition of these compounds cannot stimulate growth. However, neither theoretical considerations nor experimental results explain adequately why autotrophic bacteria, such as the ammonia-oxidizing bacteria, are solely dependent on CO_2 as a carbon source.

Fixation of CO_2 by ammonia-oxidizing bacteria is catalyzed by the enzyme, ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCo), via the reductive pentose phosphate cycle. In some species of ammonia-oxidizing bacteria, RuBisCo is found in polyhedral inclusion bodies called carboxysomes (Bock, Koops et al., 1991). The function of the carboxysomes is not clear, and not all species of ammonia-oxidizing bacteria possess them. The reduction of CO_2 is dependent on a supply of NADH. NADH is generated through the electron transport chain by reversed electron flow. The absolute dependence of *N. europaea* on CO_2 as a carbon source has been postulated to represent a control mechanism originally established to maintain the electron transport chain incapable of any other mode of operation (Wood, 1988). This hypothetical control may exist in order to prevent AMO from running out of reductant or poisoning of the cells by excess hydroxylamine (Wood, 1988).

Despite the opinion that ammonia-oxidizing bacteria are obligately autotrophic bacteria, many attempts have been made to grow or enhance their growth rate using organic carbon. For instance, some strains of ammonia-oxidizing bacteria have been shown to assimilate a limited amount of pyruvate and acetate (Bock, Koops et al., 1991). Additionally, both intact cells of *N. europaea* and proteoliposomes were shown to actively accumulate several amino acids using a high affinity transport system (Frijlink, Abee et al., 1992a). However, the stimulation of the growth rate of ammonia-oxidizing bacteria by organic carbon when demonstrated has been quite modest. Mixotrophic growth has been reported for a few strains of ammonia-oxidizing bacteria, but again the growth rate was slower than when grown autotrophically.

Recently, *N. europaea* was observed to be capable of assimilating $^{14}CO_2$ into cell material anaerobically in the presence of nitrite, pyruvate and ammonia (Abeliovich and Vonshak, 1992). Ionophores, such as nigericin and carbonylcyanide-p-trifluoromethoxypheylhydrazone (FCCP) arrested the assimilation of $^{14}CO_2$. The role of ammonia was not established, but pyruvate was suggested to serve as an electron donor and nitrite as an electron acceptor during anaerobic metabolism. However, metabolic pathways were not delineated and, although cell viability under anaerobic conditions may have been enhanced by pyruvate and nitrite, cell growth under these conditions was questionable.

1.5 Alternative substrates for AMO.

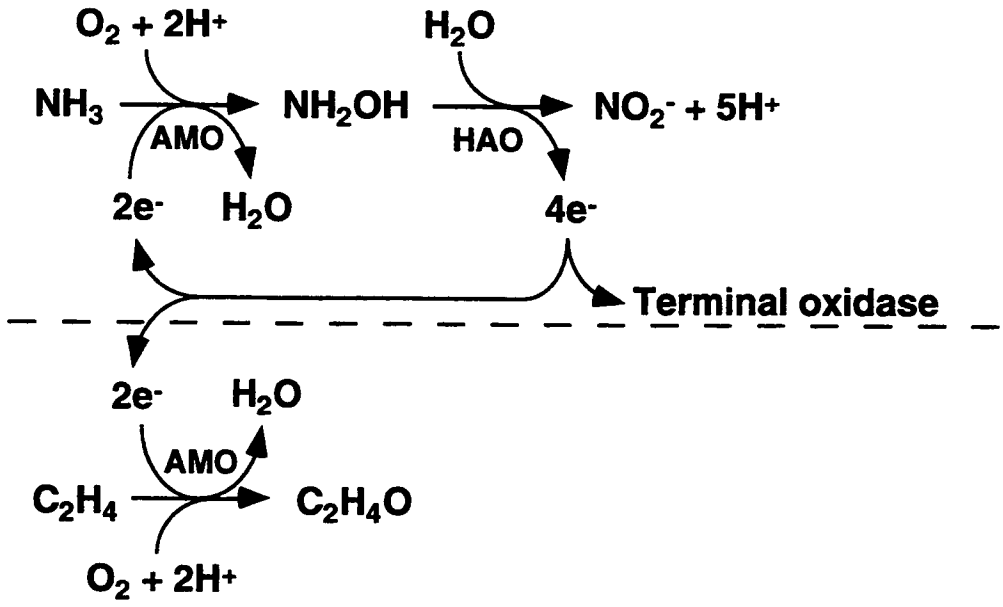
Non-polar organic hydrocarbons are inhibitors of ammonia oxidation by *N. europaea*. The mechanism for the inhibition was later established by the demonstrations that *N. europaea* catalyzed propene to epoxypropane, cyclohexane to cyclohexanol and benzene to phenol and that the transformations were prevented by inhibitors of AMO, such as allylthiourea, acetylene or ultraviolet light (Hyman and Wood, 1983; Hyman and Wood, 1984; Hyman and Wood, 1985). Additionally, the transformations were enhanced when carried out in the presence of ammonia, hydroxylamine or hydrazine. These results led to the conclusion that AMO catalyzed the oxidations. Since the initial recognition of the broad substrate specificity of AMO, the substrate range of AMO has been expanded to include many compounds, some of which are shown in Table 1.1. These substrates are referred to as alternative substrates.

Compound Type	Product Type
Alkane	Alcohol
Alkene	Epoxide
Arenes	Phenol
R-Cl	Aldehyde $+Cl^-$
Thioether	RS(O)R
Ether	Alcohol + Aldehyde

Table 1.1. Types of compounds oxidized as alternative substrates by AMO.

The oxidation of alternative substrates by AMO is referred to as cometabolism because it is fortuitous and the bacteria do not derive any apparent benefit. However, the oxidation of the alternative substrate requires a source of

reductant. The reductant source can be ammonia. However, as shown in Scheme 1.3, ammonia and the alternative substrate compete for reductant generated during the oxidation of hydroxylamine. Additionally, while the oxidation of ammonia allows regeneration of reductant the oxidation of the alternative substrate depletes reductant.



Scheme 1.3. Flow of reductant during turnover of ethylene by AMO in intact cells of *N. europaea*.

Recognition that the ammonia-oxidizing bacteria, via the catalytic activity of AMO, catalyze the oxidative dehalogenation of many halogenated hydrocarbon pollutants has spurred interest in these bacteria for use in bioremediation.

1.6. Potential role of *N. europaea* in bioremediation.

The utilization of biological organisms for the transformation and removal of pollutants from the environment is referred to as bioremediation. Biotechnological processes offer many advantages over physiochemical

processes such as lower costs and the possibility of *in situ* treatment . Additionally, many of the principles involved in bioremediation have been used for decades to treat wastes such as municipal sewage and effluents from industrial processes such as oil refining (Lees and Senior, 1995). Greater public acceptability exists for bioremediation compared to alternatives such as incineration and disposal. Current interest in *N. europaea* stems predominantly from the ability of AMO to oxidize environmental pollutants. However, both advantages and disadvantages exist with the designed use of microorganisms, including *N. europaea*, for bioremediation.

Potential limitations of biological treatments are, in general, related to their biological nature. Biological remediation requires optimization and monitoring of the environmental conditions such that indigenous or inoculated organisms can survive and degrade the contaminant(s) at sufficient rates. Both the concentration and types of contaminants, as well as the presence of heavy metals, would have to be assessed in order to implement bioremediation. Additionally, bioremediation can be slow and uncertainty exists as to whether microbial degradation can reduce the contaminant to sufficiently low concentration levels (Fetzner and Lingens, 1994). In spite of these difficulties, bioremediation is considered an economical alternative to previously utilized methods, such as incineration and pump and treat systems (National Research Council, 1994).

As a result of interest in bioremediation, many organisms have been discovered which can degrade many types of contaminants (Lees and Senior, 1995). In some cases, biodegradation requires aerobic conditions and others require anaerobic conditions. Theoretically, the most effective scheme would involve the use of the contaminant by the organism as either an energy source or electron acceptor. This situation has been observed for some contaminants and the ability to harness these organisms for bioremediation is currently being addressed. One of the most persistent and common contaminants is trichloroethylene. Trichloroethylene appears to be biodegradable only through co-metabolism (Atlas, 1995).

Co-metabolism involves the contaminant being metabolized gratuitously because of the lack of enzyme specificity. To stimulate co-metabolism in bioremediation, a cosubstrate is added to the contaminated site to induce the growth of microorganisms whose enzymes can degrade both the cosubstrate and the pollutant. For example, trichloroethylene is oxidatively dechlorinated by

AMO. The addition of ammonia would stimulate growth of ammonia-oxidizing bacteria. Using ammonia as a primary substrate, the bacteria would co-metabolize trichloroethylene via AMO. This process may be slow due to the competitive effect between ammonia and the trichloroethylene for the catalytic site of the oxygenase. Toxicity can also be a problem associated with the use of biological systems for bioremediation. For *N. europaea*, trichloroethylene is believed to be oxidized to an epoxide intermediate by AMO; subsequent hydrolysis of the epoxide leads to spontaneous dechlorination (Fetzner and Lingens, 1994). During the course of trichloroethylene oxidation a reactive intermediate is formed which can also lead to inactivation of AMO and other proteins (Rasche, Hyman et al., 1991). However, toxicity is also an issue associated with biological alternatives to *N. europaea*.

Trichloroethylene is also co-metabolically oxidized by methane-oxidizing bacteria. The rates observed for trichloroethylene oxidation by methane-oxidizing bacteria expressing the soluble form of methane monooxygenase (sMMO) are an order of magnitude greater than any rates by other bacteria which oxidize trichloroethylene. However, rapid oxidation of trichloroethylene required the presence of sMMO, which is only expressed when the concentration of Cu^{2+} is maintained below 1 μM (Hanson, 1992). This feature raises the question of whether sMMO activity would be expressed in the natural environment. Thus practical aspects of implementing bioremediation are manifold.

Advantages associated with the use of ammonia-oxidizing bacteria in bioremediation include their ubiquity, minimal growth requirements and the broad substrate specificity of AMO. Many enzymes present in microorganisms degrade only a narrow range of substrates (Atlas, 1995), while AMO oxidizes many types of compounds. This feature is advantageous because contaminated sites often contain a variety of compounds. An additional feature of the ammonia-oxidizing bacteria is that in order to degrade a variety of compounds only ammonia and O_2 are required for AMO activity. The advantage of this feature is made clearer by considering an alternative enzyme system. Toluene monooxygenase, present in the toluene-oxidizing bacteria, can also oxidize halogenated hydrocarbons. However, the presence of toluene is required to maintain enzyme expression (Thomas and Ward, 1989). At contaminated sites where toluene exists, this enzyme system would be useful, but at sites where toluene does not exist the undesirable addition of toluene would be necessary.

Some of the limitations of biological systems are being overcome through adaptations or engineering of microbial strains (Fetzner and Lingens, 1994). Although designed microorganisms may in principle be more effective catalysts for bioremediation, the introduction of microorganisms into the environment in order to enhance bioremediation has not been successfully demonstrated (Atlas, 1995). Additionally, the release of genetically modified microorganisms in the environment is controversial and may not have wide spread public acceptance, especially for *in situ* treatments. The benefits of genetically modified organisms for bioremediation will most likely be in bioreactors rather than *in situ* treatments (Atlas, 1995).

In situ treatment is a cost effective alternative to physical cleanup methods, such as pump and treat systems. *In situ* treatment involves the installation of an engineered system to stimulate the growth of indigenous or introduced microorganisms in regions of subsurface contamination, such as ground water. The process typically entails perfusion of nutrients and one or more electron acceptors in order to optimize the environment for biodegradation of contaminants. O_2 is currently the most common electron acceptor used for *in situ* bioremediation systems (National Research Council, 1994). In theory, the ammonia-oxidizing bacteria could be utilized for *in situ* treatments. The bacteria are ubiquitous and stimulation of growth would require O_2 and ammonia.

The most successful applications of bioremediation to date have been those that modify the environment to stimulate the activity of naturally occurring microorganisms (Atlas, 1995). The largest bioremediation project thus far in the United States has been the treatment of the Exxon Valdez Alaskan oil spill. Eleven million gallons of crude oil was spilled. Initial efforts to physically cleanup the oil using high-pressure water, a process which cost more than 1 million dollars per day, was both slow and left residual subsurface contamination. However, bioremediation, consisting of adding nitrogen-containing fertilizers to the contaminated shorelines, stimulated the metabolic activities of indigenous hydrocarbon-degrading microorganisms. This treatment resulted in three to five times faster degradation of the surface and subsurface oil compared to degradation at untreated test sites. Although the rates of hydrocarbon degradation were stimulated, the complete removal of the contaminating hydrocarbons was not achieved. However, treatment continued for a couple of years and the remediation of hundreds of miles of contaminated shoreline cost less than 1 million dollars (Atlas, 1995).

1.7. Cell-free ammonia-oxidizing activity.

Although there exists much interest in the catalytic activity of AMO, the purification of active enzyme has not been achieved. Reasons for the limited amount of progress in purification include both the unstable nature of ammonia-oxidizing activity in extracts and the lack of a suitable electron donor for AMO (Ensign, Hyman et al., 1993). For these reasons only a limited amount of information has been published on cell-free ammonia oxidation.

Cell-free ammonia-oxidizing activity is highly unstable (Suzuki, Kwok et al., 1981; Ensign, Hyman et al., 1993). Extracts from *N. europaea* are reported to lose 50% of their activity in 4 hours when stored at 4°C while frozen extracts (-20°C) were stable for only 1 or 2 days (Suzuki and Kwok, 1970). Additionally, the ammonia-dependent O_2 uptake activity of the extracts was variable and dependent upon the age of the cells at the time of harvest.

An artificial electron donor for AMO has not been determined. Additionally, the immediate source of reductant for ammonia oxidation *in vivo* has not been identified. Thus twenty years after initial attempts to characterize cell-free AMO activity, activity is still measured as the rate of either ammonia-dependent O_2 uptake or nitrite formation. Both methods of measuring AMO activity are dependent on the endogenous coupling of electrons from hydroxylamine oxidation to fulfill the reductant requirement of ammonia-oxidizing activity. For this reason, these assays limit our understanding of AMO activity. Despite these difficulties, ammonia-oxidizing activity has been demonstrated using spheroplasts, vesicles, cell extracts and a membrane fraction reconstituted with purified cytochrome c-554 prepared from *N. europaea*.

Initial efforts to characterize ammonia-oxidizing activity in cell-free extracts of *N. europaea* were conducted in the laboratory of Suzuki. Cell-free AMO activity, measured as the rate of either ammonia-dependent O_2 uptake or nitrite formation, was obtained by disruption of cells by a French press. In contrast, disruption of cells by sonication or osmotic rupture led to extracts incapable of oxidizing ammonia. Extracts catalyzed the stoichiometric oxidation of ammonia to nitrite at rates between 10% and 20% of the rates of intact cells. However, a stabilizing agent such as bovine serum albumin (BSA), spermine or Mg^{2+} was necessary to measure ammonia-oxidizing activity in cell extracts (Suzuki, Kwok et al., 1981; Ensign, Hyman et al., 1993). The relative effectiveness of these

activators was influenced by the concentration of phosphate buffer and thus was attributed to an ionic effect. Of the three activating agents, bovine serum albumin (BSA) was consistently the most effective activator. However, the roles of these activators in the assay were not established

Cell-free ammonia-dependent O_2 uptake activity was consistently associated with prior reduction of the endogenous cytochromes (Suzuki, Kwok et al., 1981). Cytochrome reduction occurred only in the presence of BSA, spermine or Mg^{2+}. Partial reduction of the cytochromes was a necessary precondition for ammonia-oxidizing activity. In the presence of BSA, the addition of ammonia to active extracts led to a partial reduction of c-type and a-type cytochromes prior to the initiation of ammonia-dependent O_2 uptake. A lag period was associated with initiation of activity by ammonia. The lag was suggested to be the time required for ammonia oxidation to partially reduce the cytochromes. In the absence of BSA, both the cytochrome reduction and ammonia-oxidizing activity required priming by either NADH or hydroxylamine. Both the partial cytochrome reduction and ammonia-dependent O_2 uptake activity were initiated more quickly with the addition of either NADH or hydroxylamine than with ammonia alone. However, in the absence of BSA the cytochromes did not undergo secondary reductions and activity ceased. Based on these observations, it was suggested that electrons for the hydroxylation of ammonia were supplied externally by NADH or hydroxylamine or through endogenous metabolism in the presence of BSA (Suzuki, Kwok et al., 1981).

In addition to cell-free extracts, some attempts at further purification of AMO activity were reported (Suzuki and Kwok, 1981). A membrane fraction, excluded from an anaerobic Sepharose 6B column, contained cytochrome a_1, other c-type cytochromes, a strong cytochrome oxidase activity and ammonia-stimulated NADH oxidation, but did not oxidize ammonia. However, ammonia-oxidizing activity of the membrane fraction was restored by the addition of purified cytochrome c-554. Ammonia oxidation by the membrane fraction required the same assay conditions as those required for extracts (i.e. BSA). Cytochrome c-554 was reduced by the membrane fraction in the presence of hydroxylamine, hydrazine or ammonia. The oxidation of NADH or cytochrome c-554 reduced by the membrane fraction was stimulated by alternative substrates for AMO such as methane, methanol and carbon monoxide (Suzuki, Kwoks et al., 1976). As for experiments conducted with extracts, AMO activity was measured

in the presence of other enzyme activities, which suggests that the assay was coupled.

Cell-free ammonia-oxidizing activity has also been demonstrated for the marine ammonia-oxidizing bacteria, *Nitrosocystis oceanus* (Watson, Asbell et al., 1970). However, for this marine bacterium, seawater, Mg^{2+}, and occasionally ATP were required for ammonia-dependent O_2 uptake or nitrite formation. The role of ATP was not elaborated and subsequent investigations of ammonia-oxidizing activity from extracts of *Nitrosocystis oceanus* have not been reported. However, ammonia-oxidizing activity in extracts of *N. europaea* was later reported not to be affected by ATP (Suzuki and Kwok, 1970).

In summary, an assay system for measuring ammonia-oxidizing activity in extracts of *N. europaea* was developed. Activity was associated with the maintenance of the ability of the cytochromes to be reduced, which suggest the involvement of electron transfer processes. However, much about the mechanism of ammonia hydroxylation remained unresolved. Additionally, neither the role of BSA nor the other activating agents in the assay of ammonia-oxidizing activity was explained.

1.7.1. Effects of BSA and Cu^{2+} ions on assay.

A more recent study of ammonia-oxidizing activity of cell extracts of *N. europaea* showed that Cu^{2+} ions are required in the assay in order to attain ammonia-oxidizing activity rates between 10% and 20% of the rates of intact cells (Ensign, Hyman et al., 1993). The addition of an optimal concentration of Cu^{2+} ions to the assay stimulated cell-free ammonia-oxidizing activity 5 to 15 fold (Ensign, Hyman et al., 1993). In contrast, Cu^{2+} ions did not affect the ammonia-oxidizing activity of intact cells. Metal ions other than Cu^{2+}, including Zn^{2+}, Co^{2+}, Ni^{2+}, Fe^{2+}, Fe^{3+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, Cr^{3+} and Ag^{+} were ineffective at stimulating cell-free ammonia-oxidizing activity.

In addition to Cu^{2+} ions, the assay media also required BSA in order to attain maximal rates of either ammonia-dependent O_2 uptake or nitrite formation. Activity measured in the absence of BSA with 10 μM Cu^{2+} was reported to be only 5% of the maximal activity measured in the presence of BSA. Other serum albumins could replace BSA, but gamma globulins, a common contaminant in

BSA, or ovalbumin could not (Ensign, Hyman et al., 1993). Because of the well established ability of BSA to bind copper, increasing the concentration of BSA in the assay required a corresponding increase in the concentration of Cu^{2+} in order to attain optimal rates of activity. The role of copper in the assay, but not of BSA, was established.

Intact cells of *N. europaea* undergo a time-dependent inactivation of ammonia-oxidizing activity by acetylene (C_2H_2) resulting in a progressive and saturable incorporation of $^{14}C_2H_2$ exclusively into a 27-kD polypeptide. It is thought that the attempted oxidation of C_2H_2 by AMO leads to the formation of an activated intermediate which binds to the active site. In contrast to intact cells, cell extracts catalyzed the incorporation of $^{14}C_2H_2$ into the 27-kD polypeptide only in the presence of Cu^{2+} ions (Ensign, Hyman et al., 1993). Other metals tested were ineffective at facilitating the incorporation ^{14}C-label from $^{14}C_2H_2$ into the 27-kD polypeptide. When the ^{14}C-labeling reaction was allowed to go to completion, the extent of ^{14}C-label incorporation catalyzed by either intact cells or extracts was comparable. Because the ^{14}C-label incorporation is a saturable process, this result suggested that all the AMO molecules in cell extracts were catalytically competent to undergo reduction, bind and reduce O_2, and activate C_2H_2. This result suggested that the inability to attain rates of ammonia-dependent O_2 uptake or nitrite production greater than 10 to 20% of the rates of intact cells may be due to a reduced rate of catalysis rather than denaturation or inactivation of AMO. A reduced rate of catalysis was proposed to result from a loss of coupling integrity among AMO, HAO and accessory proteins involved in electron transfer between these two enzymes (Ensign, Hyman et al., 1993).

These results demonstrated that Cu^{2+} ions were required for catalysis by AMO in cell extracts. Two reasons for the requirement of AMO for Cu^{2+} were considered. Copper ions may activate Cu^{2+}-deficient catalytically inactive molecules of AMO or Cu^{2+} may stimulate the rate of AMO turnover. A comparison of the stimulation of the rate of nitrite formation by Cu^{2+} was done for samples of an extract which were pretreated with or without acetylene. In addition, the pretreatments were conducted either in the presence or absence of Cu^{2+} ions for an extended period of time. If Cu^{2+} ions stimulated the rate, then the acetylene-treated sample without copper would be slowly inactivated. However, the rate of nitrite formation by the sample, which had been pretreated with acetylene in the absence of Cu^{2+} ions, was stimulated to the same extent as the extract which had not been exposed to acetylene. This result suggested that

in the absence of exogenous Cu^{2+} ions there existed Cu^{2+}-deficient catalytically inactive molecules of AMO.

Extracts of *N. europaea* also catalyze the oxidation of ethane, an alternative substrate for AMO, to ethanol at a rate about 10% the rate of intact cells (Ensign, Hyman et al., 1993). Assay requirements for ethanol formation by extracts included the presence of Cu^{2+}, BSA and hydrazine as a source of reductant. Hydrazine, a substrate for hydroxylamine oxidoreductase, was supplied at a concentration well above its Km such that the rate of ethanol formation was not affected by a reductant limitation. The use of either hydroxylamine or NADH as the source of reductant led to slower rates of ethanol formation (Ensign, Hyman et al., 1993).

Cell-free AMO activity can be measured by using four different assays. Activity can be measured using the ^{14}C-label incorporation, ammonia-dependent O_2 uptake and nitrite or ethanol formation. Activity measured by using the ^{14}C-label incorporation indicated that 100% of the AMO molecules in an extract were catalytically active. However, activity measured as the rate of ammonia-dependent O_2 uptake, nitrite or ethanol formation indicated that the rates were only 10% the rates of intact cells. Based on these results it has been suggested that AMO activity is slowed to 10% the rate of intact cells due to a reduction in the coupling of enzyme activities involved in AMO catalysis and not to denaturation or loss of active AMO (Ensign, Hyman et al., 1993). Because few turnovers of $^{14}C_2H_2$ are required for ^{14}C-labeling to reach saturation, this assay remains a sensitive indicator of the portion of active AMO molecules present in an extract.

Some notable differences exist between the requirements for the ^{14}C-labeling assay and the ethanol assay. These differences include that 1) hydroxylamine was an effective source of reductant for ^{14}C-label incorporation into the 27-kD polypeptide but not for ethanol formation, 2) BSA was usually required for ethanol formation but was not necessary for ^{14}C-label incorporation and 3) importantly, high concentrations of Cu^{2+} ions inhibited ethanol formation but did not inhibit ^{14}C-label incorporation (Ensign, Hyman et al., 1993). The reason(s) for the apparent differences between these assays of AMO activity has not been adequately explained. Because the ^{14}C-label incorporation into the 27-kD polypeptide catalyzed by extracts is both a reductant-requiring event and a sensitive technique, this assay system should prove useful for screening potential electron donors for AMO.

1.7.2. Stabilization of ammonia-oxidizing activity.

Even when ammonia-oxidizing activity is assayed in the presence of optimal concentrations of BSA and Cu^{2+} ions, the activity is still apparently unstable to long term manipulations such as would likely be required for purification schemes (Suzuki, Kwok et al., 1981; Ensign, Hyman et al., 1993). In addition to its use in the assay, BSA is reported to stabilize ammonia-oxidizing activity in cell extracts (Suzuki, Kwok et al., 1981). Alternative stabilizing conditions were not reported, although anaerobic conditions destabilized activity. The mechanism for the stabilizing effect of BSA on ammonia-oxidizing activity was unexplained. Therefore initial efforts were undertaken to determine the role(s) of BSA in the stability, with the expectation that results would lead to alternative methods to stabilize ammonia-oxidizing activity.

As a result of experiments described in Chapter 4, the stabilizing effect of BSA is proposed to result from its inhibition of lipolysis. Other agents which modify lipase activity, including $CuCl_2$, $HgCl_2$, lecithin, or phenylmethylsulfonyl fluoride (PMSF), are also shown to stabilize ammonia-dependent O_2 uptake activity. As a preview of material presented in Chapter 4, a comprehensive literature perspective is provided on the lipase inhibitory properties of these stabilizing agents.

Mercuric ions, like cupric ions, have previously been reported to inhibit the activity of many lipases (Wills, 1960; Nishijima, Akamatsu et al., 1974; Nishijima, Nakaike et al., 1977). Specifically, mercuric and cupric salts have both been reported to inhibit pancreatic lipase (Wills, 1960), a purified phospholipase A from *E. coli* (Nishijima, Nakaike et al., 1977) and a membrane-bound phospholipase from *Mycobacterium phlei* (Nishijima, Akamatsu et al., 1974).

Phospholipids, such as lecithin, have previously been shown to slow the decay of oxidative phosphorylation efficiency of mitochondrial membranes (Rossi, Rossi et al., 1962). Because the decay correlated with the hydrolysis of endogenous membrane lipids, it was suggested that exogenous phospholipids protected mitochondrial membranes by competing with endogenous phospholipids as substrates (Rossi, Rossi et al., 1962; Rossi, Sartorelli et al., 1964). By analogy with this mode of protection of mitochondrial membranes, lecithin may provide an alternative substrate for the activity of an endogenous lipase and thus protect *N. europaea* membranes.

PMSF has been reported to bind to an active site serine present in many lipases as well as proteases (Patkar and Bjorkling, 1994). Proteolysis was dismissed as a cause for loss of ammonia-oxidizing activity because proteins other than BSA would have provided some degree of protection from proteases but other proteins did not stabilize activity. Additionally, a progressive decrease in the molecular weights of the polypeptides present in the extracts during the loss of activity did not occur as would be expected if proteolysis was involved.

The inhibition of lipase activity by BSA is not as simply explained as the effect of the other stabilizing agents. BSA has been reported to activate or protect numerous membrane-associated enzymatic activities (Chefurka, 1966; Ko, Frost et al., 1994) and also to inhibit lipid degradation in other biological systems (Honjo, Ozawa et al., 1968; Galliard, 1974). However, in order to describe the two mechanisms by which BSA has been proposed to inhibit lipase activity, a brief description of the enzymatic behavior of lipases is necessary.

In general, lipases and phospholipases catalyze the hydrolysis of ester bonds in triacylgycerols that form aggregates such as micelles, vesicles, membranes or emulsified particles. The reactions catalyzed by lipases occur at a lipid-water interface and are critically dependent upon the binding of the enzyme to the interface (Gargouri, Pieroni et al., 1986). Therefore all amphiphilic substances are expected to influence the rate of the interfacial reaction. In this regard, BSA has been shown to reduce the activity of a few purified lipases. It has been suggested that the inhibition of lipase activity by BSA is due to its modification of the physicochemical properties of the oil-water interface (Gargouri, Pieroni et al., 1986). The ability of specific proteins to inhibit lipase activity is highly variable and may depend on the ability of a protein to bind to the lipid-water interface and on physical conditions (Conricode and Ochs, 1989).

Alternatively, BSA has been suggested to inhibit lipase activity by binding free fatty acids. This effect would result because free fatty acids at or above their critical micelle concentration activate phospholipase catalysis. By binding fatty acids, BSA reduces their concentration to below the critical micellar concentration and therefore BSA indirectly inhibits activation of the phospholipase (Scarpa and G., 1972).

With regard to the prevalence of lipase activities, many reports have described the presence of phospholipase activity in crude cell lysates of various microorganisms including bacteria (Avigad, 1976; Hazlewood and Dawson, 1976; Christie, 1987). Phospholipase activity can be associated with either membrane

or soluble fractions. The instability of ammonia-oxidizing activity of membrane preparations suggests that the lipase activity in *N. europaea* is associated with the membrane.

1.8. Other monooxygenase enzyme systems of potential relevance to AMO.

Monooxygenases represent a class of enzymes which catalyze a reductive activation of O_2, linked to the insertion of one oxygen atom into substrate. The second atom O_2 is reduced to water with the oxidation of a reductant (H_2X).

$$S + O_2 + H_2X \rightarrow SO + H_2O + X \qquad (1)$$

There exist some common elements to catalysis by monooxygenases. First, monooxygenases require cofactors capable of reacting with O_2. A limited number of strategies have evolved to circumvent the spin forbidden properties associated with O_2 activation (Malmström, 1982). Cofactors utilized for the purpose of O_2 activation in different monooxygenases include redox active transition metals, flavins and pteridines. Transition metal cofactors observed in monooxygenases include heme iron, non-heme iron, and copper. In general, activation of O_2 by metals occurs as a result of electron transfer from the reduced metal to O_2. Flavins and pteridines are organic cofactors which, when reduced by one electron, exist in a semiquinone form. The semiquinone form of flavins and pteridines can react with O_2 resulting in a covalently bound peroxide intermediate. Amino acid sequences in the regions involved in cofactor binding have been found to be conserved among similar monooxygenases (Harayama and Kok, 1992). However, the amino acid sequences of two putative polypeptide components of AMO do not have significant sequence similarity to any of the protein sequences currently present in the gene databases other than those of *pmoA* and *pmoB* from methane-oxidizing bacteria (McTavish, Fuchs et al., 1993; Semrau, Chistoserdov et al., 1995).

A second element common to monooxygenase reactions is that a reductant source is required for O_2 activation. The physiological source of reductant for cofactor activation varies among the monooxygenase enzymes. Reduced pyridine nucleotides, NADH or NADPH, are common sources of

reductant for monooxygenase catalysis, although ascorbate and o-diphenol have also been shown to function as reductants in some isolated systems. Amino acid sequences in regions of the protein which bind NADH, flavins, and iron-sulfur centers are also conserved (Harayama and Kok, 1992).

The monooxygenase reaction can be separated into two enzymatic steps, the oxidation of reductant and the hydroxylation of substrate. Although the flavoprotein hydroxylases catalyze both reactions on a single polypeptide chain, most monooxygenase activities are part of a multicomponent system with both a reductase and a hydroxylase component. Additionally, other polypeptide components may either be required for or to enhance monooxygenase activity. In general, monooxygenase turnover involves the transfer of reducing equivalents through an electron-transport chain to the cofactor present in the hydroxylase component. The cofactor activates O_2 and subsequently the activated form of O_2 hydroxylates the substrate.

Although sequencing data suggest that AMO may be a novel type of monooxygenase, a survey of other purified and partially purified monooxygenase enzymes is presented. The cofactor and reductant for the monooxygenases are discussed and a summary of the information is provided in Table 1.2.

1.8.1. Methane monooxygenases.

Methane monooxygenases catalyze the oxidation of methane to methanol. This enzyme is present in a group of bacteria which can utilize methane as their source of energy. Copper ions exert considerable influence on the nature of the monooxygenase expressed in these bacteria and also on the morphology of the bacterial cell. When Cu^{2+} is present in limiting amounts during the growth of either *Methylococcus capsulatus* Bath or *Methylosinus trichosporium* OB3b, methane oxidation is carried out mostly by the soluble form of methane monooxygenase (sMMO). When Cu^{2+} is present in nonlimiting quantities, the activity of the particulate form of methane monooxygenase (pMMO) is dominant. Increasing copper availability also leads to an increase in the content of intracytoplasmic membranes in cells of methane-oxidizing bacteria.

No significant amino acid sequence similarity exists between the soluble and the particulate forms of methane monooxygenase. Most likely each form

uses a different transition metal for catalysis. Both inhibitor profiles and substrate specificities of sMMO and pMMO are different. Although both sMMO and pMMO are inhibited by acetylene, only pMMO is inhibited by thioureas. sMMO can oxidize many other hydrocarbons as alternative substrates. These include saturated and unsaturated hydrocarbons up to approximately C_8 in size, aromatics, cyclic, heterocyclic, halogenated alkenes, and ethers. The ability of sMMO to catalyze the oxidation of many abundant and potentially toxic hydrocarbons has led to a considerable amount of interest in this enzyme. In contrast to both sMMO and AMO, pMMO does not oxidize hydrocarbons larger than butane.

1.8.1.1. Soluble methane monooxygenase.

The soluble methane monooxygenase (sMMO) catalyzes the reaction: $CH_4 + O_2 + H^+ + NAD(P)H \rightarrow NAD(P)^+ + H_2O + CH_3OH$. The soluble form of methane monooxygenase has been purified and extensively characterized and consists of three components, the hydroxylase, the reductase and component B (Lipscomb, 1994). The hydroxylase component is a dimer of α, β, γ subunit types with a Mr of 210-kD. The hydroxylase binds methane and uses reducing equivalents obtained from the reductase to activate O_2. The α subunit of the hydroxylase contains a nonheme binuclear Fe hydroxylase which activates O_2. The reductase component is a polypeptide of Mr of 42-kD which contains one flavin adenine dinucleotide molecule and an Fe_2S_2 center. The reductase shuttles electrons from NADH to the hydroxylase. Component B has a Mr of 15.7-kD, lacks prothestic groups and converts sMMO from an oxidase to an oxygenase. In the absence of component B, the hydroxylase and reductase components catalyze the $4e^-$ reduction of O_2 to H_2O.

The complete sequences of each of the components of sMMO are known and a crystal structure of the hydroxylase component has been published (Rosenzweig, Frederick et al., 1993). The active site of the hydroxylase component contains a hydroxo-bridged dinuclear iron cluster. Catalysis is thought to involve reduction to the ferrous state of the diiron center which then reacts with O_2. The O-O bond is heterolytically cleaved to yield water and an [Fe(IV)•Fe(IV)]=O species, which purportedly abstracts a hydrogen atom from

methane to yield a substrate radical and a diiron cluster-bound hydroxyl radical. Recombination of the radicals yields the product methanol. The role of the reductase and component B is not strictly related to electron transfer from NAD(P)H, they also appear to regulate catalysis by altering the catalytic properties of the hydroxylase at intermediate stages of the turnover cycle (Lipscomb, 1994).

1.8.1.2. Particulate methane monooxygenase.

Although much more information exists about the sMMO, AMO is more similar to particulate methane monooxygenase (pMMO). This enzyme also catalyzes the oxidation of CH_4 to CH_3OH, but the source of reductant is uncertain. On the basis of both the inhibitor profiles and membrane location, AMO resembles pMMO. Additionally, two putative genes for pMMO have been sequenced and have greater than 40% amino acid identity with two of the genes coding for AMO (Semrau, Chistoserdov et al., 1995). No significant sequence similarity exists between sMMO and pMMO. Similar to the ammonia-oxidizing system in *N. europaea*, the source of reductant for pMMO occurs indirectly via the oxidation of methanol by methanol dehydrogenase. However, the electron pathway remains uncertain.

Although few studies have been conducted on pMMO because of its apparent instability in extracts, it has been stated that pMMO activity in crude preparations of *Methylosinus trichosporium* OB3b is both stable and highly active (Tonge, Harrison et al., 1975). These authors noted that the methane-oxidizing activity of the extracts, as determined by the rate of methane-dependent O_2 uptake, was much lower and less stable than if determined by the rate of methanol formation.

Similar to the earlier work describing the stimulation of cell-free ammonia-oxidizing activity by purified cytochrome c-554, an isolated CO-binding cytochrome c stimulated the activity of pMMO in cell-free preparations. A release of the CO-binding cytochrome c from the membrane occurred during storage. This release coincided with the loss of pMMO activity of the membrane fraction. The addition of purified CO-binding cytochrome c reconstituted activity. Both

NADH and ascorbate were reported to function as electron donors for methanol formation (Tonge, Harrison et al., 1975).

A three-component enzyme system that catalyzed the oxidation of methane to methanol was later purified from *Methylosinus trichosporium* OB3b (Tonge, Harrison et al., 1977). The methane-oxidizing complex could be released from the membrane fractions by treatment with phospholipase C or sonication. Three protein components were isolated: a soluble CO-binding cytochrome c (Mr of 13-kD), a copper protein (Mr of 47-kD) and a small protein (Mr of 9.4-kD). Ascorbate and methanol in the presence of partially purified methanol dehydrogenase, but not NADH, functioned as electron donors for methanol formation by the purified system (Tonge, Harrison et al., 1977). The CO-binding cytochrome c had ascorbate oxidase activity and it was suggested to be the natural electron donor for pMMO activity. However, little direct evidence exists to support this role for cytochrome c in methane oxidation (Anthony, 1986). Furthermore, ascorbate-linked pMMO activity has not been observed in other studies of pMMO and many laboratories, including their own, have been unsuccessful in reproducing this work (Smith and Dalton, 1989; Dalton, 1992).

Two other reports of purification of pMMO have appeared in the literature, but neither has been verified in other laboratories. The first involved solubilization of *M. capsulatus* (strain M) membranes with sodium deoxycholate (Akent'eva and Gvozdev, 1988). The proteins were purified on DEAE cellulose and resolved into a hydroxylase component (Mr of 200 to 240-kD) of six subunits and a NADH reductase (Mr of 180-kD) of four subunits (Dalton, 1992). The proteins contained copper and iron, but it was unclear which metal was contained in which fraction (Dalton, 1992). The pMMO activity of this preparation was unstable.

A second report concerned the solubilization of pMMO from *M capsulatus* (Bath). Many detergents were tested for solubilization, but only dodecyl-ß-D-maltoside released pMMO into a form that could be subsequently activated by treatment with BioBeads first to remove the detergent, followed by the addition of lecithin to recover enzyme activity (Smith and Dalton, 1989). Subsequent purification steps resulted in an irrecoverable loss of activity. The solubilized pMMO was composed of polypeptides of Mr of 49-kD, 23-kD, and 22-kD and contained 3 nmol of copper per milligram of protein (Dalton, 1992). Enzyme activity was measured as methanol formation in the presence of 20 µM Cu^{2+} ions. Like the effect of Cu^{2+} ions on AMO activity, Cu^{2+} ions also stimulated the

cell-free activity of pMMO. NADH was used as a source of reductant. Because the preparation likely contained other enzyme activities, NADH may have functioned as an indirect electron donor through the activity of a dehydrogenase. Other electron donors such as ascorbate, *tert*-butyl peroxide and chloroperoxybenzoic acid and trimethylhydroquinone, were ineffective. Suggested reasons for the loss of activity included enzyme instability or the loss of a necessary component(s) which required reconstitution of activity from the individually purified components.

More recently, duroquinol, but not NADH, was suggested to serve as a direct electron donor for solubilized pMMO (Shiemke, Cook et al., 1995). However, only certain quinols stimulated activity. The ability of the quinol to stimulate pMMO activity did not correlate with the reduction potential of the quinol, which suggested that the specificity was likely due to a physical interaction. This result, combined with the known ability of tetra- and tri-methylhydroquinones to stimulate AMO activity in intact cells, suggests that quinones may be involved in the pathway of electron flow to AMO *in vivo* (Shears and Wood, 1986).

In summary, some information exists with regard to the possible components of pMMO. All the data so far acquired for pMMO suggest that it is a copper-containing protein. However, cell-free pMMO activity has been associated with 1) polypeptides of various sizes, 2) an inability to reproduce results and 3) the uncertainty of possible interference due to sMMO activity. For these reasons, the characterization of pMMO has been difficult.

1.8.2. Microsomal cytochrome P-450 hydroxylase.

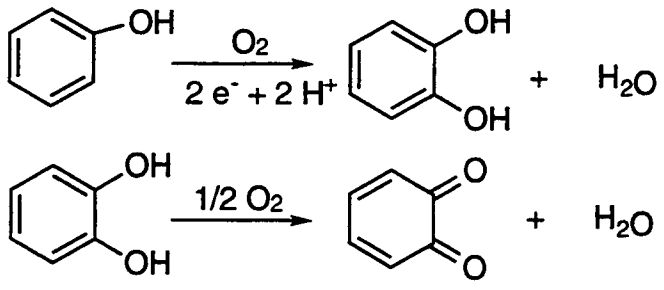
Among the metal-containing monooxygenases, cytochrome P-450 is the best characterized (Malmström, 1982). The microsomal P-450-associated monooxygenase offers an example of a well studied membrane-associated monooxygenase enzyme system. In general, the system consists of cytochrome P-450, a heme iron hydroxylase component, and NADPH-cytochrome P-450 reductase, a flavoprotein which transfers reducing equivalents from NADPH to the hydroxylase. The microsomal P-450-associated monooxygenases have broad substrate specificities in part due to the existence of multiple cytochrome

P-450 isozymes. Although the different isozymes have specificity for different substrates, considerable overlap often exists (Guengerich and Liebler, 1983). The hydroxylase has an apparent monomeric Mr between 45- to 60-kD depending on the isozyme. The reductase component contains one FAD and FMN molecule per monomer and has an apparent monomeric Mr between 74- to 80-kD depending on the source. Cytochrome P-450 catalyzes a variety of different reactions such as carbon hydroxylations, heteroatom oxygenation, heteroatom dealkylations and epoxidations . Various P-450 isozymes have been purified and reconstituted into a catalytically active unit.

Common features between AMO and microsomal P-450 monooxygenases include their inactivation by acetylenic compounds, membrane location and their ability to oxidize many types of compounds. The inactivation of cytochrome P-450 by alkynes has served as a model for the proposed mechanism for the inactivation of AMO by alkynes. Cytochrome P-450 catalyzes the oxidation of terminal acetylenes leading to both the formation of acetic acid derivatives as products and the concomitant inactivation of the enzyme. For most cytochrome P-450 isozymes, the inactivation results in the N-alkylation of the prosthetic heme group by a reactive species generated at the active site (Chan, Sui et al., 1993). It is suggested that the triple bond is enzymatically oxidized to an intermediate, such as a ketene, which either binds to the heme causing inactivation of the enzyme or is nonenzymatically hydrated leading to the formation of the observed carboxylic acid product. The loss of activity through heme alkylation leads to a notable change in absorbance of the heme. The inactivation of AMO by alkynes does not lead to any apparent spectrophotometric changes. However, inactivation of cytochrome P-450 4A1 isozyme by alkynes occurs without detectable loss of the heme chromophore. The inactivation results in the binding of the radioactive alkyne to protein. It has been suggested that the ketene intermediate binds to an active site nucleophile (Chan, Sui et al., 1993). This result may be more characteristic of AMO, where the radiolabel becomes attached to a polypeptide of an apparent M_r of 27-kD rather than a prosthetic group.

1.8.3. Tyrosinase.

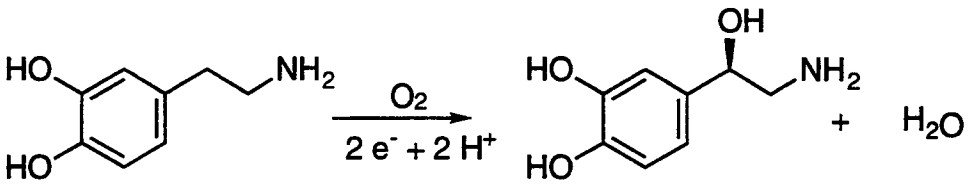
Tyrosinase is a copper-containing monooxygenase, which has been called phenolase, catechol oxidase and more officially, o-diphenol oxidoreductase. This enzyme catalyzes both the orthohydroxylation of monophenols and the oxidation of o-diphenols to o-quinones (Robb, 1984).



This enzyme is widely distributed in nature and its activity has been suggested to have different functions in various organisms. Most often its activity is associated with the formation of melanin pigments. Soluble as well as particulate forms of tyrosinase exist. The enzyme from *Neurospora crassa* has been the most extensively studied; it has a monomeric Mr of 42-kD. This enzyme contains an antiferromagnetically coupled copper pair at its active site with spectroscopic properties similar to hemocyanins. The monophenolase activity of tyrosinase derives reducing equivalents from the oxidation of o-diphenols, the product of the monooxygenation (Lerch, 1981). An initial lag in monophenolase activity is observed; the lag is shortened by the addition of o-diphenols as a source of reductant. Like AMO, tyrosinase is inhibited both by copper chelators, such as diethyldithiocarbamate, and ultraviolet light.

1.8.4. Dopamine-β-hydroxylase.

Dopamine-β-hydroxylase catalyzes the benzylic hydroxylation of phenylethylamines.



Dopamine-β-hydroxylase is of central importance in the catecholamine biosynthetic pathway in which it catalyzes the conversion of dopamine to noradrenalin, both of which act as neurotransmitters. Dopamine-β-hydroxylase is known to exist as both soluble and membrane-bound proteins. It is postulated that a precursor product relationship exists between these two forms of enzyme, although the precise nature of the postranslational modification remains unknown (Stewart and Klinman, 1988). The soluble form of dopamine-β-hydroxylase from bovine adrenal medulla is a copper-containing tetrameric glycoprotein of Mr of 290-kD with subunits arranged as pairs of disulfide-linked 75-kD monomeric species and no other cofactors (Stewart and Klinman, 1988). The copper in dopamine-β-hydroxylase is relatively weakly bound, undergoing exchange and loss of copper during the course of enzyme purification. However, the reconstitution of active enzyme by copper ions is rapid and requires only low concentrations (2 μM) of copper ions.

The enzyme catalyzes hydroxylation of many analogues of phenylethylamine. Ascorbate has been shown to be the most effective electron donor to the enzyme *in vitro*, although ascorbate analogs, 2,6-dichlorophenolindophenol and $Fe(CN)_6^{4-}$ are also highly active reductants (Ljones and Skotland, 1984). High concentrations of ascorbate are found in the chromaffin vesicles of the bovine adrenal medulla, where dopamine-β-hydroxylase is localized. It is believed that ascorbate is the natural electron donor. Catalysis is known to proceed via a redox process in which two Cu(II) centers are first reduced by ascorbate to Cu(I). However, the two Cu(II) centers are believed to be mononuclear. One copper center may act as binding site for O_2 and substrate while the other copper center functions as an electron transfer center (Blackburn, 1993). This is in contrast to the situation found in tyrosinase

and hemocyanin, where the dinuclear copper site binds O_2. The primary sequences of dopamine-β-hydroxylase from various mammalian sources are known (Blackburn, 1993). These sequences exhibit 90% identity to one another.

1.8.5. p-Hydroxybenzoate hydroxylase.

p-Hydroxybenzoate hydroxylase is part of a well characterized flavoprotein monooxygenase which catalyzes the hydroxylation of p-hydroxybenzoate with the concomitant dehydrogenation of NADPH. The hydroxylase component contains redox active flavin which is reduced, by NADPH at the N5 position of the flavin isoalloxazine ring (Harayama and Kok, 1992). Dioxygen then binds to $FADH_2$ and is reduced by a two single-electron transfers. The first electron transferred from the $FADH_2$ to O_2 may produce a superoxide anion intermediate which by the second electron may be transformed to a 4a-hydroperoxy-flavin. The net effect is that O_2 is converted to a reactive hydroperoxy-flavin, which attacks the aromatic substrate.

1.8.6. Phenylalanine hydroxylase.

Phenylalanine hydroxylase catalyzes the hydroxylation of phenylalanine to tyrosine using O_2 with tetrahydrobiopterin as a cofactor. Dihydrobiopteridine reductase catalyzes the NADH-dependent reduction of quinonoid dihydrobiopterin to tetrahydrobiopterin . Reductases of the pterin-dependent hydroxylases are very similar 25-kD polypeptides (Harayama and Kok, 1992). In addition to the pterin, the mammalian hydroxylases contain non-heme iron while the bacterial enzyme from *Chromobacterium violaceum* contains a single copper atom. The roles of these metals are not known, but it has been suggested that these metals may facilitate O_2 binding. However, reduction to Fe^{2+} and Cu^{1+} is obligatory before catalysis can proceed (Blackburn, 1993). For both enzymes, a 4a-OH tetrahydrobiopterin is produced as an intermediate and therefore it has been postulated that the active oxygen species is the 4a-peroxy pterin. Therefore, the mechanism for hydroxylation may be similar to that by $FADH_2$ in p-hydroxybenzoate hydroxylase.

1.8.7. Alkane hydroxylase.

The alkane hydroxylase from *Pseudomonas putida (oleovorans)* is a three-component enzyme which catalyzes the hydroxylation of terminal carbon of alkanes and the omegahydroxylation of fatty acids. The electron transfer from NADH to the active site of the membrane bound hydroxylase component, AlkB, is achieved by a 41-kD NADH-rubredoxin reductase and a 18-kD rubredoxin. The hydroxylase component has been purified, and Fe(II) and phospholipids were found to be required for its activity (Harayama and Kok, 1992). NADH-rubredoxin reductase bears sequence similarity to the NADH-putidaredoxin reductase of a bacterial P-450 hydroxylase system.

Most of the monooxygenase systems derive reductant for catalysis either through the direct or indirect interaction of a NAD(P)H reductase component (Table 1.2). In the case of dopamine-β-hydroxylase and tyrosinase, the direct source of reductant is ascorbate and o-diphenol, respectively. The reductant source for AMO is not known. However, reductant for AMO can be supplied indirectly via NADH, but NADH cannot maintain a steady rate of cell-free ammonia-oxidizing activity. It has been argued that NADH is unlikely to be the reductant source for AMO because of energetic reasons. It has been suggested that the oxidation of ammonia to hydroxylamine should not require a reductant of such a low potential as NADH. Additionally, the production of NADH by the ammonia-oxidizing bacteria requires a large input of energy via reverse electron flow and ammonia oxidation would consume NADH required for CO_2 fixation. Thus, it is thought that NADH is not the source of reductant for AMO. Given the ability of certain quinols to stimulate both AMO and pMMO activity *in vitro*, it can be speculated that the source of reductant for either of these two systems may involve electron flow via a quinone as suggested in Scheme 1.2.

Table 1.2. Electron donors, electron transport systems and cofactors involved in monooxygenase reactions[a]

Monooxygenase	Cofactor	Electron donor	Electron transport system[b]
pMMO or AMO	Cu, uncertain	not known	not known
Soluble methane monooxygenase	nonheme binuclear Fe (II)	NAD(P)H	NADH reductase (flavoprotein and Fe-S)
Microsomal P-450 hydroxylase	heme iron	NADPH	NADPH-cyt. P-450 reductase (flavoprotein)
Dopamine-β-hydroxylase	Cu	Ascorbate	—
Tyrosinase	Cu	o-diphenols	—
p-Hydroxybenzoate hydroxylase	FAD	NADPH	—
Phenylalanine hydroxylase	tetrahydrobiopterin and nonheme Fe	NADH	Dihydrobiopteridine reductase
Pseudomonas p. (oleovorans) Alkane hydroxylase	nonheme Fe	NADH	NADH rubredoxin reductase (flavoprotein), rubredoxin (nonheme Fe)

[a] The following abbreviations are used: Fe-S: iron-sulfur protein; NAD(P)H: reduced nicotinamide adenine dinucleotide; pMMO: particulate methane monooxygenase; AMO: ammonia monooxygenase. [b] A bar designates that the electron donors directly reduce the cofactor.

CHAPTER 2

Inhibition of the ammonia oxidation in *Nitrosomonas europaea* by sulfur compounds: Thioethers are oxidized to sulfoxides by ammonia monooxygenase

2.1. Abstract

Organic sulfur compounds are well known nitrification inhibitors. The inhibitory effects of dimethylsulfide, dimethyldisulfide, and ethanethiol on ammonia oxidation by *Nitrosomonas europaea* were examined. Both dimethylsulfide and dimethyldisulfide were weak inhibitors of ammonia oxidation and exhibited inhibitor characteristics typical of substrates for ammonia monooxygenase (AMO). Depletion of dimethylsulfide and dimethyldisulfide was observed and was prevented by acetylene, a specific inactivator of AMO. The inhibition of ammonia oxidation by the thioethers was examined in detail. Depletion of dimethylsulfide required O_2 and was prevented by specific inhibitors of AMO, allylthiourea or acetylene. Cell suspensions incubated in the presence of ammonia and dimethylsulfide led to the formation of a product identified as dimethylsulfoxide. Depletion of six other thioethers was also prevented by specific inhibitors for AMO. The oxidative product of three of the thioethers was accounted for predominantly by the formation of the corresponding sulfoxides. Using GC-MS, allylmethylsulfide was shown to be oxidized to allylmethylsulfoxide by *N. europaea* with the incorporation of a single atom of ^{18}O derived from $^{18}O_2$. This result supports the involvement of a monooxygenase in the oxidation of allylmethylsulfide. The thioethers are shown to be a new class of substrates for AMO. This is the first report of the oxidation of the sulfur atom by AMO in whole cells of *N. europaea*. The ability of *N. europaea* to oxidize dimethylsulfide is not unique among the ammonia oxidizing bacteria. *Nitrosococcus oceanus*, a marine nitrifier, was also demonstrated to oxidize dimethylsulfide to dimethylsulfoxide.

2.2. Introduction

Nitrification is the bacterially mediated process in which ammonia is oxidized sequentially to nitrite and then to nitrate. The reactions are catalyzed by specialized ammonia-oxidizing bacteria, such as *Nitrosomonas europaea*, and nitrite-oxidizing bacteria, such as *Nitrobacter winogradyski*. Nitrifying bacteria are ubiquitous components of the soil microbial population and their activities are stimulated in agricultural lands following the application of ammonia or urea-based fertilizers. Nitrification can lead to loss of nitrogen fertilizers by leaching of nitrate to ground and surface waters. Additionally, microbial denitrification of the nitrate can result in the production of N-oxides. Thus environmental and economic considerations have maintained the long term interest in nitrification inhibitors.

Nitrification in the soil is inhibited by several sulfur compounds. For example, the sulfur-containing amino acids methionine and cysteine inhibit nitrification (Jensen and Sorensen, 1952; Brown, Quastel et al., 1954; Keeney, 1986). However, because methionine did not inhibit ammonia oxidation by a pure culture of *N. europaea* the inhibitory effects of methionine were suggested to result from the microbial breakdown product(s) of methionine (Lees, 1952). Likewise, the inhibitory effects of cysteine on nitrification were also proposed to be due to degradation product(s) of cysteine (Jensen and Sorensen, 1952; Banwart and Bremner, 1975). Some of the known sulfur-containing degradation product(s) suggested to be inhibitors of nitrification include carbon disulfide, dimethylsulfide, dimethyldisulfide and alkyl thiols and subsequently these compounds were shown to be inhibitors of nitrification (Bremner and Bundy, 1974; Banwart and Bremner, 1975). While the inhibition of ammonia oxidation in pure cultures of *N. europaea* by carbon disulfide was characterized (Hyman, Kim et al., 1990), the inhibition of nitrification by dimethylsulfide, dimethyldisulfide or alkyl thiols has not been characterized in pure cultures. The purpose of this study was to examine the mechanism by which dimethylsulfide and the class of sulfur compounds known as the thioethers inhibited ammonia oxidation in pure cultures of *N. europaea*.

Ammonia oxidation in *N. europaea* and other ammonia-oxidizing bacteria is initiated by the enzyme ammonia monooxygenase (AMO) (Eqn. 1).

$$NH_3 + O_2 + 2e^- + 2H^+ \longrightarrow NH_2OH + H_2O \quad (1)$$

$$NH_2OH + H_2O \longrightarrow NO_2^- + 5H^+ + 4e^- \quad (2)$$

The hydroxylamine generated by AMO is then oxidized to nitrite by the enzyme hydroxylamine oxidoreductase (HAO) (Eqn. 2). Electrons derived from the oxidation of hydroxylamine provide a source of reductant for AMO during steady-state ammonia oxidation (Wood, 1986). The oxidation of ammonia by these bacteria therefore is susceptible to inhibition either through direct effects on AMO or indirect effects on HAO or other components involved in supplying electrons to AMO. Inhibitors of nitrification which act on AMO typically function by one of the following mechanisms 1) reversible inhibitors, such as allylthiourea (ATU) and carbon disulfide, prevent ammonia oxidation by interfering with catalysis by AMO (Hofman and Lees, 1953; Hyman, Kim et al., 1990) 2) alternate substrates for AMO, such as methane, ethylene and halogenated hydrocarbons, inhibit ammonia oxidation by competing for the flux of activated O_2 (Hyman and Wood, 1983; Hyman and Wood, 1984b; Hyman, Murton et al., 1988; Rasche, Hicks et al., 1990) 3) mechanism-based inactivators of AMO, such as acetylene, irreversibly inactivate AMO (Hyman and Wood, 1985) and 4) exposure of cells to visible light also leads to inactivation of AMO (Shears and Wood, 1985). Given this diversity of inhibitory mechanisms for AMO, it is not surprising that AMO, rather than HAO, is most commonly identified as the target enzyme for inhibitors of nitrification (Bédard and Knowles, 1989).

In this chapter, the mechanisms by which several sulfur-containing compounds inhibit ammonia oxidation by *N. europaea* were characterized. Both the target enzyme and the mechanism of inhibition were identified for the class of organic sulfur compounds known as the thioethers. Several thioethers are shown to be substrates for AMO. The products of thioether oxidations are demonstrated to be the corresponding sulfoxides. This is the first report of the oxidation of the sulfur atom by AMO.

2.3. Materials and methods.

2.3.1. Materials.

Carbon disulfide and all the organic sulfides and sulfoxides, except for tetrahydrothiophene sulfoxide, were purchased from Aldrich Chemical Co., Milwaukee, Wi. Tetrahydrothiophene sulfoxide was purchased from American Tokyo Kasei, Portland, OR. All of these reagents were greater than 97% pure by manufacturer analysis. C_2H_2 was generated in a gas-generating bottle from calcium carbide (~80%, Aldrich Chemical Co. Inc., Milwaukee, Wi) as previously described (Hyman and Arp, 1987). $^{18}O_2$ (97.7%) was supplied by MSD Isotopes, Rahway, N J.

2.3.2. Growth and preparation of the cells.

Nitrosomonas europaea was grown in 2-liter shake flasks as described previously (Hyman and Arp, 1992). Cells (1.5 l) were harvested by centrifugation (20,000 x g, 15 min) after 3 days of growth. Cells were washed and the pellet resuspended (1 ml per 1 liter harvest) with assay buffer (50 mM NaH_2PO_4 and 2 mM $MgCl_2$, pH=7.8). Cell suspensions were stored on ice and used within 24 hours of harvesting. *Nitrosococcus oceanus* was grown in batch culture (1 l) for 14 days in media consisting of seawater buffered with 25 mM HEPES (pH = 8.0) as described by Ward (Ward, 1987). Cells of *N. oceanus* were harvested by centrifugation (20,000 x g, 25 min), washed and resuspended in seawater (1 ml per 1l harvest). Cultures of *N. europaea* and *N. oceanus* were periodically checked for contamination by streaking on nutrient agar plates.

2.3.3. Analytical procedures.

Sulfur compounds were analyzed with a Shimadzu gas chromatograph (model GC-8A) equipped with a flame ionization detector. The compounds were separated by using a Teflon-lined stainless steel column (0.3 cm by 34 cm)

containing Porapak Q (80-100 mesh) (Waters Associates, Inc., Framingham, MA) or containing 17% Carbowax 1500 on Chromosorb G (45-60 mesh) operating between 45°C and 170°C. Resolution of dimethylsulfoxide from dimethylsulfone was achieved with a longer (1 m) Carbowax column . Formaldehyde was determined with an enzymatic assay as described (Rasche, Hicks et al., 1990). The protein content of the cell suspensions were determined by using the biuret assay (Gornall, Bardawill et al., 1949) after solubilizing the cells in 3 M NaOH for 45 min at 75°C. Bovine serum albumin was used as the protein standard.

2.3.4. Inhibition of nitrite production by volatile sulfur compounds.

Stock solutions of the sulfur compounds were prepared daily by addition of the compounds to glass serum vials filled with assay buffer and sealed with Teflon-lined silicone septa (Alltech Associates, Inc., Deerfield, Ill.). Solutions of carbon disulfide were prepared in dimethylsulfoxide. Incubations were conducted in serum vials (9.4 ml) containing assay buffer (0.95 ml) and either ammonium sulfate (5 mM) or hydroxylamine hydrochloride (2 mM) and sealed with Teflon-lined silicone septa. After addition of the sulfur compound to the assay vial and equilibration for approximately 2 min at 30°C in a shaking water bath, the reactions were initiated by the addition of an aliquot of the cell suspension (0.05 ml, 0.7 mg protein) to the assay vial. The assay vials were allowed to incubate (5 or 10 min) in the shaking water bath. Liquid samples (5 µl or 10 µl) of the reaction mixtures were removed for determination of the nitrite content by colorimetric analysis as described (Hageman and Hucklesby, 1971) . The rate of nitrite production from either ammonium sulfate or hydroxylamine hydrochloride in the absence of any added sulfur compound remained constant during the time course of the assays.

To examine the reversibility of any inhibition by these sulfur compounds, cells were recovered from the reaction mixture by sedimentation (14,000 x g, 5 min). After three cycles of sedimenting and washing with assay buffer (3 x 1.5 ml), the cells were resuspended in assay buffer (0.5 ml). A portion (100 µl) of this washed cell suspension (0.14 mg protein) was then added to assay buffer (1.8 ml) in the chamber of an O_2 electrode. The O_2 uptake rates coupled to ammonia oxidation were measured in the presence of ammonium sulfate (2.7 mM).

Allylthiourea (100 μM) was added to the electrode chamber to inhibit further ammonia-dependent O_2 uptake, and then hydrazine (750 μM) was added to the electrode chamber and the O_2 uptake rate was recorded. The ammonia and hydrazine-dependent O_2 uptake rates of the cells exposed to the sulfur compounds were compared to the rates obtained with cells which were not exposed to the sulfur compounds but were otherwise treated in the same manner.

2.3.5. Thioether depletion assays.

Stock solutions of the thioethers were made fresh daily as described above except buffered seawater (25 mM HEPES, pH =7.8) was used for assays involving *N. oceanus*. Incubations were conducted as described above in assay buffer (0.9 ml) with the following changes; the concentration of ammonium sulfate was 2.5 mM, butyl rubber septa were used for the assay vials containing dimethylsulfide and buffered seawater replaced the assay buffer for incubations with *N. oceanus*. After addition of the thioether to the incubation vial and equilibration for approximately 2 min at 30°C in a shaking water bath, the reactions were initiated by addition of the cell suspension (0.1 ml, 0.5 mg to 2 mg protein) to the assay vials. To determine the amount of thioether depleted from the reaction vials, a sample of the gas phase (25 μl to 50 μl) was removed prior to the addition of cells and at designated times during the assay. At 30 min a liquid phase aliquot (50 μL) was withdrawn from the reaction vials and frozen for product analysis at a later time. Control vials for depletion analysis included incubations containing cell suspensions treated either with C_2H_2 (5 to 20 μmol) or allylthiourea (100 μM) to inhibit AMO and boiled cells to assess abiotic loss.

2.3.6. Incorporation of $^{18}O_2$ into allylmethylsulfide.

Cell suspensions and the stoppered reaction vials (9.4 ml) containing assay buffer (1.2 ml) and ammonium sulfate (2.5 mM) were repeatedly evacuated and flushed with N_2 to remove O_2. A stock solution of allylmethylsulfide was made O_2-free and an aliquot (1.5 μmol, 0.4 ml) was added

to each of the N_2-filled vials. Two milliliters of either $^{16}O_2$ or $^{18}O_2$ (97.7%) was then added to the reaction vials as an overpressure. Reactions were initiated by the addition of a cell suspension (0.2 ml, 4 mg protein) to the vials. The control vial contained 2 ml of $^{18}O_2$ and cell suspensions in which AMO was inactivated by treatment with C_2H_2. The percent of mass 36 ($^{18}O_2$) relative to mass 32 ($^{16}O_2$) in the headspace of the vials was determined before the addition of the cell suspension and at the end of the incubation using a Dycor quadrupole residual gas analyzer (Ametek, Pittsburgh, PA) fitted with a fused silica capillary tubing (50 μM i.d.) as the inlet. After a 75 minute incubation in a shaking water bath, the liquid phases of the reaction vials were transferred to test tubes containing sufficient solid NaCl (~0.1 g) to saturate the aqueous phase. Two extractions with ~1 ml of CH_2Cl_2 were made to remove the organic oxidation product(s). The extracted samples were then dried with excess Na_2SO_4 in preparation for analysis by GC-MS. The CH_2Cl_2 extractions were separated on a fused silica capillary column (30 m, 0.25 mm i.d.) coated with SE-54 (film thickness 0.25 μm). Initial column temperature was 40°C for 5 min, and then increased to 275°C at a rate of 10°C per minute. The GC injector and detector temperatures were 200°C. Mass spectral analysis of the peaks separated using this column were obtained with a Finnigan-4023 GC-MS functioning in the electron impact mode at 70 eV. Allylmethylsulfoxide was prepared by oxidation of the allylmethylsulfide with hydrogen peroxide (Oae, 1991). Pure allylmethylsulfide (~0.3 mmol) at room temperature was oxidized by the dropwise addition of cold 30% H_2O_2 (~2 mmol) during vortexing (~3 min). This mixture was then diluted ~10^4 fold with water. A portion of this mixture was then extracted into CH_2Cl_2 as described for the cellular extractions and analyzed by GC-MS.

2.4. Results.

2.4.1. Inhibition of ammonia oxidation by *N. europaea* by volatile sulfur compounds.

The inhibition of nitrite production from either ammonia or hydroxylamine hydrochloride was tested for each of four sulfur compounds (Table 2.1). All four compounds were inhibitors of ammonia oxidation by *N. europaea*; dimethylsulfide

and dimethyldisulfide were only weak inhibitors while carbon disulfide and ethanethiol were strong inhibitors of the oxidation of ammonia to nitrite. Dimethylsulfoxide (73 µmol), which was used to deliver carbon disulfide, did not inhibit the oxidation of ammonia or hydroxylamine to nitrite by *N. europaea* (not shown). Carbon disulfide inhibited only the oxidation of ammonia to nitrite and not the oxidation of hydroxylamine oxidation. In contrast to the other sulfur compounds, only ethanethiol inhibited hydroxylamine oxidation to nitrite, although its effect on ammonia oxidation was greater than its effect on hydroxylamine oxidation.

Further examination of the mechanism by which each compound inhibited ammonia oxidation was obtained by examining (1) the extent of consumption of the sulfur compound during incubation with cells and (2) the reversibility of the inhibition of the activities of AMO and HAO. During the incubations, cell suspensions of *N. europaea* were found to consume 30% (dimethylsulfide), 69% (dimethyldisulfide) and 63% (ethanethiol) of the 0.5 µmoles of each sulfur compound initially present in the incubation vial. Because the consumption of dimethylsulfide and dimethyldisulfide was prevented by treating cell suspensions with acetylene (C_2H_2), a specific inactivator of AMO, the depletion of these two sulfur compounds was likely due entirely to oxidation by AMO. In contrast, depletion of ethanethiol exhibited a 20% greater loss when AMO was inactivated as compared to depletion by boiled cell suspensions. These results suggested that another mechanism of depletion for ethanethiol, other than and in addition to AMO, may be present in the cells. Carbon disulfide is not believed to act as substrate for AMO (Hyman, Kim et al., 1990), and therefore, depletion of carbon disulfide by *N. europaea* was not determined.

Table 2.1. Inhibition of ammonia oxidation in *N. europaea* by volatile sulfur compounds

Sulfur compound[a]	Amount added (μmol)	% Nitrite produced relative to uninhibited cells from[b]		% Reversibility relative to uninhibited cells for[c]		% Depletion relative to amount added[d]
		$(NH_4)_2SO_4$ (5 mM)	NH_2OH (2 mM)	AMO	HAO	
CS_2	0.1	23	95	16	105	ND[e]
DMS	0.5	86	101	103	93	30
DMDS	0.5	76	107	99	102	69
C_2H_5SH	0.5	0	64	19	102	63

[a]Abbreviations: CS_2, carbon disulfide; DMS, dimethylsulfide; DMDS, dimethyldisulfide; C_2H_5SH, ethanethiol. [b]The percentages are expressed relative to the amount of nitrite produced by uninhibited cell suspensions incubated in the presence of either ammonium sulfate or hydroxylamine hydrochloride during a fixed time assay. The relative error among the percentages range ±1-10% for the triplicate samples. [c]The cell suspensions were sedimented and resuspended three times with buffer to remove the compound. The activities of AMO and HAO were measured as the rates of ammonia- or hydrazine-dependent O_2 uptake. The activities are expressed as a percentage relative to a rate of 32.9 μmol of O_2 consumed hr^{-1} mg of protein $^{-1}$ for the activity of AMO or 8.2 μmol of O_2 consumed hr^{-1} mg of $protein^{-1}$ for the activity of HAO of the uninhibited cell suspensions. [d]The net percentage of the sulfur compound depleted by the cell suspensions during the incubation with respect to the amount of compound initially added. Background loss of the thioether was accounted for by incubation of the compounds with boiled cell suspensions. Errors ranged ±1-4% for depletion assays perfomed in triplicate. [e]ND, not determined.

Reversibility of the inhibition was determined by removing the inhibitor from the cell suspensions and measuring the residual activities of AMO and HAO, as ammonia- and hydrazine-dependent O_2 uptake, respectively. The activities of AMO and HAO following incubation with either dimethylsulfide or dimethyldisulfide were essentially identical with the activity of the uninhibited cell suspensions. In contrast, after removing carbon disulfide or ethanethiol the ammonia-dependent O_2 uptake rates of the cell suspensions were respectively 16% and 19% of the rate of cell suspensions incubated in the absence of any inhibitor. However, the activity of HAO was unaffected after removing carbon disulfide or ethanethiol, suggesting an element of specificity to the inhibition of ammonia oxidation by these compounds. Our activities for AMO and HAO following treatment with carbon disulfide corresponded well with previously published results (Hyman, Kim et al., 1990).

Our characterization of four volatile sulfur compounds with regard to ammonia oxidation revealed the following: 1) Carbon disulfide was a specific and potent inhibitor of AMO as was previously demonstrated (Hyman, Kim et al., 1990). 2) Ethanethiol was a potent inhibitor of AMO and depletion of ethanethiol suggested that it may be a substrate of AMO. However, the inhibitor properties of ethanethiol (partial reversibility to the AMO activity, potency of inhibition on nitrite production from hydroxylamine hydrochloride and depletion of ethanethiol in the absence of AMO activity) suggest a more complex interaction of this compound with *N. europaea* than can be explained simply by oxidation of the ethanethiol by AMO. These complex inhibitory effects of ethanethiol on *N. europaea* were not further investigated; however, the general toxic properties of alkyl thiols on biological systems are noted (Ziegler, 1984). 3) Dimethylsulfide and dimethyldisulfide were depleted by cells of *N. europaea*, which suggests that these sulfur compounds may be substrates of AMO. AMO is known to oxidize several hydrocarbons and might be expected to oxidized the methyl groups in these compounds. However, the sulfur atom had not been demonstrated to be a site of oxidation by AMO, although not an uncommon site for oxidation by other monooxygenases (May, Phillips et al., 1981; May and Katopodis, 1986; Ziegler, 1989).

2.4.2. Requirements for dimethylsulfide depletion and dimethylsulfoxide formation by *N. europaea*.

Two approaches were used to establish a role for AMO in dimethylsulfide consumption. First, the requirements for depletion of dimethylsulfide and those for AMO catalysis were compared. As shown in Eqn. 1, both a source of O_2 and reductant are required for AMO activity. Cell suspensions incubated anaerobically in the presence of ammonia did not consume dimethylsulfide. Upon reintroduction of O_2, the cell suspensions resumed consumption of dimethylsulfide at about the same rate as did cell suspensions in the presence of O_2 (not shown). These results indicated that O_2 was required for dimethylsulfide depletion. Cell suspensions more rapidly consumed dimethylsulfide in the presence of ammonia than in the absence of ammonia (Figure 2.1A). Presumably more reductant is available for rapid oxidation of dimethysulfide by AMO when ammonia is present because *in vivo* the reductant for AMO-catalyzed reactions is provided by hydroxylamine oxidation. Cell suspensions incubated with dimethylsulfide in the absence of a reductant source slowly consumed dimethylsulfide. A supply of endogenous reductant for AMO catalysis has been proposed for *N. europaea* (Wood, 1986). In a second approach the effect on depletion of dimethylsulfide by two specific inhibitors of AMO, ATU and C_2H_2, was examined (Figure 2.1A). Both inhibitors of AMO prevented the consumption of dimethylsulfide (results for ATU not presented). The results from both approaches were consistent with a role for AMO in dimethylsulfide consumption.

In previous studies of alternate substrate oxidations by AMO, the products of the oxidations were not metabolized by the cell suspensions and accumulated in the reaction media (Hyman and Wood, 1983; Hyman and Wood, 1984b; Rasche, Hicks et al., 1990). Based on the results with microsomal monooxygenase activities, dimethylsulfoxide seemed a likely product of dimethylsulfide oxidation by AMO (Ziegler, 1989). Analysis of reaction mixtures by GC revealed a product peak that corresponded with the elution time of dimethylsulfoxide at both 110°C and 130°C. The time course of dimethylsulfoxide production by *N. europaea* is presented in Figure 2.1B. The time course of dimethylsulfoxide production coincided with the time course for disappearance of dimethylsulfide. Dimethylsulfoxide accounted for an average of 69±11% of the dimethylsulfide oxidized.

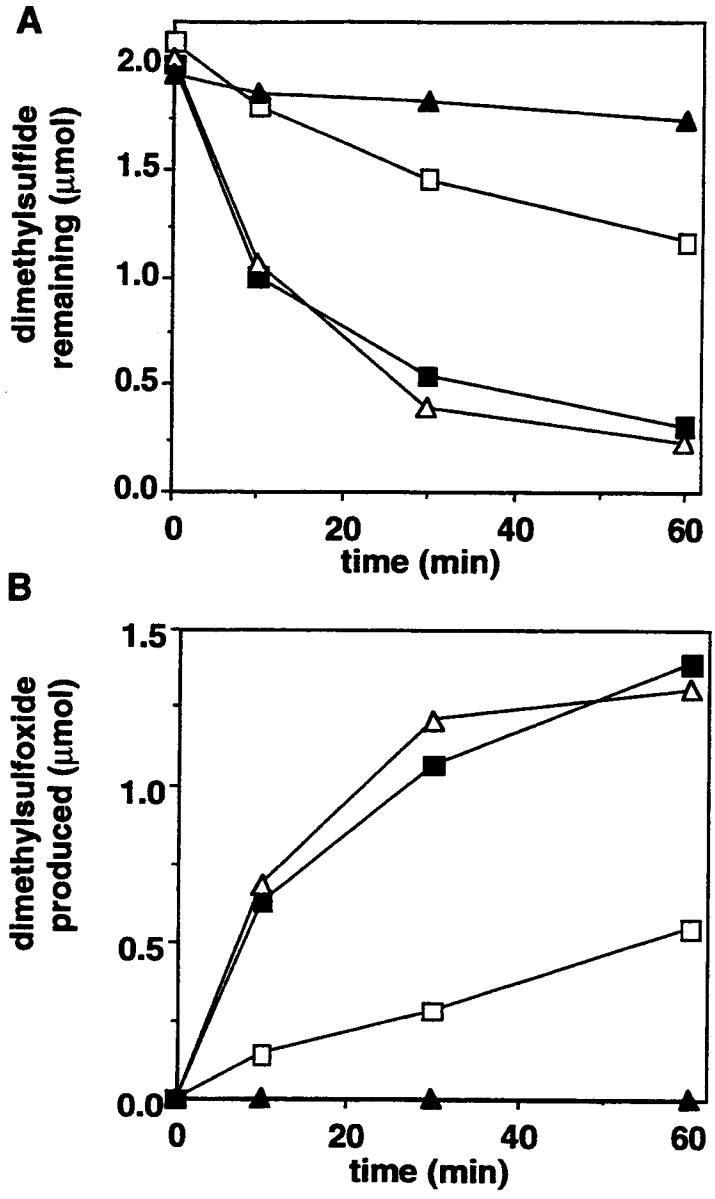


Figure 2.1. Time course of dimethylsulfide oxidation and dimethylsulfoxide formation by cells of *N. europaea.* Cell suspensions (0.1 ml, 1.4 mg protein) were incubated with dimethylsulfide (2.0 µmol) as described in Material and Methods. The µmoles of DMS remaining (**A**) or µmoles DMSO formed (**B**) *versus* time are shown for the incubations containing; (▲), C_2H_2-treated cells and 5.0 mM $(NH_4)_2SO_4$ or active cells incubated with (□), 0 mM, (■), 2.5 mM and (Δ), 5.0 mM $(NH_4)_2SO_4$. Data points represent the average of triplicate measurements. The relative error for the average data varied from 1% to 12%.

Dimethylsulfone, a more oxidized derivative of dimethylsulfide, was not detected as a product of cellular incubation with either dimethylsulfide (2 μmol) or dimethylsulfoxide (2 μmol). If the amount of dimethylsulfide depleted which is unaccounted for as dimethylsulfoxide had all been converted by further oxidation of dimethylsulfoxide, enough dimethylsulfone would have been present for detection by GC. Although formaldehyde has been observed as a biological oxidative product of dimethylsulfide (De Bont, Van Dijken et al., 1981; Suylen, Stefess et al., 1986), it was not detected as a product of dimethylsulfide oxidation by *N. europaea* .

2.4.3. Oxidative conversion of various thioethers to sulfoxides by *N. europaea.*

It was of interest to determine if other thioethers were substrates for AMO. Therefore, depletion of various thioethers by cell suspensions of *N. europaea* was examined (Table 2.2). Prior to depletion analysis, the toxicity of each thioether on the activities of AMO and HAO was determined by comparing the ammonia-and hydrazine-dependent O_2 uptake rates of cell suspensions pretreated with each thioether (1 μmol, 60 min) to the corresponding rates for cell suspensions pretreated identically except in the absence of any thioether. None of the seven thioethers we examined exhibited any irreversible effect on the activity of either AMO or HAO, under our assay conditions, except allylsulfide. Allylsulfide irreversibly inactivated the AMO activity of the cell suspensions but did not affect the HAO activity of the cell suspensions. This result indicated that allylsulfide had inhibitor properties unlike the other thioethers examined. For five of the seven thioethers examined, 66-88 % of the compound was depleted in the presence of ammonia by cells of *N. europaea* during a 30 min incubation (Table 2.2). Methylphenylsulfide and allylsulfide were depleted to a lesser extent (38% for methylphenyl sulfide and 24% for allylsulfide). Depletion of all the thioethers were substantially limited during incubations containing either boiled cells or cells treated with the AMO specific inhibitors, ATU or C_2H_2. For most thioethers, abiotic losses ranged between 5% and 22% (in 30 min) of the initial amount added. However, for methylphenylsulfide abiotic loss was higher (47% of 1 μmol in 30 min), which may account for the decreased depletion of this compound. The large standard deviations for methylphenylsulfide depletion (Table 2.2) were

primarily a result of large variation in the background losses associated with this compound.

In addition to the product of dimethylsulfide oxidation, the products generated from incubation of the cell suspensions with either tetrahydrothiophene or methylphenylsulfide were also identified and quantified by GC analysis. The oxidative product, generated from the incubation of *N. europaea* with either methylphenylsulfide or tetrahydrothiophene, had identical retention times at two GC temperatures with authentic samples of the corresponding sulfoxides. The amount of sulfoxide formed by the cells in 30 min for each of these thioethers was comparable to the amount of thioether depleted (Table 2.2). This indicated that most of the thioether oxidation was accounted for by oxidation of the sulfur atom. This result was surprising because AMO has also been demonstrated to catalyze the oxidation of a variety of carbon functional groups, including the oxidation of alkanes to alcohols, alkene functions to epoxides, and the hydroxylation of benzene (Hyman and Wood, 1984b; Hyman, Sansome-Smith et al., 1985; Hyman, Murton et al., 1988). However, our results indicated that the sulfoxides constituted the majority of the oxidized thioether product. Therefore, the sulfur atom was the primary site of oxidation by AMO for the three thioethers whose sulfoxides were quantitated. While the amount of dimethylsulfoxide detected accounted for only 69% of dimethylsulfide oxidized, the amount of the sulfoxide formed by cell suspensions during incubation with either methylphenylsulfide or tetrahydrothiophene was on the average somewhat higher than the amount of the thioether depleted. The amount of methylphenylsulfoxide formed by the cell suspensions averaged 112% of the net methylphenylsulfide depleted and the amount of tetrahydrothiophene sulfoxide formed averaged 113% of the net tetrahydrothiophene depleted. The amount of sulfoxide formed being greater than 100% of the calculated net amount of thioether depleted may have resulted from an overestimation of the amount of background loss of thioether from the sample vials relative the control vials.

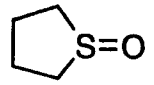
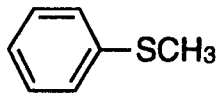
While the products of the diethylsulfide, allymethylsulfide or allylsulfide oxidations were not identified or quantified, products were detected as single GC peaks. These products were not detected in the samples containing either C_2H_2- or ATU-treated cell suspensions. During incubation of *N. europaea* with either diethylsulfide or allylmethylsulfide, the time course of product formation coincided with depletion of the parent thioether. The products of the incubations with either diethylsulfide or allylmethylsulfide were of sufficient quantity such that

it could have accounted for the amount of thioether depleted. As for allylsulfide only a small amount of product was detected (~5% of the peak area for tetrahydrothiophene sulfoxide). Treatment of diethylsulfide, allylmethylsulfide or allylsulfide with hydrogen peroxide produced compounds with retention times similar to the products generated by the cell suspensions from each of these thioethers. In the case of thiophene, no product was detected at a variety of operating temperatures using either of the two GC columns described in Materials and methods. Thiophene sulfoxide, a predicted product, is known to be unstable (Bailey and Cummins, 1954). Although hydroxylated products of the thiophene ring were also possible products, we did not attempt to identify any of these potential product(s).

Table 2.2.

[a]The amount of thioether depleted and product formed was determined as described in Materials and methods. Data represent averages of triplicates ± standard deviation. [b]The amount of thioether loss from vials containing either C_2H_2-treated or boiled cells was subtracted from the amount of thioether depleted with active cells. [c]Product peaks similar in size and retention times to the other sulfoxides were observed, but were not identified or quantified because authentic sulfoxides were unavailable.[d] Product peak was not observed for our GC conditions. [e]Identified by GC-MS, but not quantified. [f]A small peak (approximately 5% of the peak area of the other sulfoxides) was observed.

Table 2.2. Thioether oxidation by whole cells of *Nitrosomonas europaea*[a]

Compound	Structure	Amount (nmol)	Sulfide depleted[b] in 30 min (nmol)		Sulfoxide produced in 30 min (nmol)		Product detected
			No NH_4^+	5 mM NH_4^+	No NH_4^+	5 mM NH_4^+	
Methylsulfide	CH_3SCH_3	2000	502±102	1331±117	293±33	1063±124	$(CH_3)_2S{=}O$
Ethylsulfide	$CH_3CH_2SCH_2CH_3$	1000	200±96	757±59	——— *c*	——— *c*	——— *c*
Tetrahydro thiophene		1000	211±61	832±125	270±23	826±42	
Thiophene		1000	310±35	882±44	——— *d*	——— *d*	——— *d*
Methylphenyl-sulfide		1000	171±139	378±204	196±4	416±9	$Ph{-}S(O)CH_3$
Allylmethyl-sulfide	$H_2C{=}CHCH_2SCH_3$	1000	290±21	801±39	——— *c,e*	——— *c,e*	$AllylS(O)CH_3$ *c,e*
Allylsulfide	$(H_2C{=}CHCH_2)_2S$	1000	55±88	245±93	——— *f*	——— *f*	——— *f*

2.4.4. GC-MS identification of the product of allylmethylsulfide oxidation by *N. europaea.*

The oxidative products of one of the thioethers, allylmethylsulfide, was subjected to analysis using GC-MS for verification of the identity of the product formed by *N. europaea.* The product of allylmethylsulfide oxidation was chosen for this analysis because it was one for which an authentic sample of the putative product, allylmethylsulfoxide, was unavailable. However, GC analysis coupled with mass spectral analysis could allow an assignment of the oxidative product of allylmethylsulfide. The capillary GC separation of the oxidative product of allylmethylsulfide, generated by *N. europaea*, is shown in Figure 2.2A. This product, GC peak I, had a retention time of 11.1 min and was determined to represent allylmethylsulfoxide (MW=104) by interpretation of its mass spectral fragmentation pattern. The mass spectrum for allylmethylsulfoxide is shown in Figure 2.3A. The mass spectrum shows a low intensity molecular ion at m/z = 104 and a base peak at m/z = 41 which represents the stable allyl radical cation. The fragments formed at m/z = 63 and m/z = 64 are made by loss of the allyl moiety generating $CH_3S{=}^{16}O^+$ and $CH_3S^{16}OH^+$. These two mass fragments are typical of sulfoxides (Budzikiewicz, Djerassi et al., 1967). Acetylene-inactivated cells incubated with allylmethylsulfide produced no peaks at the retention time assigned to allylmethylsulfoxide (Figure 2.2C) indicating that AMO activity was required for formation of allylmethylsulfoxide.

Because neither allylmethylsulfoxide nor allylmethylsulfone are commercially available and because published mass spectral fragmentation patterns were not obtained for comparison with the mass spectrum of our product, an alternative to an authentic sample of each of these compounds was generated by oxidation of allylmethylsulfide with hydrogen peroxide. The capillary GC separation of the oxidative products of allylmethylsulfide, generated using hydrogen peroxide, is shown in Figure 2.2B. The peaks were identified to be allylmethylsulfoxide (peak I) and allylmethylsulfone (peak II) by analysis of their mass spectral fragmentation pattern. The retention time (11.2 min) and the mass spectrum for allylmethylsulfoxide, as obtained by chemical oxidation with hydrogen peroxide, had a similar retention time and an identical fragmentation pattern to the mass spectrum of the product generated by the cell suspensions.

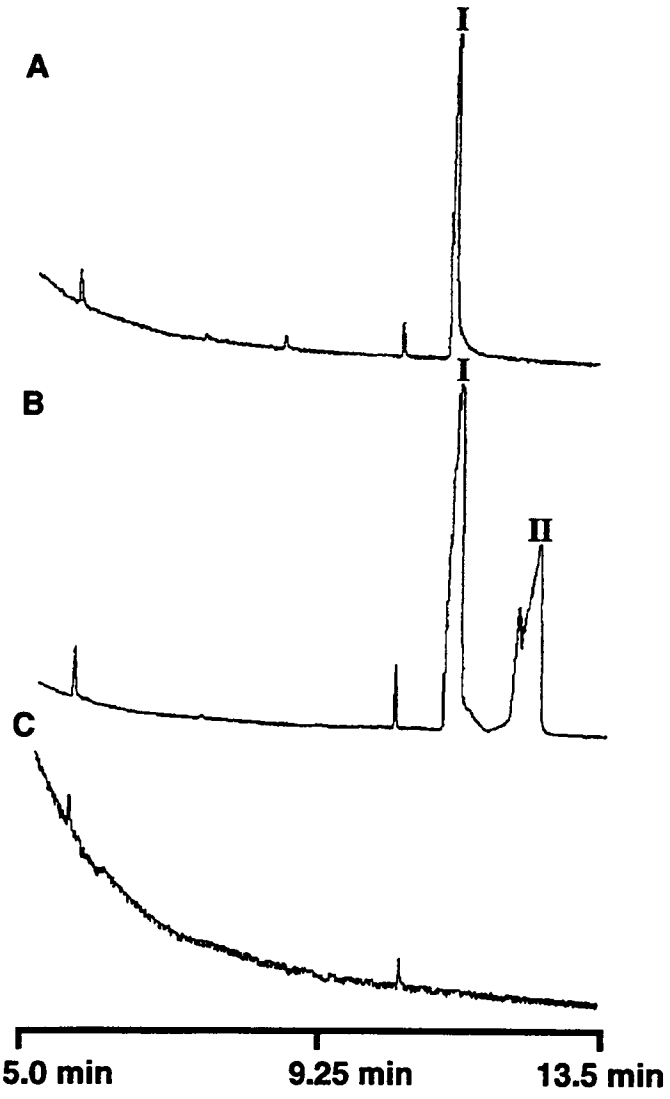


Figure 2.2. Capillary GC separation and identification of allylmethylsulfoxide and allylmethylsulfone. The capillary gas chromatograms of the CH_2Cl_2 extracted samples are shown for: (**A**) cells incubated in the presence of allylmethylsulfide, (**B**) hydrogen peroxide (H_2O_2) oxidized allylmethylsulfide and (**C**) cells which had been inactivated with C_2H_2 prior to incubation with allylmethylsulfide. The GC peaks were identified by mass spectral analyses to be allylmethylsulfoxide (**I**) (see Figure 2.3) and allylmethylsulfone (**II**) (major ions at m/z = 39, 41 and a low intensity molecular ion at m/z = 120; data not presented). Acetylene-inactivated cells incubated with allylmethylsulfide did not form allylmethylsulfoxide as shown in the chromatogram C (at increased detector sensitivity).

Because the oxidation of alkenes by peracids is a slower process than oxidation of sulfenyl sulfur, the major product should be the sulfoxide, as was suggested by

our GC-MS results, rather than an epoxide (Oae, 1991). The presence of the allylmethylsulfone as a product of hydrogen peroxide oxidation was not unexpected because of the excess oxidizing equivalents used in our protocol (Oae, 1991). However, allylmethylsulfone was not produced by cells of *N. europaea* incubated with allylmethylsulfide (compare Figures 2.2A and 2.2B).

2.4.5. The incorporation of $^{18}O_2$ into allylmethylsulfide by *N. europaea* formed ^{18}O-labeled allylmethylsulfoxide.

While the use of GC-MS provided support for results obtained by conventional GC techniques, the use of GS-MS also enabled us to demonstrate that the source of the oxygen atom incorporated into allylmethylsulfide. When cells were incubated with allylmethylsulfide and $^{18}O_2$ (97%), 82 % of the sulfoxide product was labeled with ^{18}O (based on the relative intensities of the molecular ions at m/z = 104 and 106). A comparison of the mass spectrum of $^{18}O_2$-labeled allylmethylsulfoxide with that of $^{16}O_2$-labeled allylmethylsulfoxide is presented in Figure 2.3. The molecular ion for allylmethylsulfoxide is shifted from m/z = 104 to m/z = 106 as expected for the incorporation of a single atom of oxygen derived from $^{18}O_2$ into allylmethylsulfide. The fragments at m/z = 63 and 64 in the spectrum of ^{16}O-labeled allylmethylsulfoxide were also shifted to m/z = 65 and 66 confirming the presence of an ^{18}O atom in these fragment assignments. The fragments at m/z = 65 and 66 in the spectrum of ^{18}O-labeled allylmethylsulfoxide were interpreted to represent $CH_3S{=}^{18}O^+$ and $CH_3S^{18}OH^+$. The base peaks at m/z = 41 and the second most intense at m/z = 39 are unshifted for each of the respective O-labeled sulfoxides. These results established that the requirement of oxygen for sulfoxide formation was satisfied by O_2 as expected of a reaction catalyzed by a monooxygenase.

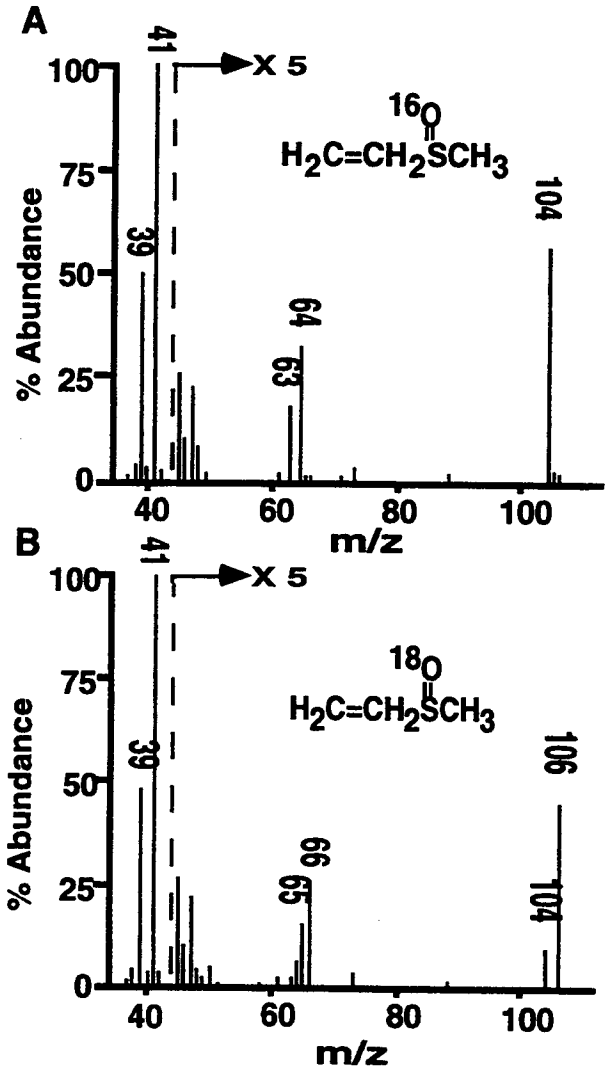


Figure 2.3. Incorporation of an atom of ^{18}O from $^{18}O_2$ into allylmethylsulfide by cells of *N. europaea*. Mass spectra are shown for allylmethylsulfoxide formed during incubations which were conducted in the presence of 20% O_2 added as an overpressure of either 100% $^{16}O_2$ (**A**) or 97.7% $^{18}O_2$ (**B**). The mass spectral fragmentation pattern presented in Figure 2.3 A corresponds to the GC peak designated (**I**) of Figure 2.2.A. ^{16}O-labeled allylmethylsulfoxide had major ions at m/z = 39, 41, 45, 47, 63, 64 and a molecular ion at m/z = 104 (Figure 2.3A). A GC peak, similar in magnitude and eluting at the same retention time as peak (**I**), was observed when the incubation was conducted in the presence with $^{18}O_2$ (chromatogram not presented). The spectral fragmentation pattern of ^{18}O-labeled allylmethylsulfoxide, had major ions at m/z = 39, 41, 45, 47, 65, 66 and a molecular ion at m/z = 106 (Figure 2.3B). The intensities of the fragments have been enhanced (5 fold) for mass fragments greater than mass 44.

Although the incorporation of $^{18}O_2$ into allylmethylsulfide was less than the theoretical value of 97% it was consistent with the isotope ratio, as determined by the gas analyzer, averaged over the time course of the experiment. The headspace gas composition of the vials were analyzed before and after the incubation using a residual gas analyzer. The amount of $^{18}O_2$ and $^{16}O_2$ was recorded as the partial pressures of mass 36 and mass 32 respectively. The fraction of $^{18}O_2$ in the headspace of the reaction vial changed from 96% $^{18}O_2$ at the start of the experiment to 77% at the end (% expressed relative to the amount of $^{18}O_2$ + $^{16}O_2$ present). The headspace compositions as measured by the residual gas analyzer were consistent with the appearance of ^{16}O-labeled allylmethylsulfoxide (molecular ion at m/z = 104) with a relative intensity of 17% in the mass spectrum of ^{18}O-labeled allylmethylsulfoxide (Figure 2.3B). The mass spectrum for ^{16}O-labeled allylmethylsulfoxide showed the presence of 5% sulfoxide component with a molecular ion at m/z= 106 and that for the $^{18}O_2$-labeled allylmethylsulfoxide has a 5% component at m/z=108 due to the 4.4% isotopic abundance of ^{34}S.

2.4.6. Oxidation of dimethylsulfide to dimethylsulfoxide by the marine nitrifier, *Nitrosoccocus oceanus*.

Because of the importance of dimethylsulfide in the oceanic sulfur cycle (Wakeham and Dacey, 1989), depletion of dimethylsulfide by an oceanic nitrifier was investigated. The time course of the oxidation of dimethylsulfide by *N. oceanus* is presented in Figure 2.4. The product was determined to be dimethylsulfoxide using GC analysis. Dimethylsulfide depletion and dimethylsulfoxide formation were prevented by treating of *N. oceanus* with C_2H_2. Treatment of the cells with ATU (100 µM) did not significantly limit the depletion of dimethylsulfide by *N. oceanus* as it did for *N. europaea*. However, differences in susceptibility to nitrification inhibitors has been noted between *N. europaea* and *N. oceanus* with acetylene strongly inhibited AMO catalyzed oxidations in both nitrifiers (Jones and Morita, 1984). The depletion of dimethylsulfide by *N. oceanus* occurred more slowly than depletion by *N. europaea*. Cell suspensions of *N. oceanus* (0.7 mg protein) consumed 1 µmol of dimethylsulfide in 20 hour, while cells of *N. europaea* (1.4 mg protein) consumed 1.3 µmol in 0.5 hour. The ammonia-oxidizing activities of the cell suspensions,

as determined by the rates of ammonia-dependent O_2 uptake, were similar (~30 µmol O_2 consumed per hour per mg of protein for *N. oceanus* and ~40 µmol O_2 consumed per hour per mg of protein for *N. europaea*). Another feature of dimethylsulfide depletion by *N. oceanus* which was distinct from the time course for depletion of dimethylsulfide by *N. europaea* was the high rate of dimethylsulfide consumption in the absence of ammonia relative to the rate in the presence of ammonia. The rate of dimethylsulfide consumption by *N. oceanus* was only slightly slower in the absence of ammonia than in the presence of ammonia (Figure 2.4). On the other hand, incubation of *N. europaea* for 20 hours with dimethylsulfide (1 µmol) in the absence of ammonia did not result in complete depletion of dimethylsulfide as it did for *N. oceanus*. Both of these results suggest that high levels of endogenous reductant may be present in *N. oceanus*. It is not unusual for bacteria, such as *N. oceanus*, which are presumably adapted to the oligotrophic conditions of seawater, to have high levels of endogenous reductant (Coley-Smith and Parfitt, 1986).

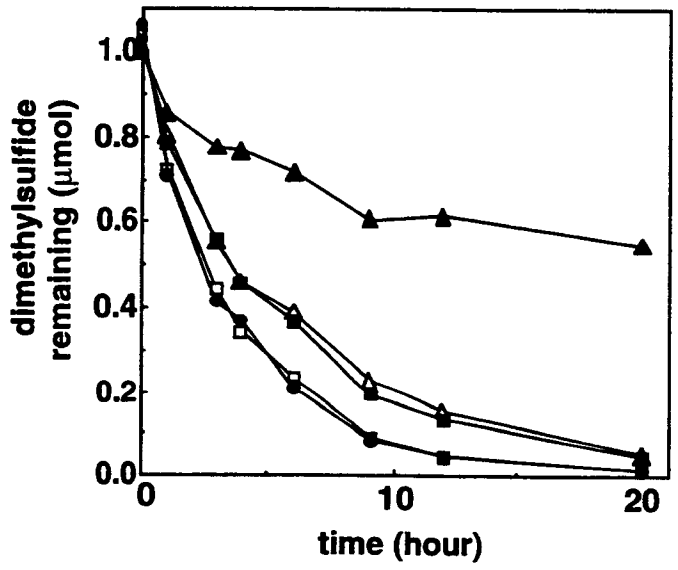


Figure 2.4. Depletion of dimethylsulfide by an oceanic ammonia-oxidizing nitrifier, *N. oceanus*. Cell suspensions of *N. oceanus* (0.05 ml, 0.7 mg protein) were incubated with dimethylsulfide (1 µmol) as described in the Methods. The µmoles of dimethylsulfide remaining *versus* time are shown for incubations containing (▲), C_2H_2-treated cells and 2.5 mM $(NH_4)_2SO_4$ or active cells incubated with (Δ), 0 mM, (■), 0.025 mM, (□), 1.2 mM, and (●), 2.5 mM $(NH_4)_2SO_4$. Data represent an average of duplicate samples. The relative error ranged from 1% to 12%.

2.5. Discussion

We investigated the inhibitory properties of dimethylsulfide on ammonia oxidation by *N. europaea* and conclude that the inhibition is a result of the oxidation of dimethylsulfide by AMO. Therefore, dimethylsulfide joins the list of alternate substrates for AMO. Depletion of several other thioethers by cells of *N. europaea* were also demonstrated (Table 2.2). The products formed by oxidation of three different thioethers were identified to be the corresponding sulfoxides. The following evidence supports the idea that AMO catalyzed the oxidation of the thioethers: 1) both a reductant source and O_2, which are required for the activity of AMO, were also required for depletion of dimethylsulfide and formation of dimethylsulfoxide 2) the AMO specific inhibitors, C_2H_2 or ATU, prevented depletion by *N. europaea* of each of the sulfides listed in Table 2.2 and 3) AMO specific inhibitors also prevented formation of the sulfoxide products. 4) Mass spectral analysis demonstrated that a single atom of ^{18}O from $^{18}O_2$ was incorporated into allylmethylsulfide producing ^{18}O-labeled allylmethylsulfoxide.

AMO catalyzes the oxidation of N-H bonds of ammonia, aryl C-H (Hyman, Sansome-Smith et al., 1985), alkyl C-H (Hyman and Wood, 1983; Hyman, Murton et al., 1988), C=C (Hyman and Wood, 1984b; Hyman and Wood, 1985; Rasche, Hyman et al., 1991) and C-O-C bonds (Hyman, Page et al.,1994). The results of this work show that AMO catalyzed the oxidation of thioethers to sulfoxides. The amount of thioether depleted by the cell suspensions in the case of dimethylsulfide, methylphenylsulfide and tetrahydrothiophene was predominantly accounted for as the sulfoxides. This indicated that AMO preferentially oxidized the sulfur atom over the alkane or phenyl moiety. Because the amount of dimethylsulfoxide formed was low (69%) in relation to the amount of dimethylsulfide depleted, we cannot exclude the possibility that a small amount of some other oxidative product(s) may have been formed. However, dimethylsulfone, a potential oxidative product, was not detected by GC. The inability of cell suspensions to further oxidize sulfoxides to sulfones was also supported by our results obtained using GC-MS, which showed that allylmethylsulfone was not a product of allylmethylsulfide oxidation by *N. europaea*. Based on the magnitude of the GC peak identified as allylmethylsulfoxide, oxidation of the sulfur atom of allylmethylsulfide was preferred over oxidation of the alkene moiety. While our data cannot rigorously

exclude the possibility that other oxidative products are formed, the data clearly support the conclusion that the majority of the products formed are sulfoxides. The oxidation of the sulfur atom of these thioethers in preference to the other carbon functional groups available for oxidation might be expected in light of the ease of oxidation of sulfenyl sulfur over oxidation at carbon atoms (Oae, 1991).

Sulfur is generally susceptible to oxidation and the oxidation of a thioether to a sulfoxide is a common reaction for a microbial or mammalian monooxygenase (Holland, 1988). Cell suspensions and cellular extracts of both bacteria and fungi have been shown to oxidize thioethers to sulfoxides and in some cases the sulfone is also formed (Ziegler, 1980; Holland, Popperl et al., 1985; Ohta, Okamoto et al., 1985). Most often the oxidation is attributed to a monooxygenase activity due to a requirement for O_2. Microsomal fractions from liver and two purified enzyme systems, cytochrome P-450 and dopamine ß-hydroxylase, have been shown to oxidize thioethers to the sulfoxides (May, Phillips et al., 1981; Ziegler, 1989). However, our GC-MS data suggest that cell suspensions of *N. europaea* do not oxidize thioethers to the oxidative state of sulfones. The lack of sulfone as a potential product is consistent with the inert behavior of dimethylsulfoxide on the ammonia metabolism of *N. europaea* (Bédard and Knowles, 1989). Dimethylsulfoxide has been used as a delivery vehicle in the study of the ammonia oxidizers for many insoluble organic compounds (Bédard and Knowles, 1989; Hyman, Kim et al., 1990).

Global mass balances for sulfur indicate that dimethylsulfide is an important volatile biogenic compound involved in the transfer of sulfur from the ocean to the atmosphere (Wakeham and Dacey, 1989). The main source of dimethylsulfide in the ocean is phytoplankton. Microbial consumption of dimethylsulfide is an important aspect of dimethylsulfide biogeochemistry in seawater. While there have been many reports of oceanic microbial consumption of dimethylsulfide (Kiene, 1990; Kiene and Bates, 1990), pure culture studies of aerobic oceanic bacterial depletion of dimethylsulfide are few (Wakeham and Dacey, 1989). Methylotrophic organisms and/or chemolithotrophs have been speculated to contribute to depletion of dimethylsulfide because depletion was sensitive to the common inhibitor of C1 metabolism, chloroform (Kiene, 1990; Kiene and Bates, 1990). However, the aerobic metabolism of dimethylsulfide by marine bacteria has not yet been documented (Wakeham and Dacey, 1989). Therefore, the ability of a marine nitrifier to consume dimethylsulfide was investigated. Results obtained using GC

analysis indicated that *N. oceanus* oxidized dimethylsulfide (Figure 2.4) and the product formed was dimethylsulfoxide. Because the oxidation of dimethylsulfide and formation of dimethylsulfoxide was prevented by C_2H_2, the oxidation most likely involved AMO. This result raises the possibility that the process of co-oxidation by marine nitrifiers may contribute to the oceanic sulfur cycle.

In summary, our results suggested that dimethylsulfide in particular and thioethers in general act as substrates for AMO. Depletion of dimethylsulfide by two ammonia oxidizers, *N. europaea* and *N. oceanus*, was prevented by treating the cell suspensions with C_2H_2, a specific inactivator of AMO. For three of the thioethers we examined, the majority of the oxidative product formed by cells of *N. europaea* was established to be the sulfoxide. The incorporation of a single atom of ^{18}O from $^{18}O_2$ into allylmethylsulfide to form ^{18}O-labeled allylmethylsulfoxide by cells of *N. europaea* also supported our conclusions of the catalytic involvement of AMO in the oxidation. The ability of dimethylsulfide to inhibit the nitrification of ammonia in the soil could be attributed to oxidation of dimethylsulfide by AMO, the enzyme responsible for initiating nitrification. The demonstration that *N. europaea* can oxidize a variety of thioethers extends the known substrate range of AMO to specifically include the sulfur atom as a potential site of oxidation in addition to oxidation at carbon and nitrogen atoms.

CHAPTER 3

Mechanism-based inactivation of ammonia monooxygenase in *Nitrosomonas europaea* by allylsulfide

3.1. Abstract

Allylsulfide caused an irreversible inactivation of ammonia monooxygenase (AMO) activity (ammonia-dependent O_2 uptake) in *Nitrosomonas europaea* . The hydroxylamine oxidoreductase activity (hydrazine-dependent O_2 uptake) of the cell suspensions was unaffected by allylsulfide. Anaerobic conditions or the presence of allylthiourea, a reversible noncompetitive AMO inhibitor, protected AMO from inactivation by allylsulfide. Ammonia did not protect AMO from inactivation by allylsulfide, but instead increased the rate of inactivation. The inactivation of AMO followed first-order kinetics, but the observed rates did not saturate with increasing allylsulfide concentrations. The time course for recovery of AMO-dependent nitrite production after complete inactivation by allylsulfide required *de novo* protein synthesis. Incubation of cells with allylsulfide prevented ^{14}C-label from $^{14}C_2H_2$ (a suicide mechansim-based inactivator of AMO) from being incorporated into the 27-kD polypeptide of AMO. Some compounds structurally related to allylsulfide were unable to inactivate AMO. We conclude that allylsulfide is a specific, mechanism-based inactivator of AMO in *N. europaea.*

3.2. Introduction

The ammonia-oxidizing bacteria, characterized by such species as *Nitrosomonas europaea*, obtain all their energy for growth solely from the rapid oxidation of ammonia to nitrite. Ammonia oxidation by nitrifying bacteria initiate environmentally and economically deleterious effects by rapidly oxidizing ammonia-based fertilizers applied to croplands. On the other hand, nitrifying bacteria also play a crucial role in the beneficial process of removal of ammonia

from sewage. Therefore, it is not surprising that there has been considerable interest in the metabolism and inhibition of ammonia oxidation by nitrifying bacteria.

The oxidation of ammonia by the species *N. europaea* is initiated by the enzyme known as ammonia monooxygenase (AMO) (Eqn. 1).

$$NH_3 + O_2 + 2e^- + 2H^+ \longrightarrow NH_2OH + H_2O \qquad (1)$$

Despite the interest in the catalytic activity of AMO, the purification of this enzyme has not been achieved. Much of what is known about the catalytic properties of AMO have been deduced from inhibitor studies using whole cells of *N. europaea* (Hofman and Lees, 1953; Hyman and Wood, 1985; Shears and Wood, 1985; Hyman, Kim et al., 1990). Many compounds inhibit AMO solely by interacting as substrates at the active site(s) of the enzyme (Hyman and Wood, 1983; Hyman and Wood, 1984); other substrates oxidized by AMO can lead to the generation of reactive intermediates (Rasche, Hyman et al., 1991). These reactive intermediates can disrupt ammonia oxidation by permanently damaging intracellular protein(s). In some cases these effects are non-specific. For example, the oxidation of trichloroethylene by cell suspensions of *N. europaea* leads to inactivation of AMO (Rasche, Hyman et al., 1991). The inactivation of AMO by trichloroethylene requires O_2 and is prevented by allylthiourea, a reversible inhibitor of AMO, which indicates that the inactivating effect of trichloroethylene requires catalytic turnover by AMO to generate a reactive species (Rasche, Hyman et al., 1991; Hyman and Arp, 1992). However, the effects of trichloroethylene oxidation are not limited to AMO because incubation of cells with ^{14}C-labeled trichloroethylene resulted in the non-specific covalent binding of the reactive species to many intracellular proteins, which indicates that the reactive species generated by AMO can diffuse away from the active site.

In contrast to the non-specific effects caused by oxidation of trichloroethylene by AMO, acetylene (C_2H_2) is a mechanism-based inactivator that is specific for AMO (Hyman and Wood, 1985; Hyman and Arp, 1992). Incubation of cells with $^{14}C_2H_2$ gave rise to the covalent attachment of ^{14}C-label almost exclusively to a polypeptide of apparent M_r of 27-kD. The inactivation of AMO by C_2H_2 is diminished in the presence of high ammonia concentrations and is prevented under anaerobic conditions when AMO is inactive. It is proposed that C_2H_2 inactivates AMO as a result of the attempted oxidation of the

acetylenic triple bond resulting in the generation of a reactive intermediate which binds to and inactivates the enzyme. The level of covalent modification of this polypeptide by ^{14}C-label from $^{14}C_2H_2$ is a saturable process and correspondes with the amount of ammonia-oxidizing activity present in the cells (Hyman and Arp, 1992). The ammonia-oxidizing activity of cells inactivated by either trichloroethylene or C_2H_2 was shown to recover by a process requiring *de novo* protein synthesis (Rasche, Hyman et al., 1991; Hyman and Arp, 1992). Acetylene-inactivated cells recovered their nitrite-producing activity faster than cells inactivated by trichloroethylene. The difference in the rate of recoveries was suggested to be due to the limited protein synthesis required when cells were inactivated by a specific inactivator like C_2H_2 in contrast to the extensive protein synthesis required following non-specific effects produced when cells are incubated with trichloroethylene (Hyman and Arp, 1992).

During our characterizations of the inhibitor properties of some sulfur compounds on the ammonia oxidation by *N. europaea*, allylsulfide was determined to be a potent inactivator of ammonia oxidation (Juliette, Hyman et al., 1993a). In contrast to a variety of other organic sulfides that were shown to be oxidized to sulfoxides by the enzyme ammonia monooxygenase (AMO), allylsulfide both specifically and potently inactivated AMO. In this chapter, the inactivation of the ammonia-oxidizing activity of cells of *N. europaea* by allylsulfide was further examined. Some of the methods previously used to unequivocally demonstrate that C_2H_2 acts as a mechanism-based inactivator of AMO were applied to our characterization of the inactivation of AMO by allylsulfide. The inactivation of the ammonia-oxidizing activity of cell suspensions by allylsulfide was time-dependent and followed pseudo first-order kinetics. The inactivation of AMO by allylsulfide required catalytic turnover of AMO. The inhibitory effects of other compounds, structurally related to allylsulfide, were also examined and the results emphasize the unique nature of allylsulfide.

3.3. Materials and methods

3.3.1. Materials.

Allylthiourea was obtained from Eastman Kodak Co. (Rochester, N.Y.). All other allylic compounds and CS_2 (spectrophotometric grade, 99+%) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Chemicals were >97% pure by manufacturer analysis except allylmercaptan (70+%) and diallyldisulfide (80%) which were supplied as technical grade reagents. Reagents for electrophoresis and fluorography were obtained from ICN Biochemicals (Costa Mesa, CA). [^{14}C]-C_2H_2 was generated from $Ba^{14}CO_3$ (specific activity = 45.8 mCi/mmol) (Sigma Chemical Co., St. Louis, MO) as described previously (Hyman and Arp, 1990). Unlabeled C_2H_2 was obtained from a cylinder (AIRCO, OR) or generated in a gas-generating bottle from calcium carbide (technical grade, Aldrich Chemical Co. Inc., Milwaukee, WI) as described previously (Hyman and Arp, 1987).

3.3.2. Growth and preparation of the cells.

Nitrosomonas europaea was grown in batch cultures (1.5 l) in Erlenmeyer flasks (2 l) in an unlit, constant temperature room (30°C) on rotary shakers in growth media previously described (Hyman and Arp, 1992). The bacteria were harvested by centrifugation (20,000 x g, 15 min), resuspended in assay buffer (1.5 ml, 50 mM sodium phosphate buffer pH=7.8 containing 2 mM $MgCl_2$) and stored on ice for use within 12 hr of harvesting.

3.3.3. O_2 electrode measurements.

The ammonia- and hydrazine-dependent O_2 uptake rates were determined by using a Clark-style O_2 electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio) mounted in a glass, water-jacketed reaction vessel containing assay buffer (1.8 ml). Ammonia-dependent O_2 uptake rates of the cell

suspensions were measured in the presence of ammonium sulfate (2.75 mM). Once a steady state rate of O_2 uptake was recorded, allylthiourea (100 μM) was then added to the O_2 electrode chamber to inhibit ammonia oxidation. Hydrazine hydrochloride (750 μM) was added to the electrode chamber. The hydrazine-dependent O_2 uptake rate was then recorded. The solubility of O_2 in air-saturated buffer at 30°C was taken as 230 μM and that of C_2H_2, in buffer equilibrated with 1 atm C_2H_2, was taken as 40 mM.

3.3.4. Requirements for AMO turnover conditions for inactivation by allylsulfide.

The requirement of O_2 for inactivation of AMO by allylsulfide was examined under the following conditions. Cell suspensions were added to assay vials (10 ml) containing assay buffer (0.7 to 0.9 ml) and ammonium sulfate (2.5 mM). The vials were sealed with Teflon-lined silicone stoppers (Alltech Associates Inc., Deerfield, Ill) and repeatedly evacuated and flushed with N_2 to remove O_2. A stock solution of allylsulfide was made O_2-free and an aliquot (0.3 μmol, 0.2 ml) was added to the appropriate vials. The vials were incubated (5 min) in a shaker bath (30°C, 160 rpm). Cell suspensions (0.1 ml, 1.3 mg of protein) were added to initiate the reaction. After 10 min, the cells were recovered from reaction mixtures by sedimentation (14,000 x g, 4 min) and washed by three cycles of sedimenting and resuspensions in assay buffer (3 x 1.5 ml). The cells were then resuspended in assay buffer (1.0 ml) and their ammonia- and hydrazine-oxidizing activities were examined by O_2 electrode as described above. The rates of both ammonia- and hydrazine-dependent O_2 uptake were compared between cell suspensions incubated: 1) anaerobically with or without allylsulfide and 2) aerobically with or without allylsulfide.

The ability of allylthiourea (ATU), a reversible AMO inhibitor, to protect AMO from inactivation by allysulfide was determined by using O_2 uptake measurements after incubating cells (30 min) with or without allylsulfide in the presence or absence of ATU (100 μM). The assay vials were prepared as described above except that; (1) the amount of allylsulfide was 0.5 μmol, and 2) all conditions were aerobic.

3.3.5. Recovery of ammonia-dependent nitrite producing activity.

Ammonia-oxidizing activity of the cell suspensions was inactivated by treatment in the presence of ammonium sulfate (2 mM) with either C_2H_2 (1.3 µmol) or allylsulfide (2.5 µmol) in butyl rubber-stoppered glass serum vials (40 ml) containing assay buffer (4.7 or 3.7 ml, respectively). The vials were placed on a rotary shaker (200 rpm) and the reactions were initiated (5 min after the addition of either allylsulfide or C_2H_2) by the addition of the cell suspensions (0.3 ml, 4 mg of protein). Uninhibited cells were preincubated in assay buffer (4.7 ml) containing ammonium sulfate (2 mM) and were treated in the same manner as the inhibited cells. Allylsulfide (1 ml) was added to the vials from a stock solution (2.5 mM) and C_2H_2 was added as a gas (2 mL) as an overpressure. After 20 min, the reaction mixtures were diluted (~8 fold with assay buffer), and then sedimented (20,000 x g, 10 min). The cells were resuspended with assay buffer (0.3 ml, 4 mg of protein) and transferred into cotton stoppered Erlenmeyer flasks (50 ml) containing growth medium (20 ml). The flasks were placed on a rotary shaker (200 rpm) in an unlit constant temperature room (30°C). Recovery of AMO-dependent nitrite producing activity was monitored by removing a sample (5 µl) of the growth medium by syringe and determining nitrite accumulation colorimetrically as described (Hageman and Hucklesby, 1971).

3.3.6. Treatment of cell suspensions with various amounts of allylsulfide prior to $^{14}C_2H_2$ labeling.

Cell suspensions of *N. europaea* were inactivated by allylsulfide under aerobic conditions in the presence of ammonium sulfate (5 mM). Allylsulfide (25 to 600 nmol, 10 µl to 240 µl) was added to sealed vials which were preincubated (5 min) in a shaking water bath (30°C, 160 rpm). Cell suspensions (0.1 ml, 1.9 mg of protein) were then added to the vials to initiate the reaction. After incubation with allylsulfide (10 min), the residual AMO and HAO activities of a portion of this suspension (50 µl, 0.2 mg of protein) were examined using O_2 uptake measurements. Another portion of the cell suspension (0.4 ml, 1.5 mg of protein) was used in the $^{14}C_2H_2$ labeling incubation.

3.3.7. $^{14}C_2H_2$ labeling incubations.

Cell suspensions (0.4 mg, 1.5 mg of protein) were exposed to $^{14}C_2H_2$ in glass serum vials (6 ml) stoppered with butyl rubber seals and capped with aluminum crimp seals (Wheaton Scientific, Milleville, NJ). Sodium phosphate buffer (1.5 to 1.7 ml) was injected into the stoppered vial to create an overpressure. Five minutes before use, an aliquot (50 μCi) of $^{14}C_2H_2$ was added to the vials. After the vials were allowed to preincubate for 5 min in a shaking water bath (30°C; 300 rpm), the labeling reactions were initiated by the addition of a cell suspension (400 μl, 1.5 mg of protein). The reactions were terminated by the addition of allylthiourea (to 100 μM) and the cells were harvested from the reaction medium by sedimentation in a microfuge (14,000 x g, 4 min). The supernatant was removed and the sedimented cells were immediately solubilized in SDS-PAGE sample buffer and stored at -20°C until further use.

3.3.8. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) and fluorography.

Protein solubilization, electrophoresis and fluorography were conducted as previously described (Hyman and Arp, 1990; Hyman and Arp, 1992). In each experiment, protein samples for analysis by SDS-PAGE and fluorography were obtained from a constant volume of cell suspension. The protein was loaded at a concentration between 150 and 200 μg per lane. Radiolabel incorporation into the 27-kD polypeptide component of AMO was determined by densitometeric scanning of the fluorograms using a GS300 densitometer (Hoefer Scientific Instruments, San Francisco) interfaced to a chart recorder. Apparent molecular weight of the ^{14}C-labeled polypeptide was determined from fluorograms by comparison with R_f values for molecular weight standards of bovine serum albumin (68,000), ovalbumin (45,000), carbonic anhydrase (30,000) and cytochrome c (12,300).

3.3.9. Effect of the various allylic compounds on ammonia oxidation by *N. europaea*.

Stock solutions of the allylic compounds were prepared daily by addition of the compound to sealed glass serum vials filled with assay buffer. Incubations were conducted in glass serum vials containing ammonium sulfate (5 mM) and assay buffer. The allylic compound (0.5 μmol, 0.25 to 0.5 ml) was added to assay vials and allowed to equilibrate for approximately 2 min at 30°C in a shaking water bath. Cell suspensions (50 μl, 1.2 mg of protein) were added to initiate the reactions. After ten minutes, a liquid sample was withdrawn from the assay vial for quantification of nitrite. The rate of nitrite production by cell suspensions in the presence of only ammonium sulfate (5 mM) and assay buffer (0.95 ml) remained constant for the time course of the assay.

The reversibility of the effects of the compounds on the activities of AMO and HAO was examined as described above. The cells were resuspended in assay buffer (0.5 ml) and a portion of the rinsed cells suspension (50 μl, 0.12 mg of protein) was added to assay buffer (1.8 ml) in the chamber of an O_2 electrode. The ammonia- and hydrazine-dependent O_2 uptake rates were recorded for the cell suspensions. These rates were compared to the rates of cell suspensions treated in the same manner except incubated only in the presence of ammonium sulfate (5 mM) and assay buffer (0.95 ml).

3.3.10. Protein determinations.

Protein concentrations were determined using the biuret assay (Gornall, Bardawill et al., 1949) after solubilizing cell protein in aqueous 3N NaOH (30 min, 60°C) and sedimenting insoluble material by centrifugation (14,000 x g, 5 min).

3.4. Results

3.4.1. Inactivation of AMO by allylsulfide: requirements for O_2 and AMO turnover conditions.

Allylsulfide was demonstrated to be a potent inactivator of ammonia oxidation in *N. europaea* (Juliette, Hyman et al., 1993a) . However, the mechanism of inactivation was not determined. Two properties of AMO-specific inactivators which have been previously used to distinguish the mode of inactivation were investigated. These properties are 1) the requirements of O_2 for the inactivation, which would indicate a requirement of AMO catalysis for the inactivation and 2) the use of allylthiourea, a reversible inhibitor of AMO, to prevent inactivation by preventing the turnover of AMO. If the inactivation of AMO by allylsulfide were prevented by either or both of these conditions, the catalytic involvement of AMO in the inactivation could be concluded.

Cell suspensions incubated with allylsulfide (0.5 μmol) and ammonium sulfate (5 mM) under anaerobic conditions retained 90% of their ammonia-dependent O_2 uptake rate (38 μmol of O_2 consumed per hour per mg of protein) compared with cell suspensions incubated anaerobically in only the presence of ammonium sulfate. In contrast, the ammonia-oxidizing activity of the cells incubated aerobically with allylsulfide was completely inactivated. These results indicated that inactivation of AMO by allylsulfide required O_2 and therefore conditions under which AMO would be catalytically active. The activity of HAO (hydrazine-dependent O_2 uptake) remained the same (7.5 μmol of O_2 consumed per hour per mg of protein) regardless of the presence of allylsulfide.

To confirm the requirement of catalytic activity of AMO in the inactivation, protection by allylthiourea, a reversible AMO inhibitor, was examined. The presence of allylthiourea protected 90% of the ammonia-dependent O_2 uptake activity from inactivation by allylsulfide. Cell suspensions were pretreated with allylthiourea in either the presence or absence of allylsulfide. The cell suspensions were rinsed to remove one or both inhibitors. Cell suspensions incubated with only allylthiourea recovered 60% of the ammonia-dependent O_2 uptake activity relative to cells incubated in the absence of any inhibitor. Cell suspensions incubated with allylthiourea and allylsulfide recovered 55% of their ammonia-dependent O_2 uptake activity relative to cells incubated in the absence

of any inhibitor. In contrast, cell suspensions incubated with only allylsulfide retained none of their ammonia-dependent O_2 uptake activity. The hydrazine-dependent O_2 uptake activity was unchanged by the presence of either allylthiourea or allylsulfide. Both the requirements of O_2 and the substantial protection from inactivation afforded by ATU suggested that allylsulfide may be a mechanism-based inactivator of AMO.

3.4.2. Inactivation of ammonia-dependent O_2 uptake by allylsulfide followed first-order kinetics.

The inactivation of enzyme activity by a mechanism-based inactivator should follow pseudo first-order kinetics (Silverman, 1988). To determine if the loss of AMO activity which occurred in the presence of allylsulfide was an apparent first-order process, the decrease in the rate of ammonia-dependent O_2 uptake in an O_2 electrode chamber was monitored. Cell suspensions were added to the O_2 electrode chamber followed by ammonium sulfate (2.7 mM). Once a constant rate of O_2 uptake was observed, various amounts of allylsulfide were then added to the electrode chamber. Figure 3.1 shows a semilogarithmic plot of the rate of O_2 uptake as a function of the time following the addition of allylsulfide to the O_2 electrode chamber. The straight lines for each concentration of allylsulfide imply a first-order rate of inactivation of the AMO activity (Figure 3.1). However, at lower concentrations of ammonium sulfate, the rates of inactivation by allylsulfide were not as clearly first order.

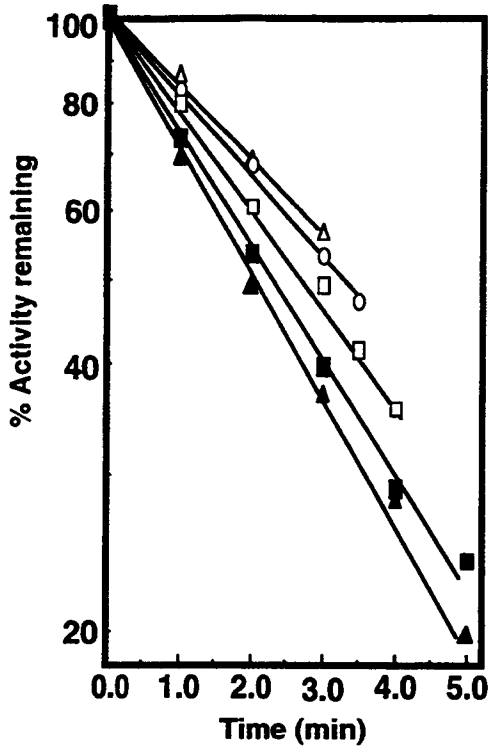


Figure 3.1. First-order inactivation of ammonia-dependent O_2 uptake by allylsulfide. A series of O_2 electrode experiments were run with cell suspensions of *N. europaea* (0.06 mg of protein) during ammonia-dependent O_2 uptake. Once a steady state rate of ammonia-dependent O_2 uptake was established in the presence of 2.25 mM ammonium sulfate (1.6 µmol of O_2 consumed per min per mg of protein), allylsulfide 0.22 (Δ), 0.26 (O), 0.31 (□), 0.35 (■) and 0.41 (▲) µmol was added to the O_2 electrode chamber. The inhibited rates of ammonia-dependent O_2 uptake were measured in relation to the time after the addition of allylsulfide to the O_2 electrode chamber. The results are presented as a semilogarithmic plot of the percent O_2 uptake rate remaining (in relationship to the preinhibited rate) versus the time after the addition of allylsulfide to the O_2 electrode chamber.

3.4.3. Dependence of the rate of inactivation of ammonia-dependent O_2 uptake by allylsulfide on the concentration of ammonia.

To characterize the dependence of the rate of inactivation of AMO by allylsulfide on the ammonia concentration a series of O_2 electrode experiments were done. The range of ammonium ion concentrations used covered the concentrations both above and below the apparent K_m for ammonium sulfate, as

determined from initial rates of O_2 uptake shown in Figure 3.2A. The effect of ammonium sulfate concentration was determined by estimating the time required for allylsulfide to inactivate the ammonia-dependent O_2 uptake activity by 50%. The results of this experiment and of comparable experiments with allylthiourea and C_2H_2 are presented in Figure 3.2B. Over the range of ammonium sulfate concentrations tested, there was essentially no change in the $t_{1/2}$ for the inhibition caused by allylthiourea, implying that the inhibition by allylthiourea was independent of ammonium ion concentration. The $t_{1/2}$ for inactivation by C_2H_2 progressively increased with successive increases in ammonium ion concentrations, as previously observed (Hyman, Kim et al., 1990). In contrast to C_2H_2, a progressive decrease in $t_{1/2}$ for allylsulfide inactivation of AMO occurred with increasing ammonium sulfate concentrations. Thus, the inactivation rate increased with increasing ammonia concentrations. The addition of sodium chloride (44 mM) to the O_2 electrode chamber containing ammonium sulfate (2.7 mM) did not increase the rate of inactivation by allylsulfide (44 nmol), suggesting that the increased rate of inactivation with higher concentrations of ammonium sulfate was probably not due to an increase in ionic strength. Furthermore, the increased rate of inactivation by allylsulfide approached a maximum as the rate of ammonia-dependent O_2 uptake approached its maximum (Figure 3.2). This confirms that the dependence of the rate of inactivation was a result of increasing ammonium sulfate concentration and hence AMO turnover rate rather than a consequence of increasing ionic strength.

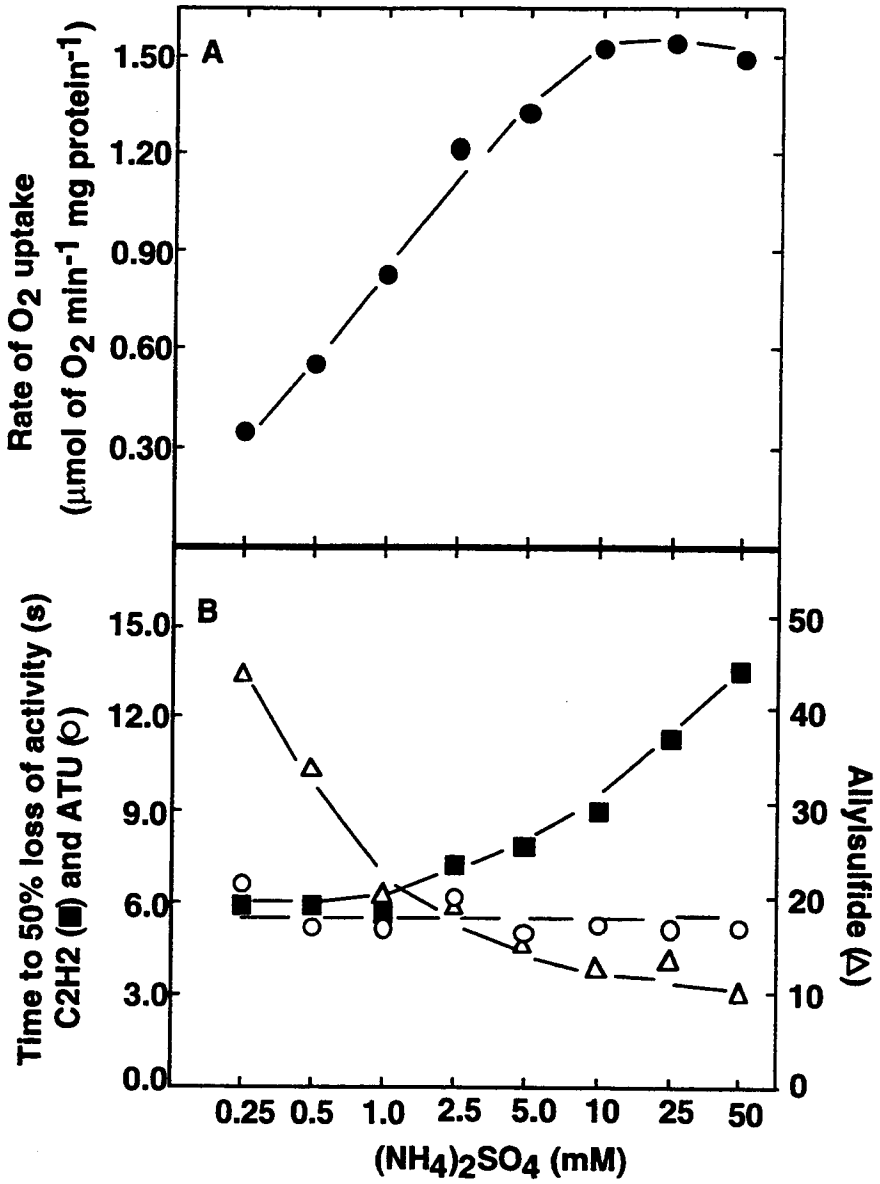


Figure 3.2. Effect of the ammonium sulfate concentration on the half-time of inhibition of ammonia oxidation by C_2H_2, allythiourea, and allylsulfide. A series of O_2 electrode incubations was performed in which cell suspensions (0.14 mg of protein per ml) were assayed for rates of ammonia-dependent O_2 uptake in the presence of a range of ammonium ion concentrations (0.25 to 50 mM). When a steady-state rate of O_2 uptake had been established for each concentration of ammonium sulfate, the inhibitor (to 200 μM) was added. The time required for the rate of O_2 uptake to decline to 50% of the preinhibited rate was then determined. (**A**) Effect of initial ammonium ion concentration on the preinhibited rate of O_2 uptake (●); (**B**) effect of ammonium ion concentration of the half-time of ammonia-dependent O_2 uptake activity of cells inhibited with 200 μM C_2H_2 (■), 200 μM allylthiourea (○), and 200 μM allylsulfide (Δ).

3.4.4. Comparison of the rate of recovery of nitrite production by allylsulfide- and C_2H_2-treated cells.

Cell suspensions of *N. europaea* can recover their ammonia-dependent nitrite producing activity after exposure to either C_2H_2 or trichloroethylene if cells are subsequently placed in growth sustaining media in the absence of the inhibitor (Rasche, Hyman et al., 1991; Hyman and Arp, 1992). The recovery of ammonia-oxidizing activity after inactivation of AMO by C_2H_2 or inactivation by trichloroethylene is known to require to require *de novo* protein synthesis (Rasche, Hyman et al., 1991; Hyman and Arp, 1992). It is also known that $^{14}C_2H_2$ binds to and inactivates predominantly one polypeptide while the covalent modifications caused by the activated ^{14}C-labeled trichloroethylene product are non-specific and result in ^{14}C incorporation into numerous polypeptides of *N. europaea* (Rasche, Hyman et al., 1991). Cells inactivated by trichloroethylene recover their nitrite-producing activity more slowly than cells inactivated by C_2H_2 (Hyman and Arp, 1992). It was suggested that the slower rate of recovery for trichloroethylene-treated cells is a consequence of the non-specific protein inactivations resulting from trichloroethylene oxidation (i.e. several proteins were modified), which is in contrast to the faster rate of recovery of cells inactivated by the specific inactivation resulting from C_2H_2 oxidation (i.e. only a single polypeptide is modified) (Hyman and Arp, 1992).

As an additional technique to assess whether the inactivation of AMO by allylsulfide was specific, we compared the recovery rates of cell suspensions whose ammonia-dependent O_2 uptake activities had been inactivated using either C_2H_2 or allylsulfide. The rate of nitrite production by uninhibited cell suspensions and by cell suspensions treated with either allylsulfide or acetylene is shown in Figure 3.3. After a 2 to 3 hour lag, the rate of nitrite production by C_2H_2-treated cells gradually increased to the rate of the uninhibited cells, demonstrating the recovery of their ammonia-oxidizing activity. Cells treated with allylsulfide also regained their ammonia-oxidizing activity but required slightly less time to recover than cells treated with C_2H_2. Because the rate of recovery after allylsulfide inactivation did not have a lag time longer than that shown for C_2H_2 suggests that the inactivation of AMO by allylsulfide did not involve the inactivation of many protein components. If, as in the case of trichloroethylene,

several proteins had been damaged by allylsulfide the cells would be expected to have a recovery rate slower than cells inactivated by C_2H_2.

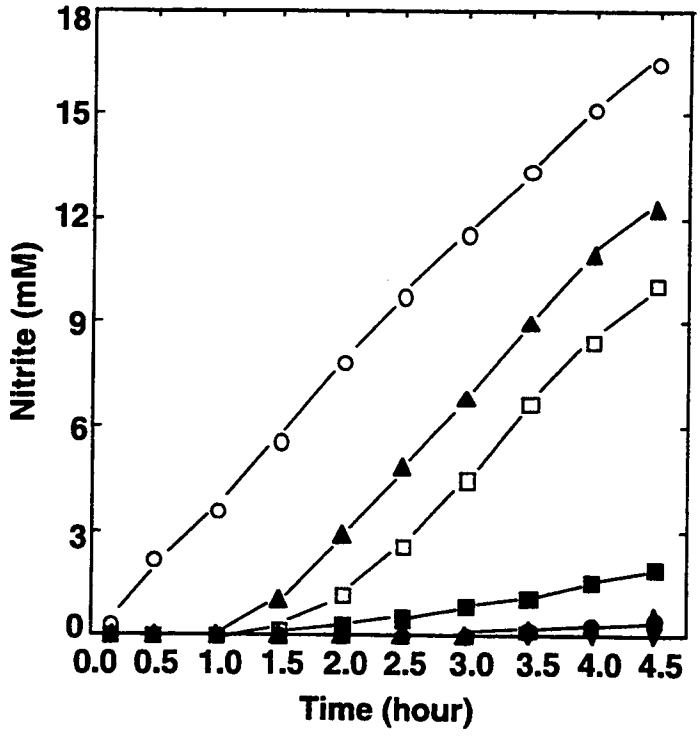


Figure 3.3. Time course of recovery of ammonia-dependent nitrite producing activity of cells after inactivation by either C_2H_2 or allylsulfide. Cells were pretreated with C_2H_2 or allylsulfide as described in Materials and methods. The time course of recovery was monitored as nitrite accumulation produced by: (1) the uninhibited cells (O), (2) cells which had been preincubated with C_2H_2 (□, ●, ▼), or (3) cells which had been preincubated with allylsulfide (▲, ■, Δ). During recovery the cells (300 µl of a 14 mg of protein per ml) were incubated in Erlenmeyer flasks (50 ml) containing 20 ml of the following medium: (1) complete growth media (O, □, ▲); (2) complete growth medium plus rifampin (100 µg/ml) (▼, ■); or (3) complete growth medium plus chloramphenicol (400 µg/ml) (▼, Δ). The time course of the recovery of uninhibited cell suspensions was unaffected by incubation in the presence of either rifampin or chloramphenicol (data not shown).

A second characteristic of the recovery of ammonia-dependent nitrite producing activity by cells of *N. europaea* after inactivation of AMO by C_2H_2 is that *de novo* protein synthesis is required. The inactivation of AMO by $^{14}C_2H_2$ is known to occur with the ^{14}C-labeling of a single 27-kD polypeptide. The

recovery time is suggested to be the period required when C_2H_2-inactivated cells resynthesize the 27-kD polypeptide a presumed component of AMO. As previously demonstrated for C_2H_2-inactivated cells, recovery of nitrite production by allylsulfide-inactivated cells was prevented by using either the transcriptional inhibitor rifampin or the translational inhibitor chloramphenicol (Figure 3.3). This result demonstrated that *de novo* protein synthesis was also required for the recovery of allylsulfide-inactivated cells and suggests that a protein component was modified as a result of incubating cells with allylsulfide.

3.4.5. Effect of allylsulfide treatment on the incorporation of ^{14}C-label from $^{14}C_2H_2$ into the 27-kD polypeptide.

It was proposed that C_2H_2 inactivates AMO as a result of the attempted oxidation of the acetylenic triple bond resulting in the generation of a reactive intermediate which binds to and inactivates the enzyme (Hyman and Wood, 1985). This interpretation was supported by the observation that the incubation of cells with $^{14}C_2H_2$ gives rise to the covalent attachment of ^{14}C-label to a membrane-bound polypeptide of apparent Mr of 27-kD, as determined by SDS-PAGE and fluorography. The incorporation of ^{14}C-label from $^{14}C_2H_2$ into the polypeptide of apparent Mr of 27-kD is well characterized (Hyman and Wood, 1985; Hyman and Arp, 1992). The level of covalent modification of this polypeptide by ^{14}C-label from $^{14}C_2H_2$ and the extent of the inhibition of ammonia-oxidizing activity are proportional. The incorporation of ^{14}C label is saturable and can be used to estimate the levels of active AMO present in cells of *N. europaea* (Hyman and Arp, 1992). The specific, time-dependent inactivation of AMO by allylsulfide resulted in a concomitant and proportional decrease in the ability of cells to incorporate ^{14}C-label from $^{14}C_2H_2$ into the polypeptide of apparent Mr of 27-kD (Figure 3.4). Thus, the levels of active AMO as measured by label incorporation decreased proportionally with the levels of active AMO measured as ammonia-dependent O_2 uptake during the treatment with allylsulfide. This result indicated that inactivation of ammonia-oxidizing activity by allylsulfide prevented catalysis by AMO.

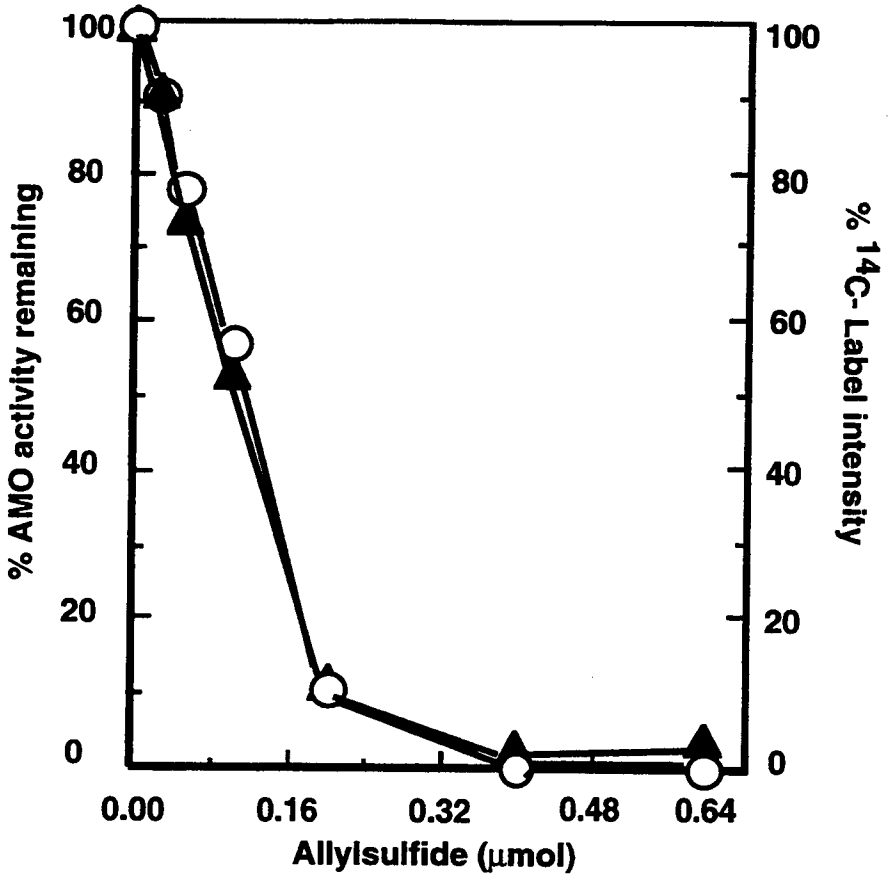


Figure 3.4. Comparison of the ammonia-dependent O_2 uptake rates of cells of *N. europaea* with their ability to incorporate ^{14}C-label from $^{14}C_2H_2$ after prior inactivation by allylsulfide. Cell suspensions were sequentially exposed to allylsulfide and $^{14}C_2H_2$ as described in Materials and methods. Shown is a plot of the residual AMO activity and the level of ^{14}C-label incorporated versus the amount of allylsulfide. The intensity of the ^{14}C-labeled 27-kD polypeptide, as determined by densitometry, of cells inactivated by allylsulfide is expressed as a percentage relative to the intensity of the ^{14}C-label which was incorporated by uninhibited cells (O).The remaining AMO activity of the cell suspensions, as determined by ammonia-dependent O_2 uptake, is expressed as a percentage relative to the AMO activity of the uninhibited cells (▲).

3.4.6. The effect of compounds with structures similar to allylsulfide on ammonia oxidation by *N. europaea*.

To determine if allylsulfide was atypical in its ability to inactivate the ammonia-oxidizing activity of *N. europaea*, we examined other compounds with

structures related to allylsulfide for their effect on the activity of AMO. The effect of these compounds on ammonia-dependent nitrite production and the reversibility of their effects on the ammonia-and hydrazine-oxidizing activities are presented in Table 3.1.

Table 3.1. Comparison of the effects of various allylic compounds on ammonia oxidation by *N. europaea*

Chemical Structure of compound	Amount added (μmol)	% Nitrite produced relative to unihibited cells from $(NH_4)_2SO_4$ (5mM)[a]	% Reversibility relative to uninhibited cells for[b]	
			AMO	HAO
$(H_2C{=}CHCH_2)_2C$	0.5	97	96	104
$(H_2C{=}CHCH_2)_2O$	0.5	92	101	108
$(H_2C{=}CHCH_2)_2NH$	0.5	101	98	100
$(H_2C{=}CHCH_2)_2S_2$	0.5	9	25	108
$H_2C{=}CHCH_2SH$	0.5	4	20	104
$(H_2C{=}CHCH_2)_2S$	0.5	10	4	110

[a]The percentages are expressed relative the amount of nitrite produced by cells incubated only in the presence of ammonium sulfate. The relative errors among the percentages ranged ±2 -10% for the triplicate samples. [b]To examine the reversibility of the inhibition, the cells were sedimented and resuspended three times with buffer to remove the compound. The activities of AMO and HAO were then measured as the rates of ammonia- or hydrazine-dependent O_2 uptake. The activities are expressed as a percentage relative to a rate of 54 μmol of O_2 consumed hr^{-1} mg of $protein^{-1}$ for the activity of AMO or 11 μmol of O_2 consumed hr^{-1} mg of $protein^{-1}$ for the activity of HAO. The errors ranged between ±1-11% among triplicate samples.

Compounds analogous to allylsulfide, but which did not contain sulfur, i.e 1,6 heptadiene, diallylamine or diallylether, did not have any irreversible effects on the AMO activity of the cell suspensions. Allylmercaptan and allyldisulfide did have irreversible effects on AMO; however, both these reagents contained sulfides and/or allylic sulfides as impurities and thus their effects could not be attributed exclusively to these compounds.

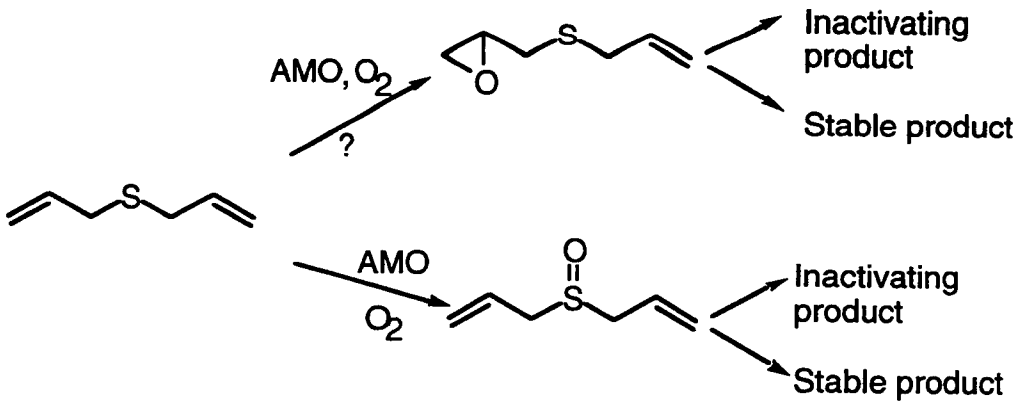
3.5. Discussion.

Two essential determinants of a mechanism-based inactivator are: 1) that the target enzyme must catalyze its own destruction and 2) that the inactivation must result from the reaction of the enzyme with a species produced within its own active site (Ortiz de Montellano, 1988). This paper provides evidence that the inactivation of AMO by allylsulfide occurred as a result of or during the process of oxidation of allylsulfide by AMO and that the inactivation was specific for AMO. As characteristic of a mechanism-based inactivator, the inactivation required conditions for catalytic turnover of the target enzyme. Incubation of cell suspensions with allylsulfide under anaerobic conditions or when the turnover of AMO was inhibited with allylthiourea, substantially prevented the inactivation of the ammonia-oxidizing activity of cells.

Inactivation of AMO activity by allylsulfide followed pseudo first-order kinetics (Figure 3.1). Increasing ammonia concentrations increased the rate of inactivation of AMO by allylsulfide (Figure 3.2). This suggests that stimulating the rate of turnover of AMO increases the rate of inactivation. The recovery of nitrite production by allylsulfide-inactivated cells required *de novo* protein synthesis indicating that an irreversible modification of protein component of AMO had occurred, as would be expected of a mechanism-based inactivation. Furthermore, the recovery rate of cells exposed to allylsulfide closely followed the recovery rate of cells exposed to C_2H_2 and was faster than the rate characterized for the recovery of cells exposed to the non-specific inactivations produced by trichloroethylene (Hyman and Arp, 1992). These results are consistent with the idea that the inactivation of ammonia-oxidizing activity by allysulfide is specific for a single essential protein component involved in ammonia oxidation. Cell suspensions inactivated with allylsulfide were incapable

of incorporating ^{14}C-label from $^{14}C_2H_2$ into the 27-kD polypeptide of AMO. This result demonstrates at the molecular level the catalytic inactivation of AMO. Furthermore, the amount of ammonia-dependent O_2 uptake which remained after treatment with progressively higher amounts of allylsulfide corresponded to the amount of ^{14}C-label from $^{14}C_2H_2$ which was incorporated into the 27-kD polypeptide.(Figure 3.4). This result verified that the level of inactivation of AMO by allylsulfide, as measured by ammonia-dependent O_2 uptake, correlated with the AMO activity remaining in the cells. Other criteria for establishing that an inactivator is mechanism-based, such as protection using either exogenous nucleophiles or alternate substrate for AMO (Silverman, 1988), were not applicable for use in our whole cell system. The most satisfying test for a mechanism-based inactivator is the demonstration of a covalent modification of an active site component such as a polypetide or prosthetic group as has been shown for the inactivation of AMO by $^{14}C_2H_2$. However, radiolabeled allylsulfide is not commercially available and therefore this remains to be determined. Nonetheless, our results indicate that the attempted oxidation of allylsulfide by AMO produces a reactive intermediate which, like C_2H_2, can specifically inactivate AMO.

Two possible oxidative sites are present in allylsulfide which may be relevant to the production of the reactive species which inactivates AMO. Oxidation of allylsulfide by AMO could occur at either the terminal alkene or the central sulfenyl moiety (Scheme 3.1).



Scheme 3.1. Two possible pathways for the oxidation of allylsulfide by AMO which may lead to the inactivation of AMO.

While oxidation at either of these sites is possible, oxidation at only one site is likely to result in the reactive species which results in the inactivation of AMO. The oxidation of terminal alkenes to stable epoxides by cells of *N. europaea* is well documented (Hyman and Wood, 1984b; Hyman, Murton et al., 1988). However the inactivation of cytochrome P-450-associated monooxygenases by terminal alkenes is also well established (Ortiz de Montellano, 1988). Given the similar catalytic natures of these two broad substrate monooxygenases and the known reactivity of epoxides, the inactivation of AMO by allylsulfide may result during the oxidation of the alkene moiety. However, previous results for the oxidation of thioethers by AMO would suggest that the oxidation of the sulfur atom would be a more frequent occurrence. Enzymatic preference for the oxidation of the sulfenyl moiety over the alkene moiety might be expected in view of the ease of oxidation of the sulfenyl moiety (Oae, 1991). None of the structurally related compounds listed in Table 3.1, which did not possess a sulfur atom and for which an epoxide might be an expected product, had any inactivating effect on AMO. If activation of the alkene moiety of allylsulfide is the initiating step which results in the inactivation of AMO, then one might expect some epoxide to be formed and therefore some inactivation to occur during the oxidation of allylmethylsulfide by *N. europaea*. However, cells were not substantially inactivated by allylmethylsulfide but rather a substantial amount of product, allylmethylsulfoxide, was produced by AMO (Juliette, Hyman et al., 1993a). While oxidation of the alkene moiety of allylsulfide is theoretically possible, results for allylmethylsulfide suggest that this is an infrequent occurrence and therefore the inactivation of AMO by allylsulfide is mostly likely to involve the attempted oxidation at the sulfenyl moiety of allylsulfide.

The inactivation of AMO by allylsulfide raises the controversial issue of allelopathic inhibition of nitrification. The ability of a plant to inhibit nutrient consumption by soil microorganisms would undoubtedly be an advantage and the effects of volatile compounds from plant residues on microorganisms is well established (Stotzky and Schenck, 1976). However, despite the fact that allelopathic inhibition of nitrification has been suggested (White, 1986b) , theoretically debated (White, 1986a), and two natural products have been investigated as potential nitrification inhibitors (Sahrawat and Keeney, 1985), the inhibition of a pure culture of a nitrifier by a plant produced compound has never been conclusively demonstrated (McCarty, Bremner et al., 1991). Interestingly,

numerous allylic sulfide compounds are known to be present in plants of the *Allium* spp., especially the garlic and chive varieties; these compounds are suspected to function either to attract or retard insects and/or pests (Block, 1992). Allylsulfide and possibly allyldisulfide may be two such compounds which inhibit microorganisms, including the ammonia-oxidizing bacteria, and are present in the microcosm surrounding bulbs of the *Allium* spp. Allylsulfide could therefore constitute a nitrification inhibitor which is both a natural product and an example of an allelopathic agent. However, despite our suggestion that exudates from plants of the *Allium* spp. may inhibit ammonia consumption by the nitrifiers, the requirements of *Allium* spp. for nitrogen fertilizer does not appear to differ from the requirements of fertilizer by other crop species (Buwalda, 1986). Although field studies suggest that nitrification may not in general be inhibited in soils containing garlic, it may be of interest in light of our findings with regards to the inactivation by AMO by allylsulfide, to determine whether the rate of nitrification in crops of the *Allium* spp. differs from that other crop species which do not produce allylsulfides.

The biological activity of allylic sulfides in the soil environment is not unknown. Germination of sclerotia by the white rot fungi *Sclerotium cepivorum* Berk. is specifically triggered only by allylic sulfur compounds produced by members of the genus *Allium*. It is suggested that the exudates containing alkyl and alkenyl-l-cysteine sulfoxides from roots of the *Allium* spp. are metabolized by the soil microflora to yield a range of volatile allylic thiols and sulfides which specifically activate the germination of sclerotia (King and Coley-Smith, 1969; Coley-Smith and Parfitt, 1986) The use of allylic sulfides to elicit the early germination of the white rot sclerotia is being investigated as an alternative method to control white rot.

The inhibition of ammonia oxidation by allylsulfide may also have a direct practical use as a tool in ecological studies of nitrification. One important inhibitor used in the study of the nitrogen cycle is C_2H_2. Through various mechanisms C_2H_2 inhibits nitrogenase, AMO, nitrous oxide reductase and nitrate reductase (Hyman and Arp, 1988). The C_2H_2 blockage assay for nitrous oxide reductase activity is the most widely used assay for denitrifying activity (Oremland and Capone, 1988). However, this assay is limited by the complication that while C_2H_2 is an electron acceptor for denitrification it also inhibits nitrification. What is needed to estimate natural nitrogen transforming activities is a series of inhibitors specific for each process. For example, methyl fluoride and dimethylether have

recently been shown to be effective inhibitors of methane oxidation and nitrification, with little effects on methanogenesis and denitrification (Oremland and Culbertson, 1992). Allylsulfide is another potential specific inhibitor for nitrification which has several attractive features for field studies. From a practical point allylsulfide is highly volatile and can be applied as effectively as C_2H_2 and other gaseous inhibitors. Perhaps more importantly, allylsulfide acts as a mechanism-based inactivator of AMO. Its effects are therefore likely to be more similar to those of C_2H_2 than methyl fluoride or dimethylether, which both appear to inhibit nitrification by acting as alternative substrates for AMO.

The unique properties of allylsulfide have also been recognized in another area of research. Both allylsulfide and its biologically more oxidized products, i.e. allylsulfoxide and allylsulfone, were shown to inhibit microsomal P-450 2E1 monooxygenase. Allylsulfide has been suggested to inhibit carcinogenesis as a result of its inhibition of microsomal monooxygenase P-450 2E1 (Brady, Ishizake et al., 1991). However, allylsulfide was concluded not to be a general inhibitor of monooxygenases because other microsomal monooxygenases activities were unaffected (Brady, Li et al., 1988). In light of the similar oxidations catalyzed by AMO and P-450 associated monooxygenases, it is also interesting to note that the bulk of the sulfur compounds which inactivate cytochrome P-450 monooxygenases have been concluded not to be mechanism-based suicidal agents but instead partition out of the active site (Ortiz de Montellano, 1988). However, our data for the inactivation of AMO by allylsulfide, in cells of *N. europaea*, suggest that allylsulfide is a mechanism-based inactivator and that the inactivating species is unlikely to diffuse far from the catalytic site.

CHAPTER 4

Roles of bovine serum albumin and copper in the assay and stability of ammonia monooxygenase activity *in vitro*

4.1. Abstract.

We investigated the effects of bovine serum albumin (BSA) on both the assay and the stability of ammonia-oxidizing activity in cell extracts of *Nitrosomonas europaea*. Ammonia-dependent O_2 uptake activity of freshly prepared extracts did not require BSA. However, a dependence on BSA developed in extracts within a short time. The role of BSA in the assay of ammonia-oxidizing activity apparently is to absorb endogenous free fatty acids which are present in the extracts because: 1) only proteins which bind fatty acids, e.g. BSA or ß-lactoglobulin, supported ammonia-oxidizing activity, 2) exogenous palmitoleic acid completely inhibited ammonia-dependent O_2 uptake activity, 3) the inhibition caused by palmitoleic acid was only reversed by proteins which bind fatty acids, and 4) the concentration of endogenous free palmitoleic acid increased during aging of cell extracts. Additionally, the presence of BSA (10 mg/ml) or $CuCl_2$ (500 µM) stabilized ammonia-dependent O_2 uptake activity for 2 to 3 days at 4°C. The stabilizing effect of BSA or $CuCl_2$ was apparently due to an inhibition of lipolysis because both additives inhibited the increase in concentrations of free palmitoleic acid in aging extracts. Other additives which are known to modify lipase activity were also found to stabilize ammonia-oxidizing activity. These additives included $HgCl_2$, lecithin, or phenylmethylsulfonyl fluoride.

4.2. Introduction.

Nitrosomonas europaea is a lithoautotrophic organism which obtains all of its energy for growth from the oxidation of ammonia to nitrite. Ammonia is initially oxidized to hydroxylamine (NH_2OH) by a membrane-bound monooxygenase

enzyme, ammonia monooxygenase (AMO). The electrons required to support both AMO activity and ATP generation are provided by the further oxidation of hydroxylamine to nitrite by the complex multiheme enzyme, hydroxylamine oxidoreductase (HAO) (Wood, 1986). HAO has been purified and studied in considerable detail, at both the biochemical and genetic level. In contrast, less is known about AMO and it has not yet been purified with activity. There are two features of this enzyme system which have hindered progress in this area. First, the requirements and conditions for a suitable *in vitro* assay remain poorly defined. Second, ammonia-oxidizing activity in cell-free extracts is very unstable (Suzuki, Kwok et al., 1981; Ensign, Hyman et al., 1993). Purification and characterization of AMO will require that these issues are investigated and resolved.

In vitro activity of AMO in cell extracts was previously demonstrated to be highly dependent on assay conditions (Ensign, Hyman et al., 1993). Early studies by Suzuki's group demonstrated that *in vitro* assays of ammonia-dependent O_2 uptake activity required a variety of additives including Mg^{2+}, spermine or bovine serum albumin (BSA) (Suzuki, Kwok et al., 1981; Ensign, Hyman et al., 1993). The most consistently effective additive was BSA (Ensign, Hyman et al., 1993). Recently it was demonstrated that copper ions specifically activate AMO in cell-free extracts although BSA was still required for enzyme activity (Ensign, Hyman et al., 1993). A high concentration of copper ions (250 μM) was required for maximal ammonia-oxidizing activity because of the copper binding capability of BSA. However, it has been shown that other serum albumins were capable of replacing BSA and that ovalbumin, a non-serum albumin, was ineffective. Importantly, the effective serum albumins included proteins both with and without high affinity copper-binding sites (Ensign, Hyman et al., 1993). These results suggest that the mechanism of action of serum albumins is independent of the copper-binding abilities of these proteins. However, the role of serum albumins in the assay was not established.

In contrast to the progress made with *in vitro* assays of ammonia-oxidizing activity, much less attention has been given to stabilization of activity in cell extracts. In general, ammonia-oxidizing activity is extremely labile and fresh extracts lose 50% of their activity within 2 hours when stored at 4°C (Suzuki and Kwok, 1970). Enzyme activity is also lost slowly over 1 to 2 days in extracts stored at -20°C. However, full activity can be retained for greater than 3 months when extracts are rapidly frozen and stored at -196°C (Ensign, Hyman et al.,

1993). Although low temperature storage allows for stockpiling of active extracts, stabilization strategies which are effective at higher temperatures will be needed before AMO can be purified. Because determination of stability requires measurement of activity, it is important to consider that all current *in vitro* assays of ammonia-oxidizing activity require the coupling of electron transfer from HAO to AMO. Instability of ammonia-oxidizing activity may result from the loss of catalytic competence of the individual enzymes. Alternatively, instability may result from a loss of coupling of electron transfer between HAO and AMO while the enzymes remain catalytically competent.

In the present report we describe experiments which elucidate the role of BSA in the *in vitro* assay of ammonia-oxidizing activity. We have demonstrated that *in vitro* ammonia-oxidizing activity can be assayed using freshly prepared cell extracts without BSA. However, the extracts subsequently developed a requirement for BSA. This acquired BSA-dependence apparently arises from the need to absorb and remove inhibitory free fatty acids which accumulated as a result of lipolysis in the cell extracts. We also demonstrate for the first time that BSA and copper ions can independently stabilize ammonia-oxidizing activity for up to 2 or 3 days at 4°C. Our results suggest that the stabilizing effects involve the inhibition of the activity of an endogenous phospholipase. This understanding of the mechanism of stabilization of ammonia-oxidizing activity by BSA or copper ions has also led us to develop alternative stabilizing treatments which should be useful in future attempts at purification of active AMO. An irony of our results is that while copper ions and BSA serve different roles in the *in vitro* assays of ammonia-oxidizing activity they play a similar role in the stabilization of ammonia-oxidizing activity.

4.3. Materials and Methods.

4.3.1. Materials.

Bovine serum albumin (BSA) and pig serum albumin (PSA) (fraction V, 98%), ovalbumin (99%), ß-lactoglobulin, myoglobin (98%), gamma globulin (99 %), lactalbumin, L-α- egg yolk lecithin, spermine, phenylmethylsulfonyl fluoride (PMSF) and leupeptin were obtained from Sigma and were used without further

purification. Ultrapure $CuCl_2$ (99.99%), $HgCl_2$ (99.5+ %), triethylamine, and 2,4'-dibromoacetophenone were obtained from Aldrich Chemical Co. Inc., Milwaukee, Wis. C_2H_2 was generated from calcium carbide (~80%, Aldrich) in a gas-generating bottle as previously described (Hyman and Arp, 1987).

4.3.2. Growth of bacteria.

N. europaea (ATCC 19178) cultures were grown in a 100-liter carboy at 30°C with mixing resulting from forced aeration. Growth medium consisted of 25 mM $(NH_4)_2SO_4$, 3 mM KH_2PO_4, 750 µM $MgSO_4$, 200 µM $CaCl_2$, 10 µM $FeCl_3$, 16 µM EDTA, and 1 µM $CuSO_4$. The medium was buffered with phosphate (pH 8.0) consisting of KH_2PO_4 (18 mM) and NaH_2PO_4 (1.6 mM). The pH of the culture was maintained at 7.8 by the addition of a sterile solution of K_2CO_3 (20% wt/vol) with a pH stat (Cole Palmer Instrument Co., Chicago Ill.). When the nitrite concentration of the culture was 35-40 mM, the cells were harvested by filtration with a Pellicon Ultrafiltration Tangential Flow System (Millipore, Bedford, Mass.). The concentrated cells were collected by centrifugation, washed twice with buffer (0.1 M KH_2PO_4, pH 7.8, 1 mM $MgCl_2$) and resuspended in 150 ml of this buffer. The resuspended cells were rapidly frozen in liquid N_2 and stored at -80°C.

4.3.3. Preparation and storage of cell-free extracts.

Frozen cells were thawed at 30°C and diluted 2 fold with buffer (0.1 M KH_2PO_4, pH 7.0). Typically, frozen cells (20 to 30 ml, 10 to 15 mg of protein per ml) were disrupted by one passage through a French Pressure cell operated at 7500 pounds/ $inch^2$. Unbroken cells were removed by centrifugation (7000 x g, 10 min). The extract was used immediately for experiments. For stability experiments, an extract was divided into several portions and stored with or without the various stabilizing agents either at 4°C with no agitation or at 30°C with vigorous agitation in a shaking water bath (170 cycles per min).

4.3.4. O_2 electrode measurements.

Ammonia-dependent O_2 uptake was measured with a Clark-type oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio) mounted in a water-jacketed reaction vessel (1.7- or 1.8-ml volume) maintained at 30°C. Except where indicated, BSA (10 mg/ml), $CuCl_2$ (230 µM) and $(NH_4)_2SO_4$ (2.8 mM) were routinely added to the buffer (0.1 M KH_2PO_4, pH 8.0) in the O_2 electrode chamber and then the extract (200 µl, 2.2 to 2.7 mg of protein) was added. In order to measure BSA- independent, ammonia-dependent O_2 uptake activity, $CuCl_2$ (12 to15 µM) and $(NH_4)_2SO_4$ (2.8 mM) were added to the buffer (0.1 M KH_2PO_4, pH 8.0) in the O_2 electrode chamber immediately before the extract was added. The HAO activity of the extract (200 µl, 2.2 to 2.7 mg of protein) was measured as hydrazine (1 mM)-dependent O_2 uptake after inactivation of AMO by acetylene in the presence of $(NH_4)_2SO_4$ (2.8 mM), $CuCl_2$ (14 µM) and C_2H_2 (0.23 mM).

4.3.5. Extraction, derivatization, and quantitation of free fatty acids from extracts of *N. europaea*.

Chloroform was chosen as the extraction solvent because it is both a poor solvent for complex lipids and a good solvent for simple lipids such as free fatty acids (Christie, 1982). An authentic fatty acid stock (0.5 to 3 mM) was prepared daily and diluted as needed in the same buffer used for the extract. Free fatty acids were extracted from an aliquot (500 µl) of either the extract or the stock solution of authentic fatty acid with $CHCl_3$ (300 µl) in the following manner. The samples were vigorously vortexed (10 sec) and then subjected to microcentrifugation (14,000 x g, 2 min). The upper aqueous and interfacial layers were suctioned off, and an aliquot (100 or 50 µl) of the $CHCl_3$ layer was placed in a screw-cap vial. The $CHCl_3$ was removed *in vacuo* affording a residue containing fatty acids . The free fatty acids were derivatized with 2,4'-dibromoacetophenone (Christie, 1987). Aliquots (25 µl) of both triethylamine (10 mg/ml in acetone) and 2,4'-dibromoacetophenone (10 mg/ml in acetone) were added to the screw-cap vials. The samples were reacted in a heating block (100°C, 30 minutes) and then quenched with glacial acetic acid (3.5 µl) and heated (100°C, 15 minutes). Acetone was removed *in vacuo* and the p-

bromophenacyl derivatives of the fatty acids were dissolved in acetonitrile. Because this derivatization procedure requires the fatty acid to be in the unesterified state, it is assumed that only free fatty acids were quantified and that abiotic hydrolysis of phospholipids did not occur. Not all of the free fatty acids present in the extract or in the fatty acid stock were extracted into the $CHCl_3$ layer. However, we assumed that a constant fraction of free fatty acids was extracted, as judged by the linearity of our standard curves.

The p-bromophenacyl derivatized fatty acids were subjected to high-pressure liquid chromatography (HPLC). The samples were separated on an octadecylsilica column (250 by 4.4 mm, 5-µm particle size; Vydac, Hesperia, Calif.) and eluted isocratically with 100% acetonitrile at a flow rate of 1 ml/minute. The p-bromophenacyl derivatized palmitoleic acid was detected at a wavelength of 254 nm. Palmitoleic acid in the extract was identified by comparison of its retention time with that of authentic palmitoleic acid which had been similarly derivatized with 2,4'-dibromoacetophenone. Palmitoleic acid was quantified by peak height on a chart recorder. To account for the effect of $CuCl_2$ (500 µM), BSA (10 mg/ml), or $HgCl_2$ (100 µM) on the extraction, derivatization and quantitation processes, separate standard curves for authentic palmitoleic acid were prepared for each of these amended conditions.

4.3.6. Protein determination.

The protein contents of cell suspensions and extracts were determined using the biuret assay (Gornall, Bardawill et al., 1949) after solubilizing the cells or extracts in 3 M NaOH (45 min, 75°C). Bovine serum albumin was used as the protein standard.

4.4. Results.

4.4.1. Role of BSA in the assay of ammonia-dependent O_2 uptake activity.

It was previously demonstrated that maximal ammonia-oxidizing activity in previously frozen cell extracts requires the presence of both BSA and copper ions (Ensign, Hyman et al., 1993). In the present study, we have found that ammonia-oxidizing activity can be assayed also in the absence of BSA. This BSA-independent activity is observed only in freshly prepared extracts with low protein concentrations (less than 15 mg of protein per ml). In general, these BSA-independent activities range from 60% to 90% of the activities of the same extracts assayed with BSA. Consistent with our previous observations, copper ions were still necessary for ammonia-oxidizing activity to be observed (Ensign, Hyman et al., 1993). However, in assays without the competitive binding of copper ions by BSA, maximal ammonia-oxidizing activity was observed with low concentrations of copper ions (13 to 18 μM).

We have also observed that BSA-independent ammonia-oxidizing activity is transient and that over time extracts develop a requirement for BSA (Figure 4.1). An aliquot of an extract was incubated at 30°C, and samples were assayed in either the absence or the presence of BSA (10 mg/ml). Ammonia-dependent O_2 uptake activity was lost within 30 min in the assays without BSA, but activity was observed for up to 2 hours in the assays with BSA (Figure 4.1). Once BSA-independent activity was lost, BSA (0.5 to 10 mg/ml) could reinstate activity. As the extract aged, greater BSA concentrations were required to reinstate activity. These results suggest that the composition of the extract undergoes at least two changes during storage. One change could be mitigated by adding BSA to the assay medium. Another change involved the loss of ammonia-oxidizing activity which occurred at 2 hours. This loss was not recoverable by the addition of more BSA (20 mg/ml).

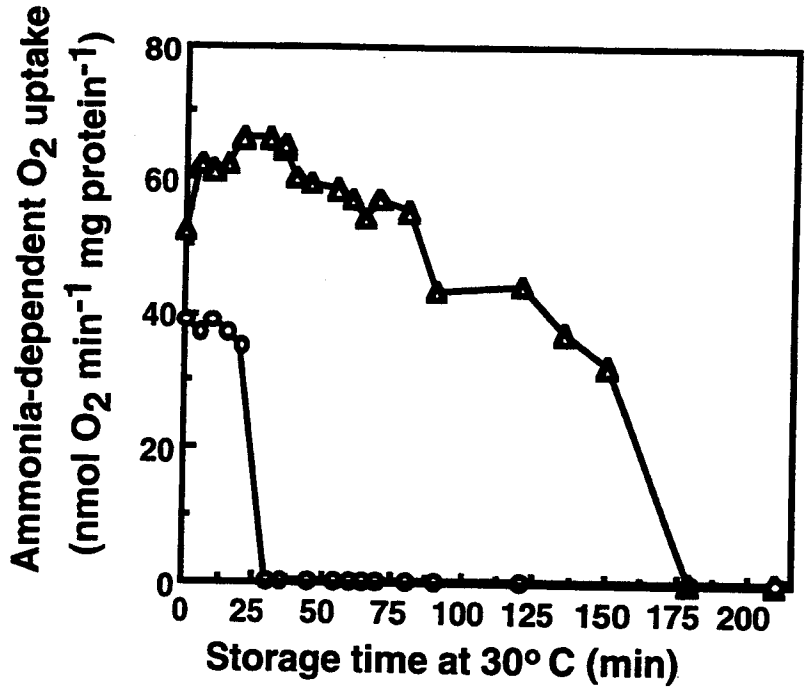


Figure 4.1. Ammonia-dependent O_2 uptake activity measured in the presence and absence of BSA. The activity of an extract (200 µl, 2.2 mg of protein) was assayed at the indicated times as described in Materials and Methods in the presence of either (O) $CuCl_2$ (14 µM) or (Δ) BSA (10 mg/ml) and $CuCl_2$ (230 µM). Rates of ammonia-dependent O_2 uptake were recorded if initiated within 5 min of addition of extract to the O_2 electrode chamber.

To further investigate the role of BSA in the assay of ammonia-oxidizing activity, we considered whether other proteins could substitute for BSA. In mammalian systems, a major role for serum albumins other than metal binding is fatty acid binding (Goodman, 1958; Spector, John et al., 1969). Three fatty acid binding proteins, BSA, pig serum albumin or ß-lactoglobulin (2 mg/ml) reinstated ammonia-dependent O_2 uptake activity in aged extracts (Figures 4.2A to D). ß-lactoglobulin provides the first example of a protein other than serum albumins replacing BSA in the assay of ammonia-oxidizing activity. In contrast, neither ovalbumin (2 mg/ml, Figure 4.2E) nor a higher concentration (8 mg/ml) of other proteins which do not bind fatty acids, such as myoglobin or lactalbumin (Glatz and Veerkamp, 1983; Puyol, Perez et al., 1991) was effective at reinstating ammonia-dependent O_2 uptake (not shown). Our results suggested that the

ability to bind fatty acids is an important aspect of the role of BSA in the assay of ammonia-oxidizing activity.

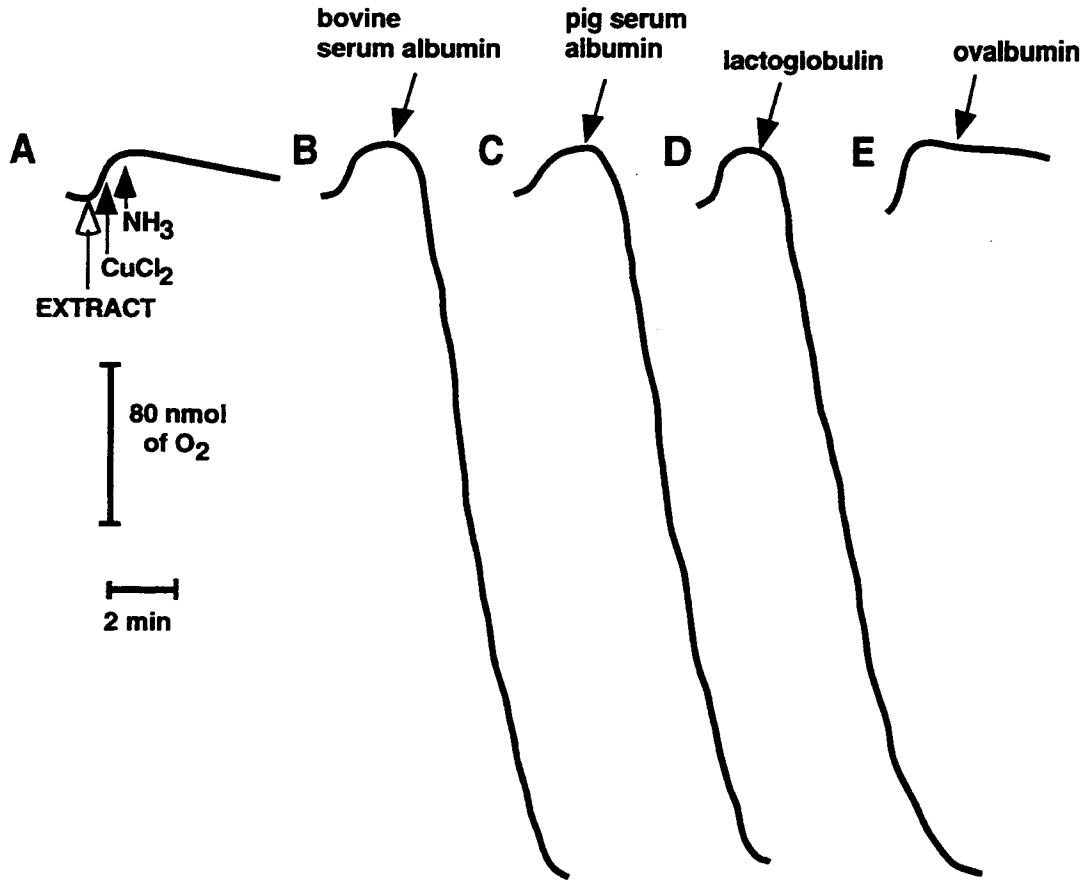


Figure 4.2. Loss of ammonia-dependent O_2 uptake activity as measured in the absence of BSA was reversed by proteins which bind fatty acids. Ammonia-dependent O_2 uptake was measured as described in Materials and Methods except that BSA was not added prior to the addition of extracts. Extracts (200 µl, 2.2 mg of protein), $CuCl_2$ (13 µM) and $(NH_4)_2SO_4$ (2.8 mM) were added to each reaction as shown in trace A. Additional proteins (2 mg/ml) were added as indicated in traces B through E. Rates of ammonia-dependent O_2 uptake activities ranged between 38-42 nmol O_2 min^{-1} mg of $protein^{-1}$.

More direct evidence that the role of BSA in the assay involved fatty acid binding was established by considering the effects of free fatty acids on the assay of ammonia-dependent O_2 uptake. Palmitoleic acid is the predominant fatty acid in *N. europaea* (Blumer, Chase et al., 1969). The addition of

palmitoleic acid (240 µM), to the assay medium completely inhibited BSA-independent, ammonia-dependent O_2 uptake (Figures 4.3A and B). However, the inhibition of ammonia-dependent O_2 uptake by palmitoleic acid was reversed by 100% by either BSA or pig serum albumin (5 mg/ml) and reversed by 70% by ß-lactoglobulin (5 mg/ml, Figures 4.3C to E). In contrast, the inhibition was not reversed by ovalbumin (5 mg/ml, Figure 4.3F), myoglobin, gamma globulin, or lactalbumin (5 mg/ml, not shown).

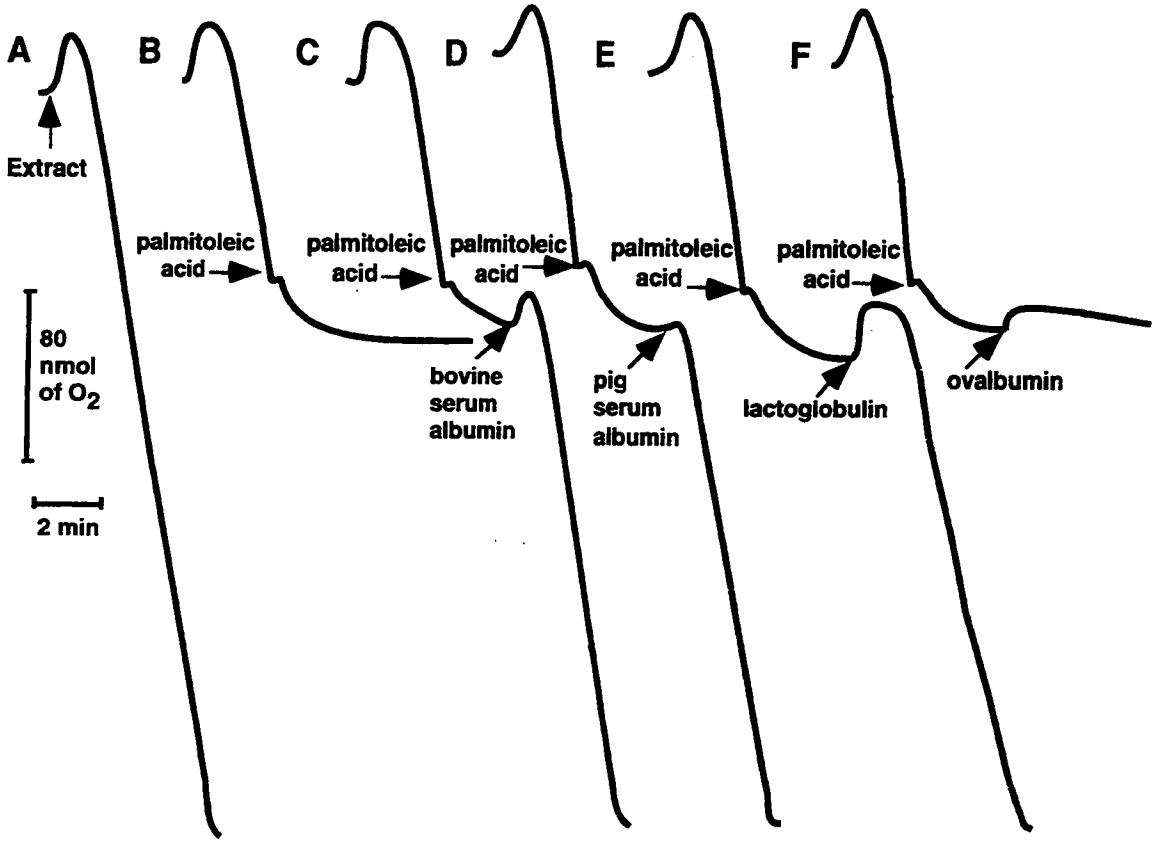


Figure 4.3. Inhibition of ammonia-dependent O_2 uptake by exogenously added palmitoleic acid and relief of the inhibition by either serum albumins or ß-lactoglobulin. Ammonia-dependent O_2 uptake was measured as described in Materials and methods except that BSA was not added prior to the addition of extracts. The O_2 electrode chamber contained cell extracts (200 µl, 2.2 mg of protein), $CuCl_2$ (13 µM) and $(NH_4)_2SO_4$ (2.8 mM). Ammonia-dependent O_2 uptake was measured in the absence of BSA as shown in trace A. Palmitoleic acid (240 µM) was added at the indicated times to the reactions shown in traces B through F. Subsequently, proteins (5 mg/ml) were added to the reactions as indicated in trace C through F.

In addition to palmitoleic acid, both palmitic and oleic acids also completely and reversibly inhibited ammonia-dependent O_2 uptake (not shown). The concentration of fatty acid required for complete inhibition of ammonia-dependent O_2 uptake varied from 20 to 400 μM among different extracts. This variability may reflect differences in the age and preparation of extracts. Neither palmitoleic acid (400 μM) nor BSA (10 mg/ml) affected the rate of hydrazine-dependent O_2 uptake (not shown), which suggested that the inhibition by fatty acids was specific for a component(s) associated with AMO activity.

4.4.2. Stabilization of ammonia-dependent O_2 uptake activity by either BSA or $CuCl_2$.

A high concentration of either BSA (10 mg/ml) or $CuCl_2$ (500 μM) stabilized ammonia-dependent O_2 uptake activity for up to 2 days at 4°C (Table 4.1). In contrast, extracts stored without either BSA or $CuCl_2$ lost all ammonia-dependent O_2 uptake activity within 8 hours (Table 4.1). Partial stabilization was achieved by using a lower concentration of either BSA or $CuCl_2$, but storage with both BSA and $CuCl_2$ did not have an additive effect on stability (Table 4.1). However, the lack of an additive effect may reflect the binding of Cu^{2+} ions by BSA. Pig serum albumin (10 mg/ml) also stabilized ammonia-dependent O_2 uptake activity (not shown), but ovalbumin (10 mg/ml) did not (Table 4.1). Our results with BSA and $CuCl_2$ provides both the longest recorded stabilization of ammonia-oxidizing activity to date and the first report of an effect of $CuCl_2$ on stability.

Table 4.1. Stabilization of ammonia-dependent O_2 uptake by either $CuCl_2$ or BSA[a]

Agent(s) added (conc)	Ammonia-dependent O_2 uptake (nmol O_2 min^{-1} mg of $protein^{-1}$) after the indicated storage time (hour)[b]										
	0	4	8	12	21	28	36	44	50	62	72
none	65	75	0	0	0	0	0	0	0	0	0
$CuCl_2$ (50 μM)	59	65	52	41	0	0	0	0	0	0	0
$CuCl_2$ (500 μM)	73	75	67	58	57	62	60	54	54	0	0
BSA (1 mg/ml)	63	73	56	52	0	0	0	0	0	0	0
BSA (10 mg/ml)	63	69	62	62	58	50	49	43	41	34	0
$CuCl_2$ (500 μM) + BSA (10 mg/ml)	71	78	65	71	62	67	65	60	58	0	0
ovalbumin (10 mg/ml)	59	65	49	0	0	0	0	0	0	0	0

[a]Samples of an extract were stored at 4°C in the absence or presence of potential stabilizing agents. At the indicated times an aliquot of extract (200 μl, 2.2 mg of protein) was assayed for ammonia-dependent O_2 uptake in the presence of BSA (10 mg/ml) and $CuCl_2$ (230 μM) as described in Materials and methods. For a given sample, the rates are reproducible to within 5%. Ammonia-independent O_2 uptake rates ranged from 2 to 4 nmol O_2 min^{-1} mg of $protein^{-1}$. [b]The onset of activity for all storage conditions was characterized by the development of a lag which increased as the extracts aged. If activity was not initiated within 5 minutes following addition of the extract to the O_2 electrode chamber, then a value of 0 was recorded.

Free palmitoleic acid was detected in our extracts (Table 4.2). Consistently, lower concentrations of free palmitoleic acid were observed in extracts that had been stored with either BSA or $CuCl_2$ than in extracts stored without BSA or $CuCl_2$. The concentrations of free palmitoleic acid in the extracts were determined by HPLC for each of the extracts stored with or without either BSA or $CuCl_2$ at the start and at the end of the 1.75 hours incubation at 30°C (Table 4.2). The free palmitoleic acid concentrations were 2.3 to 3 times higher in the unamended extract than in the amended extracts (Table 4.2). Additionally, the ammonia-dependent O_2 uptake activity of each extract was measured at the start and at the end of the 1.75 hours incubation. The extracts stored with either BSA or $CuCl_2$ retained nearly 100% of their original ammonia-dependent O_2 uptake activity, while the unamended extract had lost all of its activity after 1.75 hours (Table 4.2). The inhibition of the accumulation of free palmitoleic acid by BSA or $CuCl_2$ was also observed in extracts stored at 4°C, indicating that this process was not associated solely with storage at 30°C.

Because these results suggested that the stabilizing effect of $CuCl_2$ was not due to the catalytic requirement of AMO for $CuCl_2$ but instead to the inhibition of lipolysis (Table 4.2), we tested various other metals as stabilizing agents. Of the metals tested, only $HgCl_2$ (100 μM) stabilized the ammonia-dependent O_2 uptake activity (Table 4.2). Additionally, $HgCl_2$ (100 μM) inhibited lipolysis (Table 4.2). Because PMSF is reported to inhibit lipases (Patkar and Bjorkling, 1994), it was tested as a potential stabilizing agent. PMSF (0.5 mM) stabilized ammonia-dependent O_2 uptake activity as effectively as either $CuCl_2$ (500 μM) or BSA (10 mg/ml)(Table 4.3). Because PMSF is also a well-known protease inhibitor, another protease inhibitor, leupeptin, was tested for stabilization. However, leupeptin did not stabilize ammonia-dependent O_2 uptake activity (not shown). Lecithin was reported to stabilize mitochondrial activities by protecting membranes from lipolysis by providing an alternative substrate for phospholipases (Rossi, Rossi et al., 1962). Ammonia-dependent O_2 uptake activity was stabilized by egg yolk lecithin (10 mg/ml); however, lecithin was less effective than BSA, $CuCl_2$, or PMSF (Table 4.3). Stabilization of ammonia-oxidizing activity by lecithin may result from protection of *N. europaea* membranes rather than protection from the products of phospholipid hydrolysis. Consistent with this mechanism, incubation of an extract with exogenous palmitoleic acid (1.4 mM) did not induce a loss of ammonia-oxidizing activity.

Table 4.2 Stabilization of ammonia-dependent O_2 uptake activity and the inhibition of lipolysis by $CuCl_2$, BSA or $HgCl_2$.

Storage time (hour)	Ammonia-dependent O_2 uptake (nmol O_2 min^{-1} mg $protein^{-1}$) stored with the following additions[a]				Palmitoleic acid concentration (mM) stored with the following additions[b]					
	none	$CuCl_2$ (500 µM)	BSA (10 mg/ml)	$HgCl_2$ (100 µM)	none	$CuCl_2$ (500 µM)	none	BSA (10 mg/ml)	none	$HgCl_2$ (100 µM)
0	59	63	53	57	0.3	0.2	0.4	0.3	0.2	0.2
1.75	0	58	63	57	1.8	0.5	2.8	0.9	1.6	0.5
Ratio[c]					6	2.5	7	3	8	2.5

[a]Ammonia-dependent O_2 uptake rates were determined as described in Materials and methods. [b]The extracts were stored with or without the various additions at 30°C with vigorous shaking. [b]The averages for duplicate samples are shown. The deviation from the mean fell between 0% and 16% of the mean with an average of 5%. To account for the effects of additions on experimental procedures, $CuCl_2$, BSA or $HgCl_2$ was added to extracts stored with no additions prior to $CHCl_3$ extraction, and separate standard curves were prepared for each of these amended conditions. The concentration of palmitoleic acid varied among the samples stored without additions as a result of using separate standard curves. The palmitoleic acid concentrations reported for extracts without additions are for the purpose of comparison with the respective concentration of the amended samples. [c]Ratio of the concentration of palmitoleic acid at 1.75 hour to that at 0 hour.

Table 4.3 Stabilization of ammonia-dependent O_2 uptake activity by either phenylmethylsulfonyl fluoride or lecithin[a]

Agent added (concentration)	Ammonia-dependent O_2 uptake (nmol O_2 min^{-1} mg of $protein^{-1}$) after the indicated storage time (hour)[b]										
	0	4	8	12	24	32	37	48	58	72	96
none	74	73	62	0	0	0	0	0	0	0	0
$CuCl_2$ (500 µM)	72	82	82	68	78	60	72	70	68	64	0
BSA (10 mg/ml)	76	76	82	78	76	78	72	68	64	56	0
lecithin (10 mg/ml)	56	70	62	50	50	41	37	0	0	0	0
phenylmethylsulfonyl fluoride (PMSF) (0.5 mM)	70	72	74	74	72	70	68	54	62	56	0
dimethylsulfoxide (0.14 M)[c]	66	68	52	0	0	0	0	0	0	0	0

[a]Samples of an extract were stored at 4°C in the absence or presence of various compounds. At the indicated times an aliquot of extract (200 µl, 2 mg of protein) was removed and assayed for ammonia-dependent O_2 uptake in the presence of BSA (10 mg/ml) and $CuCl_2$ (230 µM) as described in Materials and methods. For a given sample, the rates are reproducible to within 5%. Ammonia-independent O_2 uptake ranged between 2 and 4 nmol of O_2 min^{-1} mg of $protein^{-1}$. [b]The onset of activity for all storage conditions was characterized by the development of a lag which increased as the extracts aged. If activity was not initiated within 5 minutes following addition of the extract to the O_2 electrode chamber, then a value of 0 was recorded. [c]Solvent for PMSF stock solution.

4.5. Discussion.

4.5.1. Role of BSA in the *in vitro* assay of ammonia-oxidizing activity.

Our experiments have demonstrated four important points with regard to the *in vitro* assay of ammonia-oxidizing activity. First, BSA is not essential for substantial rates of ammonia-dependent O_2 uptake using freshly prepared extracts. However, during aging of extracts, BSA becomes a necessary additive for ammonia-oxidizing activity to be observed (Figure 4.1). Second, only proteins capable of binding fatty acids support ammonia-dependent O_2 uptake (Figure 4.2). Third, exogenous palmitoleic acid completely and specifically inhibited ammonia-dependent O_2 uptake activity and the inhibition was reversed only by proteins capable of binding fatty acids (Figure 4.3). Fourth, an increase in the concentration of endogenous palmitoleic acid occurred within a time frame compatible with the onset of activity becoming dependent on BSA (Figure 4.1 and Table 4.2). On the basis of these results, we propose that the role of BSA in the *in vitro* assay of ammonia-oxidizing activity is to bind inhibitory free fatty acids and that endogenous lipolysis is responsible for the release of free fatty acids during aging of the extract. In addition, we conclude that the requirement for proteins which bind fatty acids is realized once lipolysis has generated sufficient fatty acid concentrations to be inhibitory when the fatty acids are subsequently transferred, along with extracts, into enzyme assays.

Although we demonstrated reversible inhibition of ammonia-oxidizing activity by exogenous fatty acids, the exact mode of inhibition by fatty acids was not determined. A variety of inhibitory mechanisms are possible. For example, fatty acids alter membrane fluidity and therefore could alter protein associations and are also reported to uncouple energy-dependent processes (Rottenberg and Hashimoto, 1986; Skulachev, 1991). Additionally, fatty acids may bind at the active site of AMO since AMO is known to oxidize straight chain hydrocarbons such as n-octane (Hyman, Murton et al., 1988) and is inactivated by acetylenic fatty acids such as undecynoic acid (M. Hyman, unpublished results).

4.5.2. Stabilization of ammonia-oxidizing activity.

Results presented in this study have demonstrated for the first time that either BSA or copper ions stabilize ammonia-oxidizing activity in cell extracts of *N. europaea*. Under appropriate conditions, enzyme activity can be maintained for 2 to 3 days at 4°C (Tables 4.1 and 4.3). The present increase in stability represents an approximate 20-fold improvement in stability over that reported by Suzuki and Kwok (Suzuki, Kwok et al., 1981). This increased level of stabilization may provide sufficient time for future attempts at purification of AMO.

In addition to the stabilizing effect of BSA on ammonia-oxidizing activity, the presence of BSA during storage inhibited an increase in the concentration of free palmitoleic acid (Table 4.2). Furthermore, exogenous palmitoleic acid did not induce instability of ammonia-oxidizing activity. Both these results suggest that the stabilizing effect produced by BSA is due to inhibition of lipolysis rather than an indirect effect in which BSA simply absorbs the product of lipolysis.

Inhibition of lipolysis by BSA has been observed in other biological extracts (Honjo, Ozawa et al., 1968; Galliard, 1974). It is also well documented that BSA can activate and/or protect many other membrane-associated enzymatic activities (Chefurka, 1966; Ko, Frost et al., 1994). In particular, BSA has been added to numerous sub-cellular preparations involving membrane-bound enzyme complexes and electron transfer systems. Accordingly, BSA is generally regarded as essential for the production of fully active mitochondria and chloroplasts (Galliard, 1974; Papageorgiou, 1980). In the case of mitochondria, BSA had two effects on membrane-bound ATPase activity. First, BSA stimulated activity when added to the assay. Second, BSA attenuated the loss of activity during aging of the mitochondria (Chefurka, 1966). The first effect was concluded to result from the binding of free fatty acids by BSA. The second effect may have resulted from either the binding of free fatty acids or the inhibition of lipolysis (Galliard, 1974). The dual effects of BSA on ATPase activity and stability are similar to the dual effects of BSA on ammonia-oxidizing activity and stability in cell extracts.

Our results also demonstrate for the first time that $CuCl_2$, $HgCl_2$, lecithin, or PMSF can stabilize ammonia-oxidizing activity in cell-free extracts. In view of previous studies demonstrating the activating effect of Cu^{2+} on *in vitro* AMO activity (Ensign, Hyman et al., 1993), the simplest interpretation of this result is

that Cu^{2+} exerts its stabilizing effect directly on AMO. However, as was the case for BSA, our results demonstrate that either $CuCl_2$ or $HgCl_2$ inhibits the accumulation of fatty acids during storage of extracts (Table 4.2). Moreover, both Hg^{2+} and Cu^{2+} ions are known inhibitors of lipases (Wills, 1960; Nishijima, Nakaike et al., 1977); therefore, a more reasonable interpretation is that these metal ions, like BSA, stabilize enzyme activity by their inhibition of lipolysis.

The idea that stabilization of ammonia-oxidizing activity in extracts can be achieved by the inhibition of lipolysis led us to consider alternative procedures which might be useful in future attempts at purification of AMO. The partial stabilizing effect of lecithin (Table 4.3) suggests that some degree of stabilization can be achieved by adding an alternative lipase substrate. Lecithin has been suggested to stabilize mitochondrial membranes by diverting lipase activity away from cell membranes (Rossi, Rossi et al., 1962). Treatment of cell extracts with PMSF is probably another example of direct inhibition of lipase activity given that PMSF is known to inhibit lipases (Patkar and Bjorkling, 1994), in addition to having a more traditionally recognized role as a protease inhibitor. However, we did not unequivocally eliminate proteolysis as a cause for the loss of activity. Certainly the use of PMSF is the most promising stabilizing treatment we have developed, since PMSF stabilizes as well as either BSA or Cu^{2+} ions (Table 4.3) and does not involve the use of additional proteins, metals, or lipids in potential purification schemes.

CHAPTER 5

Cell-free ammonia-dependent O_2 uptake by extracts of *Nitrosomonas europaea*: Reversible inhibition by palmitoleic acid and n-heptylhydroxyquinoline N-oxide

5.1. Abstract.

Ammonia-oxidizing activity in extracts of *Nitrosomonas europaea* is completely inhibited by palmitoleic acid (240 μM) when added during the course of ammonia-dependent O_2 uptake (Juliette, Hyman et al., 1995). The addition of palmitoleic acid (50 to 125 μM) to the assay medium prior to the initiation of ammonia-dependent O_2 uptake introduced a lag. Increasing the concentration of palmitoleic acid introduced an increasing lag in the initiation of activity. Coincidentally, an increasing lag in the onset of ammonia-dependent O_2 uptake activity was also associated with aging extracts, which suggested that endogenous fatty acids may play a role. An increase in fatty acids is reported to occur during aging of extracts of *N. europaea* (Juliette, Hyman et al., 1995). However, the physiological reason for the lag was not determined. The possibility that palmitoleic acid uncoupled activity by acting as a protonophore was eliminated by the demonstration that neither 2,4 dinitrophenol (100 μM) nor carbonyl cyanide m-chlorophenylhydrazone (11 μM) inhibited the rate of ammonia-dependent O_2 uptake. However, the electron transfer inhibitor, n-heptylhydroxyquinoline N-oxide (HQNO) (200 μg/ml) completely inhibited ammonia-dependent O_2 uptake. Similar to the inhibition of ammonia-dependent O_2 uptake by palmitoleic acid, the inhibition by HQNO was relieved by either BSA or hydroxylamine. The inhibition of activity by HQNO suggested that electron transport processes may be required for ammonia-dependent O_2 uptake activity. In a separate experiment on the stabilization of ammonia- and hydroxylamine-oxidizing activities by agents which modify lipase activity, both activities were stabilized or lost concurrently in cell extracts. This result provides the first evidence that the loss of ammonia-oxidizing activity results from the loss of coupling integrity among enzymes involved in the ammonia oxidation pathway.

This result also emphasizes the need for establishing an AMO assay which is independent of HAO activity.

5.2. Introduction.

Nitrosomonas europaea is an autotrophic bacterium which obtains all of its energy for growth from the oxidation of ammonia to nitrite. The oxidation of ammonia is initiated by the membrane-associated enzyme known as ammonia monooxygenase (AMO) (Eqn. 1).

$$NH_3 + O_2 + 2e^- + 2H^+ \longrightarrow NH_2OH + H_2O \qquad (1)$$

$$NH_2OH + H_2O \longrightarrow NO_2^- + 5H^+ + 4e^- \qquad (2)$$

The hydroxylamine generated by AMO is then oxidized to nitrite by the enzyme hydroxylamine oxidoreductase (HAO)(Eqn. 2). The oxidation of hydroxylamine by HAO is thought to provide a source of reductant *in vivo* for AMO (Wood, 1986). Although HAO has been purified and studied in considerable detail, much less is known about AMO. To date, the purification of AMO with activity has not been achieved. Two features of AMO which have hindered progress in this area include the apparent instability of ammonia-oxidizing activity in extracts and the lack of a suitable electron donor for AMO (Ensign, Hyman et al., 1993). Known electron donors for AMO include: substrates for HAO, hydroxylamine and hydrazine; NADH, which can act as a priming agent to initiate activity but does not sustain activity in isolated membranes; and tetra- and tri-methylhydroquinones have been shown to enhance AMO-dependent propane oxidation (Wood, 1986). The direct electron donor to AMO is unknown. During steady state ammonia oxidation, reductant is provided via the oxidation of hydroxylamine by HAO.

Currently, neither NADH nor tetra- and tri-methylhydroquinone can serve as adequate reductant sources for AMO *in vitro*. Cell-free ammonia-oxidizing activity is believed to rely on a high degree of coupling: for example, the

hydroxylamine formed during ammonia oxidation must be oxidized by HAO, and the electrons must be donated to AMO for the continued oxidation of ammonia. Therefore, the ability to measure ammonia-dependent O_2 uptake *in vitro* may be dependent on the integrity of an electron transfer complex composed of AMO, HAO and any components involved in supplying electrons to AMO. A better understanding of this assay feature may lead to a more reliable assay system for AMO.

Fatty acids (20-400 µM) are reported to completely inhibit ammonia-dependent O_2 uptake of cell extracts when added during O_2 uptake activity (Juliette, Hyman et al., 1995). The inhibition of ammonia-dependent O_2 uptake activity by fatty acids was reversed by proteins which bind fatty acids, such as bovine serum albumin (BSA) or ß-lactoglobulin. Neither palmitoleic acid (400 µM) nor BSA (10 mg/ml) affected the HAO activity, measured as hydrazine-dependent O_2 uptake, which suggested that the inhibition by fatty acids was specific for component(s) associated with AMO activity (Juliette, Hyman et al., 1995). However, the mechanism for the reversible inhibition of ammonia-dependent O_2 uptake activity by fatty acids was not addressed.

In this manuscript, the inhibition of ammonia-dependent O_2 uptake by palmitoleic acid was investigated. The possibility that palmitoleic acid uncoupled activity by acting as a protonphore was eliminated by the demonstration that neither 2,4 dinitrophenol nor carbonyl cyanide m-chlorophenylhydrazone inhibited ammonia-dependent O_2 uptake. However, n-heptylhydroxyquinoline N-oxide (200 µg/ml) (HQNO), a electron transport inhibitor, completely inhibited ammonia-dependent O_2 uptake activity. Our results suggested that the mechanism for the inhibition of ammonia-dependent O_2 uptake by fatty acids may be related to the inhibition of electron transport processes by fatty acids reported for other bioenergetic systems (Rottenberg and Hashimoto, 1986; Skulachev, 1991). In addition to our investigation of the inhibition of activity by fatty acids, evidence which suggests that cell-free ammonia-dependent O_2 uptake activity is dependent on HAO activity is also presented.

5.3. Materials and methods.

5.3.1. Materials.

All reagents were obtained from Sigma except that ultrapure $CuCl_2$ (99.99%), $HgCl_2$ (99.5+ %), triethylamine, and 2,4'-dibromoacetophenone were obtained from Aldrich. C_2H_2 was generated in a gas-generating bottle from calcium carbide (approximately 80%, Aldrich Chemical Co. Inc., Milwaukee, WI) as previously described (Hyman and Arp, 1987).

5.3.2. Growth of bacteria and preparation of cell-free extracts.

N. europaea (ATCC 19178) cultures were grown in a 100 l carboy and cell extracts were prepared as previously described (Juliette, Hyman et al., 1995) For experiments comparing ammonia- and hydrazine-dependent O_2 uptake rates, samples of an extract were stored (2 days, 4°C) with or without the various stabilizing agents as previously described (Juliette, Hyman et al., 1995).

5.3.3. O_2 electrode measurements.

Ammonia-dependent O_2 uptake measurements were determined with a Clark-type oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio) mounted in a water-jacketed reaction vessel (1.7 or 1.8 ml volume) maintained at 30°C. Ammonia-dependent O_2 uptake activity was measured in buffer (0.1 M KH_2PO_4, pH 8.0) with $(NH_4)_2SO_4$ (2.8 mM), either in the presence of BSA (10 mg/ml), $CuCl_2$ (230 µM) or in the absence of BSA with only $CuCl_2$ (12-15 µM). Unless indicated otherwise, the extract (200 µl, 2.2 to 2.7 mg of protein) was added to the assay buffer after $(NH_4)_2SO_4$ (2.8 mM). Hydroxylamine oxidoreductase activity of the extract (200 µl, 2.2 to 2.7 mg of protein) was measured in buffer (0.1 M KH_2PO_4, pH 8.0) as hydrazine (1 mM)-dependent O_2 uptake. Stock solutions of inhibitors were prepared daily in either buffer (0.1 M KH_2PO_4, pH 7.5) or dimethylsulfoxide.

5.3.4. Analytical procedures.

To test whether either palmitic or palmitoleic acid were substrates for AMO, samples of an extract were incubated (2 hr, 30°C) with $CuCl_2$ (15 μM), and hydrazine (2 mM) as a reductant source, in the presence of either palmitic or palmitoleic acid (0.5 mM) with or without the AMO specific inactivator, acetylene (C_2H_2). After the incubation, the fatty acids present in the suspensions were extracted, derivatized and analyzed by HPLC as previously described (Juliette, Hyman et al., 1995).

5.4. Results.

5.4.1. Palmitoleic acids increased the lag associated with the onset of ammonia-dependent O_2 uptake.

Addition of low concentrations of exogenous palmitoleic acid to the assay media prior to the start of ammonia-dependent O_2 uptake induced a lag in the onset of activity (Figure 5.1). The lag time increased with increasing concentrations of palmitoleic acid; however, the maximal rates of ammonia-dependent O_2 uptake were similar (Figure 5.1). The addition of BSA, a fatty acid binding protein, to the assay chamber shortened the lag (Figure 5.1E). Similar to the effect of BSA, the addition of a low amount of hydroxylamine (20 μM) (Figure 5.1F) or hydrazine (not shown) initiated, but did not sustain, ammonia-dependent O_2 uptake activity.

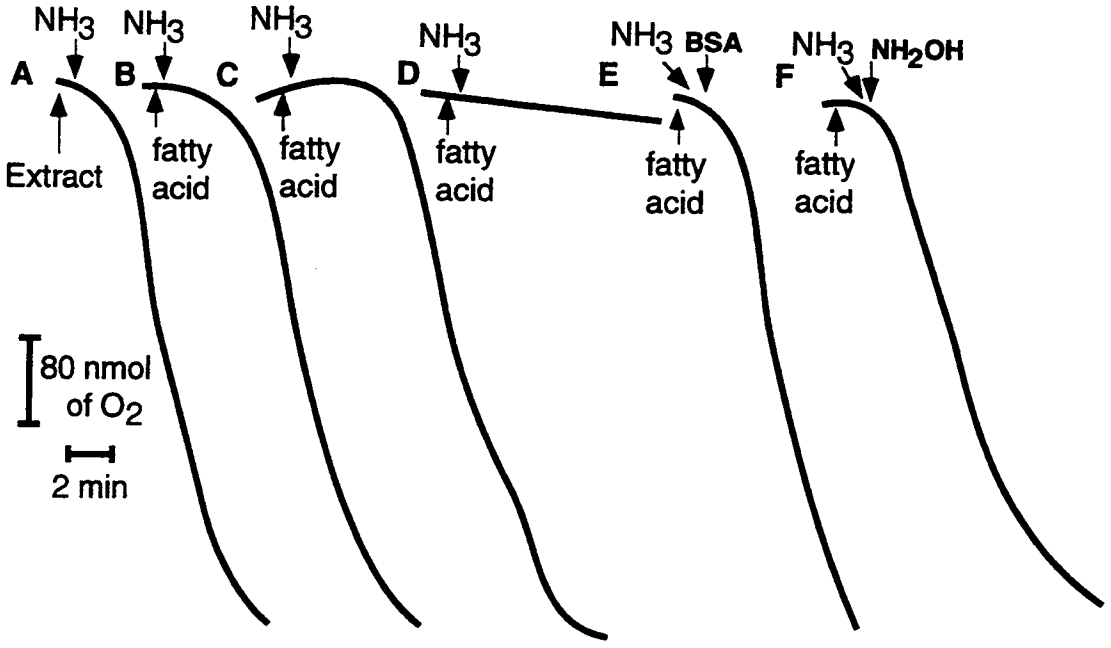


Figure 5.1. Effect of palmitoleic acid concentration on the initiation of ammonia-dependent O_2 uptake. Ammonia-dependent O_2 uptake was measured in the absence of BSA as described in Materials and methods. Sequentially, $CuCl_2$, extract (200 µl, 2.2 mg of protein), palmitoleic acid, and $(NH_4)_2SO_4$ were added to the O_2 electrode chamber buffer. Trace A is the rate of ammonia-dependent O_2 uptake in the absence of exogenous palmitoleic acid. Increasing concentrations of palmitoleic acid (50, 100, and 125 µM) were added respectively at the indicated times in traces B through D. Traces E and F were identical to trace D, except that either BSA (5 mg/ml) or NH_2OH (20 µM) was added at the indicated times.

5.4.2. The onset of ammonia-dependent O_2 uptake developed an increasing lag as the extracts aged.

Previously, it was reported that the onset of ammonia-dependent O_2 uptake activity measured in the presence of BSA was associated with a lag which increased as extracts of *N. europaea* aged (Juliette, Hyman et al., 1995). The initiation of BSA-independent ammonia-dependent O_2 uptake activity also developed an increasing lag as the extract aged (Figure 5.2). Despite the lag, the rates of ammonia-dependent O_2 uptake were comparable. Once ammonia-dependent O_2 uptake activity was apparently lost (Figure 5.2E), initial rates of ammonia-dependent O_2 uptake could be reinstated by the addition of either BSA

or a low amount of a reductant source for AMO such as hydroxylamine (Figures 5.2F and G), trimethylhydroquinone (Figure 5.2H), or hydrazine (not shown). However, in contrast to BSA, the initiation of activity afforded by either hydroxylamine or trimethylhydroquinone was of limited duration and the rates declined (Figures 5.2G and H). Storage of the extract with BSA has previously

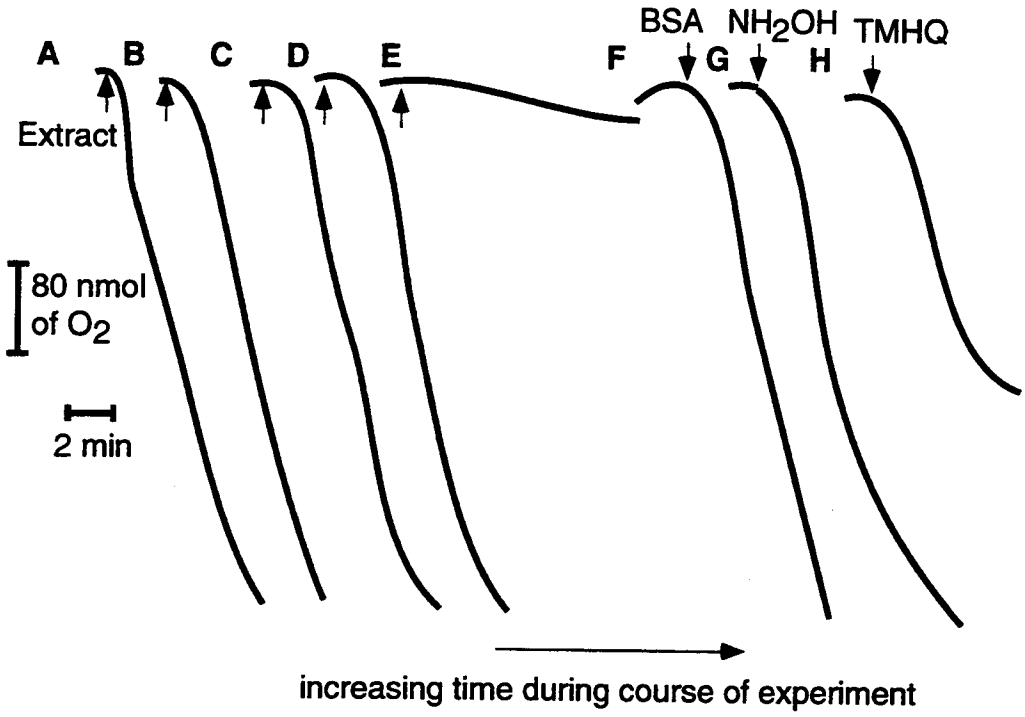


Figure 5.2. The increase in time to the onset of ammonia-dependent O_2 uptake during storage. Samples of an extract were assayed in the absence of BSA as described in Material and methods. Ammonia-dependent O_2 uptake was measured immediately after preparation of cell extract (trace A) and after 1, 2, 3 and 3.5 hours of storage (respectively, traces B through E). Traces F through H were similar to that of trace E, except that BSA (5 mg/ml), NH_2OH (20 μM) or TMHQ (20 μM) was added to the respective reactions at the indicated times.

been reported to stabilize activity (Juliette, Hyman et al., 1995). For extracts stabilized with BSA, the lag in the onset of activity developed more slowly (not shown). For example, the lag progressively increased from 0.5 to 25 minutes over the course of several days before ammonia-dependent O_2 uptake activity was apparently lost. The addition of a low amount of a reductant source for AMO also reinstated activity (not shown).

5.4.3. Effects of protonophores on ammonia-dependent O_2 uptake.

Although the addition of exogenous fatty acids to the assay of ammonia-oxidizing activity induced a lag in the onset of activity, the mechanism by which fatty acids reversibly inhibited activity remained unclear. Two possible mechanisms were considered. First, cell-free ammonia-oxidizing activity may require a membrane potential gradient to support activity. Fatty acids are reported to uncouple energy-dependent processes (Rottenberg and Hashimoto, 1986; Skulachev, 1991) and the accumulation of free fatty acids in submitochondrial particles has been proposed to lead to the loss of oxidative phosphorylation efficiency (Pressman and Lardy, 1956; Wojtczak and Lehninger, 1961; Chefurka, 1966; Chefurka and Dumas, 1966). Therefore, the effect of two uncouplers on BSA-independent ammonia-dependent O_2 uptake was examined. Neither 2,4 dinitrophenol (DNP) (100 μM) nor carbonyl cyanide m-chlorophenylhydrazone (CCCP) (11 μM) affected the initial rate of ammonia-dependent O_2 uptake when added during (Figure 5.3) or before the start of activity (not shown). The addition of ten fold higher concentrations of either of these protonphores did not inhibit the initial rates when added during O_2 uptake; however, both DNP and CCCP inhibited activity when added prior to the onset of O_2 uptake (not shown). The significance of this inhibition was not investigated. Our results from using DNP and CCCP suggested that the ammonia-dependent O_2 uptake is not dependent on a proton motive force.

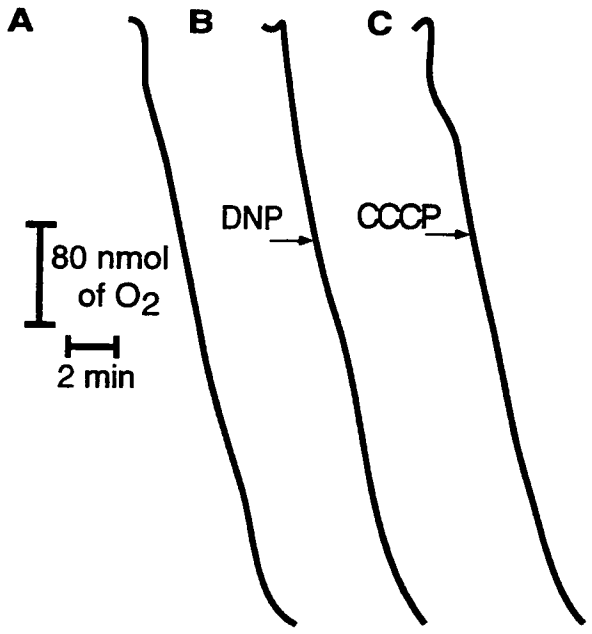


Figure 5.3. The rate of ammonia-dependent O_2 uptake is unaffected by either 2,4 dinitrophenol or carbonyl cyanide m-chlorophenylhydrazone. Ammonia-dependent O_2 uptake was measured in the absence of BSA (trace A) as described in Materials and methods. The O_2 electrode chamber contained cell extracts (200 µl, 2.2 mg of protein), $CuCl_2$ (13 µM) and $(NH_4)_2SO_4$ (2.8 mM). Dinitrophenol (DNP) (100 µM) or carbonyl cyanide m-chlorophenylhydrazone (CCCP) (11 µM) was added at the indicated times to the reactions shown in traces B and C, respectively.

5.4.4. Effects of n-heptylhydroxyquinoline on ammonia-dependent O_2 uptake.

To test whether electron transport processes were required for ammonia-dependent O_2 uptake, we considered whether electron transfer inhibitors affected ammonia-dependent O_2 uptake. The electron transfer inhibitor, n-heptylhydroxyquinoline N-oxide (200 µg/ml) (HQNO) profoundly inhibited ammonia-dependent O_2 uptake (Figure 5.4). The addition of a higher concentration of HQNO (400 µg/ml) caused a more rapid loss of activity (Figures 5.4B and C). Similar to the inhibition of ammonia-dependent O_2 uptake by palmitoleic acid, the inhibition by HQNO was relieved by BSA (Figure 5.4D) or a low amount of a reductant source for AMO such as hydroxylamine (Figure 5.4E). As before, hydroxylamine did not sustain activity but only temporarily relieved the

inhibition after which BSA reinstated activity (Figure 5.4E). The effect of HQNO on the onset of activity was not addressed. The inhibition of ammonia-dependent O_2 uptake by HQNO was similar to that by palmitoleic acid, which suggested that fatty acids inhibited activity by affecting electron transport. However, the mechanism of fatty acid inhibition of ammonia-dependent O_2 uptake remains uncertain.

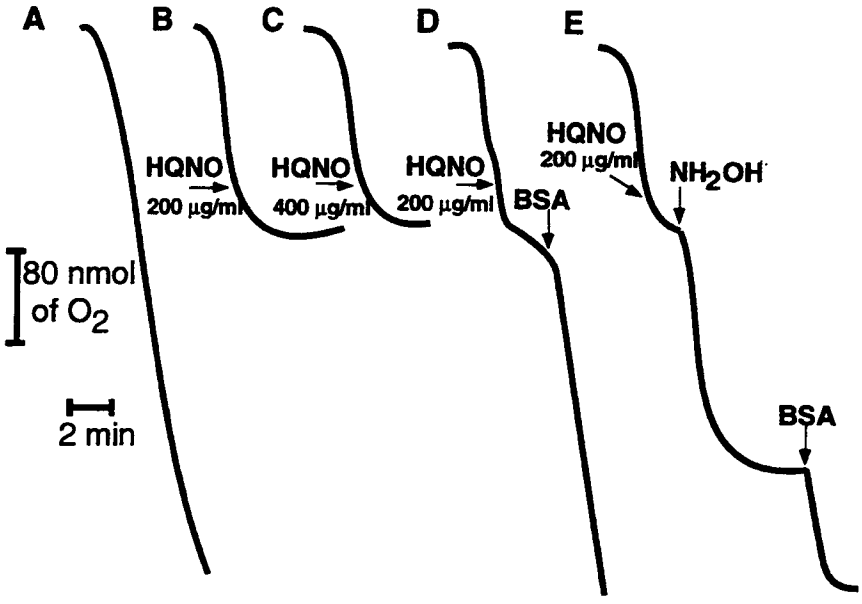


Figure 5.4. Inhibition of ammonia-dependent O_2 uptake by n-heptylhydroxyquinoline N-oxide (HQNO) and relief of the inhibition by either BSA or hydroxylamine. Ammonia-dependent O_2 uptake was measured as described in Materials and methods in the absence of BSA. The O_2 electrode chamber contained cell extracts (200 µl, 2.2 mg of protein), $CuCl_2$ (13 µM) and $(NH_4)_2SO_4$ (2.8 mM). Ammonia-dependent O_2 uptake was measured in the absence of BSA as shown in trace A. HQNO (200 or 400 µg/ml) was added at the indicated times to the reactions shown in traces B and C, respectively. HQNO (200 µg/ml) was added to the reactions shown in traces D and E at the indicated times and subsequently BSA (5 mg/ml) (trace D) or both hydroxylamine (20 µM) and BSA (5 mg/ml) (trace E) were added at the indicated times.

5.4.5. Fatty acids as substrates for AMO.

Another mechanism by which fatty acids might have reversibly inhibited ammonia-dependent O_2 uptake is if they were substrates for AMO thereby displacing ammonia from the active site. However, preliminary experiments suggested that neither palmitic nor palmitoleic acid was a substrate for AMO. Extracts were incubated with either palmitic or palmitoleic acid (0.5 mM) in the presence or absence of C_2H_2. The HPLC profiles of the derivatized fatty acid products resulting from these incubations were similar, which indicated that AMO-dependent fatty acid oxidation products were either below the limits of detection or not present (not shown).

5.4.6. Effect of agents which stabilize ammonia-dependent O_2 uptake on hydrazine-dependent O_2 uptake.

Agents which modify or inhibit lipase activity have been shown to stabilize ammonia-dependent O_2 uptake activity (Juliette, Hyman et al., 1995). The effect of these stabilizing agents on cell-free HAO activity, measured as hydrazine-dependent O_2 uptake, was examined because it has been suggested that disruption of the membrane, by lipolysis, results in a loss of coupling integrity between AMO and HAO, which leads to the loss of ammonia-dependent O_2 uptake activity (Juliette, Hyman et al., 1995). In support of these suggestions, evidence is presented that stabilization of ammonia-dependent O_2 uptake activity by BSA, $CuCl_2$, or lecithin, agents which modify or inhibit lipase activity, also stabilized HAO activity (Figure 5.5). More importantly, the ammonia- and hydroxylamine-oxidizing activities were stabilized or lost concurrently (Figure 5.5). The concurrent loss and stabilization of both ammonia- and hydrazine-dependent O_2 uptake activities suggested that these activites are interdependent. Furthermore, these results suggested that loss of coupling among these activities occurred during storage of extracts and may result in the apparent loss of ammonia-dependent O_2 uptake activity.

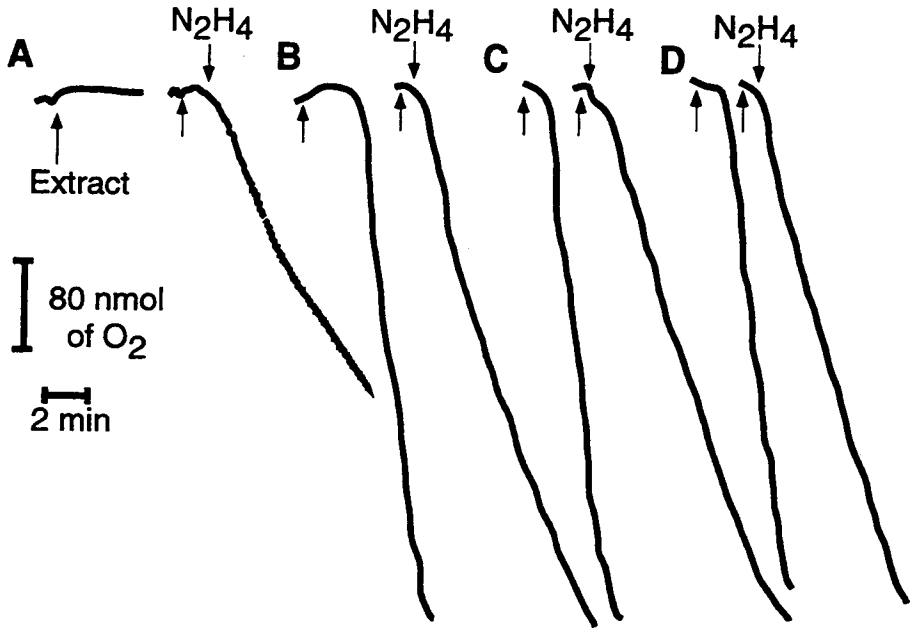


Figure 5.5. Hydrazine-dependent O_2 uptake activity for cell extracts which have lost or retained ammonia-dependent O_2 uptake activity. Samples of an extract were stored (2 days, 4°C) in the absence (traces A) or presence of the following stabilizing agents: BSA (10 mg/ml) (traces B), $CuCl_2$ (500 μM) (traces C), or lecithin (10 mg/ml) (traces D). Two traces are shown for each of the four storage conditions. The first trace is the rate of ammonia-dependent O_2 uptake measured in the presence of BSA (10 mg/ml) and $CuCl_2$ (230 μM) and the second trace is the rate of hydrazine-dependent O_2 uptake measured as described in Material and methods. An aliquot of each sample (200 μl, 2.2 mg of protein) was added as indicated at the arrow.

5.5. Discussion.

5.5.1 Inhibition of cell-free ammonia-dependent O_2 uptake by palmitoleic acids.

Our results suggested that the lag associated with the ammonia-dependent O_2 uptake of aged extracts may be due to the accumulation of endogenous fatty acids because 1) increasing concentrations of exogenous palmitoleic acid added to the assay media prior to the start of activity induced an increasing lag in the onset of activity (Figure 5.1) and 2) both BSA and reductant sources for AMO, such as hydroxylamine, hydrazine or TMHQ, activate the onset of ammonia-

dependent O_2 uptake for either aged extracts or extracts treated with palmitoleic acids. A rise in the concentration of endogenous fatty acids occurred as extracts of *N. europaea* age (Juliette, Hyman et al., 1995). Additionally, BSA both inhibited lipolysis in extracts (Juliette, Hyman et al., 1995) and slowed the development of a lag in the onset of ammonia-dependent O_2 uptake activity (not shown).

Hydroxylamine also relieved a lag associated with the oxidation of ammonia by resting cells of *N. europaea* (Hooper, 1969). However, the reason(s) for the lag in ammonia oxidation by intact cells has not been determined. Whether this lag is physiologically relevant to the lag we observed using either aged extracts or fatty acids is uncertain. Additionally, a lag was previously observed during the reduction of cytochrome c-554 by ammonia when using a membrane fraction of *N. europaea*. The lag period was shortened by increasing the concentration of ammonia (Tsang and Suzuki, 1982). It was speculated that the lag period associated with cytochrome reduction by ammonia may be time required for the build up of a reductant source necessary for cytochrome reduction (Suzuki, 1974). However, the cause of this lag is not known. In summary, the inhibition of cell-free ammonia-dependent O_2 uptake by fatty acids was associated with a lag in the onset of activity; however, the mechanism for the inhibition remained unclear. Further investigation of the underlying reason(s) for the lag may provide insight into the mechanism of fatty acid inhibition.

A substantial amount of information exists with regard to the inhibition of other enzyme activities by fatty acids (Wojtczak, 1976; Rottenberg and Hashimoto, 1986). Free fatty acids uncouple oxidative phosphorylation in chloroplasts, mitochondria and submitochondrial particles (Pressman and Lardy, 1956). The uncoupling was reversed by BSA. It seems likely that ammonia-dependent O_2 uptake activity requires an intact electron transport chain. However, neither 2,4 dinitrophenol nor CCCP changed the rate of ammonia-dependent O_2 uptake, which suggested that fatty acids do not inhibit activity by acting as protonophores. Although fatty acids are described as uncouplers, it is not expected that long-chain fatty acids would have pronounced protonophoric activity nor is the mechanism by which fatty acids uncouple oxidative phosphorylation precisely understood (Rottenberg and Hashimoto, 1986; Skulachev, 1991).

At present, none of the proposed mechanisms for the uncoupling of oxidative phosphorylation by fatty acids is entirely satisfactory (Rottenberg, 1990). In general, it has been recognized that fatty acids uncouple oxidative phosphorylation without a significant reduction in the proton electrochemical potential gradient. As a result of this characteristic, it is suggested that fatty acids be referred to as decouplers rather than uncouplers (Rottenberg, 1990). Other characteristics of the uncoupling effect of oxidative phosphorylation by fatty acids include 1) ATP synthesis is inhibited by fatty acids when driven by substrate oxidation but not when driven by an artifically imposed proton gradient 2) fatty acids enhanced the oligomycin sensitivity of the ATPase and 3) the generation of a proton gradient by ATP hydrolysis was inhibited (Rottenberg, 1990). These last two characteristics have led to the suggestion that fatty acids have a direct effect on ATPase activity (Rottenberg and Hashimoto, 1986). Alternatively, the uncoupling has also been suggested to be mediated by the ADP/ATP translocator. However, this model involves the dissipation of the protonic potential (Skulachev, 1991).

In contrast to either DNP or CCCP, the electron transfer inhibitor, HQNO, strongly inhibited ammonia-dependent O_2 uptake activity. Similar to the inhibition by palmitoleic acid, the inhibition by HQNO was relieved by either BSA or hydrazine, which suggested that the mode of inhibition by HQNO may be similar to that by palmitoleic acid. While the relief of the inhibitory effect of palmitoleic acids and HQNO by BSA could be understood in terms of the ability of BSA to bind these hydrophobic molecules, the relief of inhibition by reductant sources for AMO is not understood.

The inhibition of ammonia-dependent O_2 uptake by HQNO suggested that electron transfer processes may be involved in activity. HQNO inhibits both bacterial and mitochondrial bc_1 complexes by blocking electron transfer from the heme b center to ubiquinone (von Jagow and Link, 1986; Anraku, 1988). Interestingly, duroquinol has recently been used as an electron donor for cell-free particulate methane monooxygenase (pMMO) activity (Shiemke, Cook et al., 1995). This enzyme system has many features in common with AMO. Furthermore, the potential involvement of electron transfer processes suggested by the inhibitory effect of HQNO, supported the idea that the mechanism by which fatty acids inhibit electron transport processes in other bioenergetic systems may be similar to the mechanism by which fatty acids inhibit ammonia-dependent O_2 uptake. The concentration of palmitoleic acid used to completely

inhibit ammonia-dependent O_2 uptake was approximately 5 times higher than the concentration of fatty acids used to partially inhibit oxidative phosphorylation in submitochondrial particles (Rottenberg and Hashimoto, 1986; Juliette, Hyman et al., 1995). The fact that fatty acids have been reported to inhibit energy-associated processes in chloroplasts, mitochondrial electron transport particles and cell-free ammonia-oxidizing activity is intriguing.

Additionally, we considered whether fatty acids were substrates for AMO. AMO has a broad substrate specificity and is specifically and reversibly inhibited by many alternative substrates which compete with NH_3 for turnover. Long-chain hydrocarbons up to C_8 are alternative substrates for AMO (Hyman, Murton et al., 1988) and undecynoic acid, a C_{11} alkyne, inactivated AMO (M. Hyman, unpublished results), which suggested that fatty acids could be accessible to the active site of AMO. Fatty acids have also been described as substrates for other monooxygenases (Kupfer, 1980). Although we did not unequivocally eliminate the possibility that fatty acids are alternative substrates for AMO, our results do not support that either palmitic or palmitoleic acids were substrates for AMO. However, the profound inhibitory effect of fatty acids on ammonia-dependent O_2 uptake activity is not typical of other alternative substrates and therefore it seemed unlikely that the inhibition resulted from their behavior as substrates for AMO.

In summary, the mechanism by which fatty acids inhibit cell-free ammonia-oxidizing activity remains uncertain. Theoretically, the inhibition by fatty acids may be simply due to a physical change in the membrane, such as an alteration in the fluidity of the membrane which could lead to a reversible dissociation of proteins and/or polypeptides necessary for activity. BSA, by removing both fatty acids and lysophospholipids from the membranes, could reverse the physical change(s) in the membrane structure and thus restore ammonia-dependent O_2 uptake. However, HAO activity is not affected by fatty acids and therefore the temporary relief of inhibition by fatty acids afforded by reductant sources for AMO, such as hydrazine and hydroxylamine, is inconsistent with an inhibition caused by a structural change. In contrast to BSA, these reductant sources would not be expected to change membrane structure. Therefore, a physical change in the membrane caused by fatty acids a seemed less than satisfactory explanation for the inhibition of activity by fatty acids. The mechanisms by which BSA and reductant sources relieve the inhibition of ammonia-dependent O_2

uptake by fatty acids is likely to be different and may involve separate pathways of electron flow to AMO.

5.5.2 Stabilization of both ammonia- and hydrazine-dependent O_2 uptake.

The cause for the loss of ammonia-dependent O_2 uptake activity was also investigated. Previously reported agents which modify or inhibit lipase activity preserved cell-free ammonia-dependent O_2 uptake activity (Juliette, Hyman et al., 1995). The effect of these stabilizing agents on cell-free HAO activity was examined because it has been proposed that disruption of the membrane by lipolysis results in the loss of coupling among enzymes necessary for ammonia-dependent O_2 uptake (Juliette, Hyman et al., 1995). This suggestion implies that when cell-free ammonia-dependent O_2 uptake is lost then cell-free HAO activity would likely be affected. Our results suggest that the loss of coupling between AMO and HAO activities occurred in extracts and that protein associations necessary for ammonia- and hydrazine-oxidizing activity are stably maintained by agents which modify lipase activity. Importantly, the cell-free ammonia- and hydrazine-oxidizing activities were either stabilized or lost concurrently, which implies that they are interdependent and therefore coupled in extracts active for ammonia-dependent O_2 uptake. This result provides the first evidence that the loss of ammonia-oxidizing activity may result from the loss of coupling integrity among enzymes involved in the ammonia oxidation pathway. This result also emphasizes the need for establishing an AMO assay which is independent of HAO activity.

Chapter 6

Summary

6.1. Introduction.

Characterization of the ammonia-oxidizing bacteria and the enzyme, ammonia monooxygenase (AMO), is of importance for many reasons. The predominant reasons are environmental. The ammonia-oxidizing bacteria catalyze critical reactions involved in the nitrogen biogeochemical cycle. AMO initiates these reactions by catalyzing the oxidation of ammonia. Because ammonia is the sole source of energy for ammonia-oxidizing bacteria, its oxidation by AMO is a critical step in their bioenergetic pathway. However, little is known about AMO. AMO has not been purified with activity. Additionally, results from sequencing of the genes coding for the two putative polypeptide components of AMO suggest that AMO may be a novel monooxygenase with no significant sequence homology with other monooxygenases (McTavish, Fuchs et al., 1993) except pMMO. This result further enhances the mystery surrounding this interesting enzyme.

6.2 Inhibition of the ammonia oxidation in *N. europaea* by sulfur compounds: Thioethers are oxidized to sulfoxides by AMO.

In contrast to other broad substrate monooxygenases, AMO had not been previously shown to oxidize the sulfur atom. Both carbon disulfide and allylthiourea are specific inhibitors of AMO; however, they are not thought to be substrates for AMO (Hyman, Kim et al., 1990). The substrate range of AMO was expanded to include oxidation at the sulfur atom of thioethers.

Dimethylsulfide specifically inhibited ammonia oxidation but did not affect hydroxylamine oxidation. Dimethylsulfide was depleted by cells of *N. europaea*. Depletion required O_2 and was prevented by specific inhibitors of AMO, which suggested that it was a substrate for AMO. Dimethylsulfide depletion occurred concomitantly with the formation of dimethylsulfoxide. By using a similar set of

criteria, AMO was shown to oxidize the sulfur atom of six other thioethers. For three of the six thioethers, the amount of sulfoxide formed corresponded to the amount of thioether depleted, which suggested that sulfoxides were the only products formed. Allylmethylsulfide was shown to be oxidized to allylmethylsulfoxide by *N. europaea* with the incorporation of a single atom of ^{18}O derived from $^{18}O_2$ into the sulfoxide. This result supported the involvement of a monooxygenase in the oxidation. In contrast to some other monooxygenases (Renwich, 1989), the oxidation of sulfoxide to sulfone was not catalyzed by AMO.

Similar to the S-oxygenation reactions catalyzed by cytochrome P-450 (Watanabe, Iyanagi et al., 1980), future kinetics studies on the thioether oxidation by isolated AMO may prove useful in determining enzymatic steps in AMO catalysis. For example, the enzymatic oxidation of some thioethers by cytochrome P-450 monooxygenases yield substantial amounts of both S-dealkylated and S-oxygenated products; this result has led to the suggestion that the sulphenium cation radical is an intermediate in the reaction (Ziegler, 1989). Conversely, it has been suggested that radical intermediates are not involved in thioether oxidation by flavin-containing monooxygenases because sulfoxides are the only products obtained (Ziegler, 1984). A potential application for enzymatic sulfoxidation by AMO includes the generation of highly desirable stereo- and regioselective sulfoxides (May and Katopodis, 1986). However, preliminary results suggested that allymethylsulfoxide produced by *N. europaea* were not regiospecific (Eric Block, personal communication).

Dimethylsulfide is an important volatile biogenic compound involved in the transfer of sulfur from the ocean to the atmosphere (Wakeham and Dacey, 1989). In the troposphere dimethylsulfide is photochemically oxidized to sulfuric acids. Sulfuric acids attract water molecules promoting cloud formation, these events profoundly influence climate (Taylor and Gilchrist, 1991). The primary source of dimethylsulfide is oceanic phytoplankton, which use the dimethylsulfide precursor dimethyl propiothetin as an osmoticum.

Microbial consumption of dimethylsulfide is an important aspect of dimethylsulfide biogeochemistry in seawater. However, the influence of microbial processes on the flux of dimethylsulfide to the atmosphere is poorly understood (Kiene, 1990; Kiene and Bates, 1990). The oxidation of dimethylsulfide by oceanic bacteria has been suggested to significantly compete with the flux of dimethylsulfide to the atmosphere (Kiene and Bates, 1990). However, the

aerobic metabolism of dimethylsulfide using pure cultures marine bacteria had not previously been documented (Wakeham and Dacey, 1989).

The marine ammonia-oxidizing bacterium, *Nitrosococcus oceanus*, was shown to oxidize dimethylsulfide, a volatile form of sulfur, to dimethylsulfoxide, a relatively nonvolatile form of sulfur. The oxidation was prevented by C_2H_2, which suggested that AMO catalyzed the oxidation. These results raised the possibility that the process of co-oxidation of dimethylsulfide by marine ammonia-oxidizing bacteria may contribute to the oceanic sulfur cycle. However, because the reduction of dimethylsulfoxide back to dimethylsulfide is also a common occurrence in the marine environment (Taylor and Kiene, 1989), the overall significance of the activity of ammonia-oxidizing bacteria on the global sulfur cycle is tenuous.

6.3. Mechanism-based inactivation of AMO in *N. europaea* by allylsulfide.

In contrast to six other thioethers, which were alternative substrates for AMO, allylsulfide caused an irreversible inactivation of AMO activity in intact cells of *N. europaea* (Juliette, Hyman et al., 1993a). The HAO activity was unaffected by allylsulfide, which indicated that the inactivation was specific for ammonia oxidation. As expected for a mechanism-based inactivators, conditions under which enzyme turnover was prevented, such as anaerobic conditions or the presence of a reversible inhibitor of AMO, protected AMO from inactivation by allylsulfide (Juliette, Hyman et al., 1993b). The inactivation of AMO followed first-order kinetics. However, in contrast to the properties expected of a mechanism-based inactivator, the observed rates did not saturate with increasing allylsulfide concentrations, and high concentrations of ammonia did not protect AMO from allylsulfide inactivation. Instead, high concentrations of ammonia increased the rate of inactivation. The results remain unexplained but may be due to the use of intact cells or to the requirement of reductant for AMO turnover of allylsulfide.

Inactivation of AMO by allylsulfide prevented the turnover of C_2H_2 by AMO. Increasing concentrations of allylsulfide progressively prevented ^{14}C-label from $^{14}C_2H_2$ from being incorporated into the 27-kD polypeptide component of AMO. This result verified that allylsulfide caused a concentration-dependent inactivation of AMO. The sulfur atom of allylsulfide was required for the

inactivation because compounds structurally similar to allylsulfide, but lacking sulfur, did not inactivate AMO.

The inactivating species of a mechanism-based inactivator is expected to covalently attach to a component of the enzyme which catalyzes its activation. Therefore, it is possible that allylsulfide may specifically and irreversibly bind to a protein component associated with AMO activity. Consistent with this hypothesis, recovery of AMO activity by intact cells after complete inactivation by allylsulfide required *de novo* protein synthesis. On the basis of the recovery rate, the inactivating species is unlikely to be non-specific or diffuse far from the catalytic site. These results further supported the conclusion that allylsulfide is a specific, mechanism-based inactivator of AMO. However, the possibility exists that a protein component required for turnover of AMO was the actual site of inactivation. Future studies of allylsulfide inactivation of AMO may include identification of the polypeptide(s) labeled by exposure to radiolabeled allylsulfide.

The inactivation of AMO by allylsulfide raised the possibility of allelopathic inhibition of nitrification. Allylsulfide is a volatile component of *Allium* spp. Thus soils containing *Allium* spp. may thus inhibit ammonia-oxidizing bacteria. Allelopathic inhibition of nitrification has been suggested (White, 1986b) , theoretically debated (White, 1986a), and two natural products have been investigated as potential nitrification inhibitors (Sahrawat and Keeney, 1985). However, the inhibition of a pure culture of ammonia-oxidizing bacteria by a plant-produced compound had never been conclusively demonstrated (McCarty, Bremner et al., 1991). Therefore, allylsulfide could constitute the first nitrification inhibitor which is both a natural product and an example of an allelopathic agent.

Because allylsulfide acts as a mechanism-based inactivator of AMO, it may have practical applications as a specific inhibitor of nitrification in ecological studies involving nitrogen transformation. For example, acetylene (C_2H_2) has been used in the study of the nitrogen cycle for determining denitrifying activity (Oremland and Capone, 1988). However, the use of C_2H_2 is complicated because it inhibits many enzymes involved in the nitrogen cycle, including nitrogenase, AMO, nitrous oxide reductase and nitrate reductase (Hyman and Arp, 1988). Therefore, inhibitors specific for each process in the nitrogen cycle could be useful in the estimation of nitrogen transformations (Oremland and Culbertson, 1992).

Apart from being an inactivator of AMO and a possible allelopathic agent, allysulfide is also interesting because it has been shown to prevent carcinogenesis in some model systems. Allylsulfide and its oxidized products, allylsulfoxide and allylsulfone, are proposed to prevent carcinogenesis by their inhibition of the microsomal monooxygenase P-450 2E1 (Brady, Ishizake et al., 1991). Allylsulfide was initially thought to inactivate cytochrome P-450 2E1, but it was later suggested that the inactivation by allylsulfide resulted from its metabolic conversion to allylsulfone by other unidentified microsomal oxidizing enzymes (Brady, Li et al., 1988; Brady, Ishizake et al., 1991). The mechanism for the inactivation of P-450 2E1 monooxygenase by allylsulfone is being investigated (Brady, Ishizake et al., 1991).

The inactivation of AMO activity by allylsulfide is mechanistically interesting. Allylsulfide has not been previously shown to be an inactivator of other monooxygenases and it is one of only a few characterized inactivators of AMO activity. Because sulfones are not formed as products of thioether oxidations by *N. europaea* (Juliette, Hyman et al., 1993a), it is unlikely that allylsulfone is involved in the inactivation of AMO. Of possible relevance to the mechanism of AMO inactivation by allylsulfide is the ability of cytochrome P-450 monooxygenases to oxidize thioethers concomitant with the formation of sulfoxides and S-dealkylation products. The sulfoxide and S-dealkylation products are formed by cytochrome P-450 via a common intermediate during the oxidation of thioethers which contain highly electronegative substituents (Ziegler, 1989). It has been proposed that cytochrome P-450 abstracts a single electron from the divalent sulfur, forming a sulphenium cation radical which can partition between sulfoxide formation and carbon-sulfur bond cleavage. For carbon-sulfur bond cleavage, a proton on the carbon alpha to the sulfur is lost followed by radical rearrangement with the generation a α-carbon-centered radical. Oxygen addition on the carbon radical yields the α-hydroxyl thioether which readily dealkylates to give the thiol and the corresponding aldehyde (Ziegler, 1989). The inactivation of AMO by allylsulfide may involve S-dealkylation. Although the allyl substituent is not considered highly electronegative, the predicted α-carbon radical formed during S-dealkylation of allylsulfide is an allylic radical. The formation of allylic radicals is favorable because they are stabilized by resonance. The predicted S-dealkylation products of allylsulfide, acrolein and allyl thiol, are highly reactive compounds and intermediates species may also be

reactive. Future studies on the inactivation of AMO by allylsulfide may prove useful in characterizing AMO catalysis.

6.4. Roles of bovine serum albumin and copper in the assay and stability of AMO activity *in vitro*.

6.4.1. Role of BSA in the assay.

The function of BSA in the cell-free assay of ammonia-oxidizing activity is to bind endogenous free fatty acids which accumulation in extracts. Evidence to support this conclusion includes: 1) only proteins which bind fatty acids supported activity, 2) exogenous palmitoleic acid completely inhibited activity, 3) the inhibition caused by exogenous palmitoleic acid was reversed only by proteins which bind fatty acids and 4) the concentration of endogenous free palmitoleic acid increased during aging of cell extracts.

In efforts to determine the role of BSA in the assay of ammonia-oxidizing activity, a BSA-independent assay was developed. It was determined that BSA was not required in the assay when freshly prepared extracts were maintained at protein concentrations of less than 15 mg/ml. However, over time activity became dependent on BSA. The development of a BSA-independent assay is beneficial for future studies of cell-free AMO activity. For example, the presence of BSA in assays to screen potential electron donors for AMO is undesirable because BSA binds many hydrophobic compounds.

6.4.2. Stabilization of ammonia-dependent O_2 uptake.

Efforts undertaken to stabilize ammonia-oxidizing activity revealed that Cu^{2+} ions had a stabilizing effect on ammonia-oxidizing activity in extracts of *N. europaea*. BSA also stabilized ammonia-oxidizing activity. The mechanism for the stabilizing effects of Cu^{2+} ions and BSA is proposed to result from their inhibition of the activity of an endogenous phospholipase activity. In support of this proposal, other agents which modify lipase activity, including $HgCl_2$, lecithin,

or phenylmethylsulfonyl fluoride (PMSF), also stabilized ammonia-oxidizing activity. In contrast to extracts stored with either BSA or $CuCl_2$, ammonia-oxidizing activity of extracts stored with PMSF can be assayed without BSA. Phenylmethylsulfonyl fluoride may prove useful and convenient as a stabilizing agent in future attempts at purification of active AMO.

Irreversible loss of ammonia-oxidizing activity was slowed by agents which modified lipase activity. The irreversible loss of ammonia-oxidizing activity has been previously suggested to be due to the loss of coupling integrity between AMO and HAO (Ensign, Hyman et al., 1993). Ammonia-oxidizing activity may be dependent on the association of many proteins and a loss of membrane integrity, caused by the activity of an endogenous phosopholipase, is suggested to lead to the disassociation of the proteins required for activity.

In support of this conclusion, the loss of many other membrane associated enzymatic activities has previously been recognized to occur as a result of extensive lipolysis (Avigad, 1976; Ko, Frost et al., 1994). Loss of membrane-associated enzyme activities has been suggested to result from either the lipolytic products or the direct loss of membrane integrity. The loss of membrane integrity is supported as a cause for the irreversible loss of ammonia-oxidizing activity because an irreversible loss of activity was not induced by exogenous fatty acids. In addition, the mechanism for stabilization by lecithin provided support that the loss of membrane structure, rather than the lipolytic products, is a likely cause for the irreversible loss of ammonia-oxidizing activity (Juliette, Hyman et al., 1995).

6.5. Cell-free ammonia-dependent O_2 uptake by extracts of *N. europaea*: Reversible inhibition by palmitoleic acid and n-heptylhydroxyquinoline N-oxide.

The inhibition of ammonia-oxidizing activity by fatty acids was investigated. Endogenous fatty acids are suggested to be the cause of the development of a lag in aging extracts because exogenous fatty acids caused a similar lag and an increase in free fatty acids is reported to occur during aging of extracts of *N. europaea* (Juliette, Hyman et al., 1995). The lag, associated with the activity of either fatty acids-treated or aging extracts, was shortened by BSA

or reductant sources for AMO. It was suggested that the underlying reason(s) for the lag may provide insight into the mechanism of fatty acid inhibition.

Because fatty acids have been described as uncouplers, the effects of two uncoupling agents on ammonia-dependent O_2 uptake were examined. Neither 2,4 dinitrophenol nor carbonyl cyanide m-chlorophenylhydrazone inhibited the rate of ammonia-dependent O_2 uptake. This result suggested that activity was not associated with a proton gradient. However, the electron transport inhibitor, n-heptylhydroxyquinoline N-oxide (HQNO) completely inhibited ammonia-dependent O_2 uptake activity. This result suggested that ammonia-oxidizing activity may require electron transport processes. Similar to inhibition of activity by palmitoleic acid, the inhibition by HQNO was relieved by either BSA or hydroxylamine. In conclusion, it was speculated that the mechanism by which fatty acids inhibit electron transport processes in other bioenergetic systems may be similar to the mechanism by which fatty acids inhibit ammonia-dependent O_2 uptake.

In addition, the cause of the irreversible loss of ammonia-dependent O_2 uptake activity was investigated. It has been proposed that disruption of the membrane by lipolysis results in the loss of coupling among enzymes necessary for ammonia-dependent O_2 uptake (Juliette, Hyman et al., 1995). This suggestion implies that if cell-free ammonia-dependent O_2 uptake was lost, then hydrazine-dependent O_2 uptake activity may also be affected. Therefore, the effects of some agents, which stabilize ammonia-dependent O_2 uptake activity by modifying lipase activity (Juliette, Hyman et al., 1995), on hydrazine-dependent O_2 uptake activity were examined. Cell-free ammonia- and hydrazine-dependent O_2 uptake activities were both stabilized by agents which modify lipase activity. Importantly, the activities were either stabilized or lost concurrently. This result suggested that these activities are interdependent and therefore coupled in extracts active for ammonia-dependent O_2 uptake. This result provides the first evidence that loss of ammonia-oxidizing activity may result from the loss of coupling integrity among enzymes involved in the ammonia oxidation pathway. Because of the dependence of AMO activity on the integrity of a complex involving HAO, the use of ammonia-dependent O_2 uptake and nitrite formation as a measurement of AMO activity limits our understanding of AMO activity *in vitro*.

Establishment of an assay which does not require coupling between AMO and HAO would simplify our system. AMO activity should be determined by

product formation rather than O_2 uptake or nitrite formation. However, an adequate source of reductant for AMO activity which does not require HAO catalysis has not been determined. The stabilization and characterization of cell-free AMO activity has led to the conclusion that an alternative assay method and/or a direct electron donor for AMO is necessary.

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