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Hydrogenase catalyzes the reaction $H_2 \rightleftharpoons 2H^+ + 2e^-$. The *Azotobacter vinelandii* hydrogenase has a catalytic heterodimer consisting of a large subunit (HoxG) and a small subunit (HoxK), and an integral membrane *b*-type cytochrome (HoxZ). Eight histidines in HoxZ were replaced with alanine or tyrosine to determine the ligands to the hemes and the role of the histidines in hydrogen oxidation. Mutants H33A/Y, H74A/Y, H194A, H208A/Y, and H194,208A lost hydrogen oxidation activity with O_2 as the electron acceptor, and H194Y and H136A had partial activity. These results suggest that histidines 33, 74, 194, and 208 are the ligands to the hemes, tyrosine can serve as an alternate ligand in position 194, and histidine 136 plays a role in hydrogen oxidation. In mutants H194A/Y, addition of imidazole (Imd) resulted in full recovery of hydrogen oxidation activity, which suggests that imidazole acts as an exogenous ligand. The heterodimer activity, quantitatively measured by methylene blue (MB) reduction under anaerobic conditions, indicated that the heterodimers of all mutants were catalytically active. However, except for H33A/Y, none of the enzymes with replacements had wild-type levels of MB reduction. Imidazole was able to reconstitute full MB reduction activity in mutants H194A/Y(Imd) and H208A/Y(Imd), and partial activity in H194,208A(Imd). These results indicate that structural and functional integrity of HoxZ is required for physiologically relevant hydrogen oxidation, and that the structural integrity of HoxZ is necessary for full catalytic heterodimer H_2 oxidation.

Hydrogenase in *Azotobacter vinelandii*: The Role of the Heme Ligands in HoxZ

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Laura Meek, Author

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Hydrogenase in *Azotobacter vinelandii*: The Role of the Heme Ligands in HoxZ

Introduction

The enzyme hydrogenase catalyzes the reaction $H_2 \rightleftharpoons 2H^+ + 2e^-$. Hydrogenase is vital to microorganisms that use H_2 as an energy source and valuable to those that use the enzyme to carry out other important metabolic processes. Industrially, H_2 is potentially an inexpensive and non-polluting energy source, and the hydrogenase enzyme is the model for biomimetic catalysis.

In the bacterium *Azotobacter vinelandii*, the [NiFe] hydrogenase consists of three proteins: a large subunit, a small subunit and a *b*-type cytochrome (HoxZ). The large subunit contains the active site, in which nickel and iron play key roles. The small subunit contains [Fe-S] clusters, and transfers electrons between the large subunit and the cytochrome *b*. Structure-function studies of the large and small subunits have determined residues essential for catalysis and electron transport. When this research was begun, the ligands to the hydrogenase *b*-type cytochrome had not been determined.

In *b*-type cytochromes, one or two hemes are ligated by histidine residues. The *b*-type cytochrome in the bc_1 complex of the electron transport chain consists of two hemes and eight α-helices with ligands on helices B and D. The *b*-type cytochromes of succinate:quinone reductase (SQR) and fumarate:menaquinone oxidoreductase (QFR) have single ligands on each of four helices. The cytochrome *b* in [NiFe] hydrogenases is an integral membrane protein with four transmembrane α-helices (Menon et al., 1990). Dross et al. (1992) separated hydrogenase proteins in *Wolinella succinogenes* and analyzed the protein and heme content. They aligned the sequences of seven [NiFe] hydrogenase *b*-type cytochromes and identified conserved histidines in their alignment. From protein and heme analyses, they concluded that this cytochrome contains a single heme, which would have two histidine ligands. They

suggested one histidine on helix A as a ligand and another on helix D. Sequence analysis of *A. vinelandii*, and a new sequence alignment indicated that four histdines may serve as ligands to two hemes (Laura Meek, unpublished). Determining which histidines serve as ligands to the heme(s) will indicate the number of hemes and how [NiFe] hydrogenase cytochrome *b* compares to the models of other integral membrane, electron transport *b*-type cytochromes.

Roles for the hydrogenase cytochrome *b* have been suggested from studies with a partial deletion mutant (Sayavedra-Soto and Arp, 1992). The roles suggested for HoxZ include transfer of electrons from the large and small subunits to the electron transport chain, anchoring of the large and small subunits to the membrane, activating the catalytic site, and stabilizing the enzyme. Site-directed substitutions of HoxZ heme ligands may be able to separate these roles and define them more specifically.

The goals of this research are to determine the ligands to the hemes and the role of these ligands in hydrogenase activity. This thesis tests the hypothesis that the ligands to the hemes in the hydrogenase cytochrome *b* are essential for physiologically relevant hydrogenase activity. Determination of the ligands to the heme will provide a better understanding of heme binding in HoxZ, and hydrogenase assays of mutants and their interaction with imidazole may provide information on the roles of the ligands. Characterization of the structure-function relationship in HoxZ will provide a model for *b*-type cytochromes in homologous membrane-bound [NiFe] hydrogenases and in similar proteins interacting with quinones.

Literature Review

Hydrogenase catalyzes the reversible reaction $H_2 \rightleftharpoons 2H^+ + 2e^-$ (EC 1.18.99.1 and 1.12.2.1) (Friedrich and Schwartz, 1993). Its biological function is critical to many organisms and is also of interest industrially. The enzyme occurs in all three kingdoms of life, but is primarily found in diverse groups of prokaryotic organisms. The role that hydrogenase plays depends on the physiology of the organism as well as the direction of catalysis. Depending on the catalytic direction, hydrogenase can be important in energy production, reductant regeneration, or electron carrier reoxidation. In H_2 oxidation, hydrogenase can be a supplemental or the sole means of energy generation for the cell. In aerobic systems, reductant is needed for a variety of cellular processes, and hydrogenase can feed the pool of reductant and contribute to the proton gradient. In fermentative systems, excess reductant is a problem, and hydrogenase provides a solution by coupling protons and electrons, and the hydrogen gas can diffuse out of the cell.

In nitrogen fixing organisms, such as *Azotobacter vinelandii*, hydrogenase plays additional roles. The nitrogen fixation reaction, $N_2 + 8e^- + 8H^+ + 16\ Mg\ ATP \rightarrow 2NH_3 + H_2 + 16\ Mg\ ADP + 16\ P_i$ (Rees, 1993), indicates that H_2 is generated if N_2 is bound and reduced. However, H_2 can be produced from the di- and tri-hydride complexes of nitrogenase, and this does not require the binding of N_2, nor the production of NH_3 (Dalton and Mortenson, 1972). Hydrogenase, acting in the oxidative direction, can reduce end-product inhibition of nitrogenase, and can indirectly recycle protons and reductant for nitrogen fixation. If oxidation occurs in the periplasm (the space between the cytoplasmic membrane and the cell wall), hydrogenase contributes to the proton gradient. Under certain conditions, such as carbon and phosphorus limitation, H_2 oxidation may lead to energy generation (Arp, 1990), but the primary value is probably the reduction of oxygen, preventing oxygen damage to the oxygen-sensitive enzymes, hydrogenase and nitrogenase.

Since the enzyme can provide a renewable, non-polluting source of energy, H_2, hydrogenase has been viewed as a biological model for energy production. The industrial catalyst for H_2 generation is platinum, which is effective, but expensive. Because hydrogenase can generate H_2 with nickel and iron in the catalytic site, this enzyme is a good model for biomimetic catalysts.

Hydrogenases

Classification

Hydrogenases are categorized by metal content, catalytic direction, or cellular location. Based on metal content, hydrogenases are grouped into metal-free, iron-only ([Fe]), and nickel-iron ([NiFe]) hydrogenases. The metal-free group is found only in methanogenic archaea. Although they are hydrogenases by definition, they function rather as dehydrogenases by catalyzing a reversible hydride transfer from H_2 to N^5, N^{10}-methenyl tetrahydromethanopterin to form N^5, N^{10}-methylene tetrahydromethanopterin. Unlike [NiFe] and [Fe]-only hydrogenases, the metal-free hydrogenases don't activate H_2 nor catalyze electron transport (Geierstanger et al., 1998). The monomeric [Fe]-only hydrogenases have two irons in the catalytic center and three or five iron-sulfur clusters, depending on the species (Cammack, 1999). Originally named for the nickel in the large subunit and iron in the iron-sulfur clusters of the small subunit, the [NiFe] hydrogenases have both nickel and iron in the catalytic site. Although both metal-containing hydrogenases have features in common, the structures are quite different (Cammack, 1999).

Physiologically, most hydrogenases preferentially catalyze either the formation or consumption of H_2. However, artificial electron donors and acceptors can be used to evaluate functionality and ability to catalyze a reversible reaction (Adams et al., 1981). [Fe]-only hydrogenases primarily evolve H_2 (Cammack, 1999) and have a high specific activity, but a medium to low affinity for H_2. [NiFe] hydrogenases primarily

catalyze H_2 consumption, and are termed "uptake" hydrogenases. They have a lower specific activity than Fe hydrogenases, but a medium to high affinity for H_2 (Grahame, 1988; Mortenson, 1988). In addition to uptake and production, hydrogenase catalyzes a third type of reaction, isotope exchange, with D_2 and H_2O as substrates (McTavish et al., 1996).

Hydrogenases are also classified by cellular location: soluble (cytoplasmic), membrane-bound, and periplasmic. Cytoplasmic hydrogenases have four heterologous subunits, contain flavin mononucleotide, reduce NAD^+, and are found only in *Alcaligenes* sp. and in *Nocardia opaca* (Tran-Betcke et al., 1990). The catalytic subunits of periplasmic and membrane bound [NiFe] hydrogenases are both located on the periplasmic side of the cytoplasmic membrane (Volbeda et al. 1995, and Bernhard et al., 1996). In these, a large and small subunit form a catalytic heterodimer and pass electrons to an electron acceptor, which may or may not be considered part of the enzyme, depending on the type of hydrogenase and acceptor. In periplasmic hydrogenases, the reported acceptor is a freely mobile, four heme cytochrome c_3, (Bianco et al., 1992) which is not considered part of the enzyme. In membrane-bound hydrogenases, the electron acceptor is an integral membrane cytochrome *b*, and electrons are passed to a quinone (Bernhard et al., 1996).

Metal content, catalytic direction and cellular location are fundamental to the physiological role that hydrogenase has in a particular organism. Uptake hydrogenases provide the cell with reductant, which can be used to reduce substrates or generate energy. Two particularly important substrates include CO_2 (e.g. for CO_2 fixation in photosynthetic bacteria) and O_2 (the reduction of O_2 is important in protecting oxygen sensitive enzymes). Evolution of H_2 enables cells to dispose of excess reductant when other electron acceptors are not available. Anaerobic organisms, which generate energy by fermentation, can regenerate oxidative carriers by using protons as a terminal electron acceptor (Adams et al., 1981; and Albracht, 1994).

Even though the bacteria expressing hydrogenase vary in terms of physiological functions and regulation, the hydrogenase genes and organization are highly conserved, particularly in membrane-bound [NiFe] hydrogenases (Kortlüke et al., 1992, Du et al., 1994, Friedrich and Schwartz, 1993). The genes are usually arranged in clusters of structural, accessory and pleiotropic genes, and in some species are plasmid encoded (Friedrich and Schwartz, 1993). The structural genes of [NiFe] hydrogenases code for a large and small subunit, and in some, a cytochrome *b* (Friedrich and Schwartz, 1993). The same subunits of several [NiFe] hydrogenases are immunologically related. The large subunit is more conserved than the small subunit, and the large and small subunits are not immunologically related (Kovacs et al., 1989, Arp et al., 1985, and Lorenz et al., 1989). The accessory genes code for proteins with functions such as nickel incorporation, processing/maturation, membrane attachment, and H_2-dependent electron transport, many of which are required for forming an active hydrogenase (Bernhard et al., 1996). Products of pleiotropic genes are involved with general nickel metabolism (Friedrich and Schwartz, 1993). In membrane-bound uptake hydrogenases, the gene nomenclature varies from species to species. Gene names for the structural genes include *hya* (hydrogenase isoforms a,b, and c, e.g., *Escherichia coli hyaC*), *hup* (hydrogen uptake, e.g., *Rhodobacter capsulatus HupM*), and *hox* (hydrogen oxidation, e.g., *A. vinelandii* and *Alcaligenes eutrophus hoxZ*), and for processing functions, *hyp* (hydrogenase pleiotropic functions, e.g., *E. coli hypB*) (Friedrich and Schwartz, 1993, Lutz et al., 1991).

Regulation

Regulation of the synthesis of hydrogenase gene products varies according to the organism's physiological needs. The presence of aerobic, photosynthetic, and nitrogen fixing capabilities can influence the types of regulatory regimes. No single manner of regulation pertains to all hydrogenases, and multiple regulatory mechanisms may be operating (Brito et al., 1997). Hydrogenase gene expression may

be part of a global regulatory system such as an energy-dependent regulation, or be independently regulated such as a substrate-mediated regulation (Lenz et al., 1997). Expression may also be influenced by other environmental factors in additon to H_2, such as Ni^{2+} and O_2 (Toussaint et al., 1997, and Black and Maier, 1995).

In *A. eutrophus*, hydrogenase genes are derepressed when the growth rate is nutrient limited so that H_2 can be used a supplemental energy source (Lenz et al., 1997). In nitrogen fixing bacteria, particularly in symbiosis, transcription of the hydrogenase structural genes may be regulated by the same transcriptional activator, NifA or FnrN, as the nitrogenase genes (Gutiérrez et al., 1997, Brito et al, 1997). Nitrogen fixation is energy intensive and the nitrogenase genes are repressed when better nitrogen sources are available (Burris and Roberts, 1993). Since hydrogenase recycles the H_2 generated by nitrogenase, coregulation enables synthesis of hydrogenase coordinated with that of nitrogenase (Brito et al., 1977).

In contrast to global control, H_2 induces hydrogenase synthesis in several bacteria, such as *Alcaligenes hydrogenophilus, Alcaligenes latus, Azotobacter chroococcum, A. vinelandii, Bradyrhizobium japonicum, Paracoccus denitrificans*, and *R. capsulatus* (Lenz et al., 1997; Doyle and Arp, 1987; Prosser et. al., 1988, and Colbeau and Vignais, 1992). However, the culture conditions, at least for *A. vinelandii*, affect whether exogenous H_2 stimulates hydrogenase expression. The addition of H_2 to NH_4^+-grown cultures enhanced transcription of hydrogenase genes, but did not increase hydrogenase in N_2-fixing, H_2-generating cultures (Prosser et al., 1988). Parts of a two-component (sensor/response) regulatory system specific for hydrogenase have been identified in several bacteria (Lenz et al., 1994, Durmowicz and Maier, 1997, Elsen et al., 1996, and Toussaint et al., 1997), including free-living bacteria that can fix nitrogen (Van Soom et al., 1997). The gene coding for the protein that senses H_2 may be mutated in some species, such as in *A. eutrophus* and *Rhizobium leguminosarum* (Lenz et al., 1977, and Brito et al., 1997). However, the response protein, a transcriptional activator, is essential for hydrogenase gene expression in both the globally regulated *A. eutrophus* and the H_2-dependent regulation of *A. hydrogenophilus* (Lenz et al., 1997). This type of positive regulation occurs by

phosphorylation and may be controlled by a negative regulator, a phosphatase, that deactivates the response protein (Toussaint et al., 1997 and Lenz et al., 1994). Other factors, like RpoN (σ^{54}) and integration host factor may be involved in transcription (Brito et al., 1977; Black and Maier, 1995; and Toussaint et al., 1997). Nickel is an integral component of the [NiFe] enzyme active site and positively influences transcription of the structural genes (Black and Maier, 1995). In addition to H_2 and Ni^{2+}, microaerobic conditions have been shown to derepress transcription of hydrogenase genes (Palacios et al, 1990; Doyle and Arp, 1987, and Black and Maier, 1995). Both nitrogenase and hydrogenase are sensitive to O_2, and high levels of O_2 inhibit expression (Doyle and Arp, 1987). In most cases, the expression of H_2 uptake genes is strictly regulated (Van Soom et al., 1997), but a low level of constitutive hydrogenase synthesis has also been at found in the cyanobacterium, *Anabaena variabilis*, along with H_2-induced hydrogenase synthesis (Troshina et al., 1996).

Structure and Function

The present understanding of hydrogenase structure is a culmination of protein purification, sequencing, mutagenesis, electron paramagnetic resonance (EPR), extended x-ray absorption fine structure (EXAFS), and x-ray crystallography studies (Hatchikian et al, 1978; Seefeldt and Arp, 1986; Menon et al., 1990; Sayavedra-Soto and Arp, 1992; Guigliarelli et al., 1995; Volbeda et al., 1995; and Happe et al., 1997). In periplasmic and membrane-bound [NiFe] hydrogenases, the large subunit (HoxG in *A. vinelandii*) is a protein of about 60-67 kDa and contains the site of H_2 activation (Volbeda et al., 1995; Sayavedra-Soto and Arp, 1993; and Seefeldt and Arp, 1986) (Fig.1). The active site contains both Ni and Fe, which are ligated by cysteines (Volbeda et al., 1995), and the iron also has the unusual, non-protein ligands, CN^- and CO (Happe et al., 1997). The large subunit does not have a leader peptide (Fu and Maier, 1993), but processing, by means of proteolytic cleavage of the C-terminal 15-25 residues, enables structural reorganization and formation of the NiFe center (Volbeda et al., 1995). Nickel processing proteins are required for an active

hydrogenase and the lack of Ni^{2+} incorporation can delay the maturation of the large subunit. Impaired modification or active site assembly may affect the ability of the large subunit to associate with the membrane (Menon et al., 1991, and Bernhard et al., 1996). Oxidation of H_2 starts as a heterolytic cleavage of the H_2 molecule and results in protons being eliminated from the large subunit through a proton channel of histidines and a glutamine, while the electrons are passed to the small subunit (Volbeda et al., 1995).

In close association with the large subunit is the small subunit, a ~26-40 kDa protein that generally contains three [FeS] clusters: two [4Fe4S] clusters and a [3Fe4S] cluster (Volbeda et al, 1995; Seefeldt and Arp, 1986; Menon et al., 1991; and McTavish et al., 1993). These clusters are bound by conserved cysteines, except for a single conserved histidine, and are thought to be along the electron pathway from the large subunit to the electron acceptor (Volbeda et al., 1995). Four of these cysteines occur in the proximal and central amino acid motifs of Cys-X-X-Cys, and mutation of the cysteines in these motifs resulted in a loss of activity. In whole cells, replacement of the central [3Fe4S] cluster ligands with serine resulted in complete loss of activity when methylene blue was used as an electron acceptor. The proximal cluster had a cysteine in the third position: Cys-X-Cys-Cys. This neighboring cysteine appeared to substitute partially for the first and last ligands, since replacement of cysteine by serine in either of these positions gave 2% of wild-type activity in whole cells. When cysteines in both the third and fourth positions were replaced with serine, loss of activity was complete (Sayavedra-Soto and Arp, 1993). Substitution of serine for cysteine in the ligands to the central [3Fe4S] cluster generated strains that, unlike wild-type, were no longer sensitive to O_2 inhibition in H_2 oxidation, nor H_2 inhibition of H_2 evolution (McTavish et al., 1995). The distal [4Fe4S] has the histidine ligand, and in the periplasmic hydrogenase in *Desulfovibrio gigas*, this ligand is partially exposed on the surface of the small subunit. This ligand is surrounded by acidic residues, and Volbeda et al. (1995) suggested that this combination of amino acids may be the recognition site for the periplasmic hydrogenase redox acceptor. This distal [FeS] cluster is thought to be the direct electron donor to the membrane-bound hydrogenase acceptor. In *Wolinella succinogenes*, a membrane-bound hydrogenase,

replacement of the small subunit histidine ligand, H188, resulted in a total loss of H_2-dependent activity (Gross et al., 1998).

Expression, localization to the membrane, and processing of the small subunit are all required for an active hydrogenase (Menon et al., 1991). In membrane-bound hydrogenases, the small subunit has a N-terminal leader peptide that is not found in soluble hydrogenases. This leader peptide directs the protein, and the large subunit as well, through the membrane and then is cleaved to form the mature protein (Bernhard et al., 1996; and Sayavedra-Soto and Arp, 1993). Mutants lacking a major portion of the leader lack hydrogenase activity, and the large subunit is missing from the membrane fraction. Apparently, the ability of the small subunit to translocate through the membrane is required for the large subunit to be membrane-bound to the periplasmic side of the cytoplasmic membrane (Bernhard et al., 1996). Processing of the large and small subunit occurs concurrently and only after the subunits are localized on the membrane (Menon et al., 1991; and Fu and Maier, 1993). In membrane fractions of wild-type, the predominant form of the large and small unit is the processed form. Unprocessed small and large subunits have been found bound to the membrane in deletion mutants of various hydrogenase genes, such as accessory genes (Menon et al., 1991). The C-terminal portion of membrane-bound small subunits, unlike periplasmic hydrogenases, contains a transmembrane segment, which in *W. succinogenes* HydA, was found to be integrated into the membrane (Gross et al., 1998). Attachment to the membrane cannot occur solely by this segment, but may occur in conjunction with the integral membrane cytochrome *b* (Bernhard et al., 1996). Replacement of *W. succinogenes* H305, which is in the C-terminal segment integrated into the membrane, resulted in a complete loss of hydrogenase activity, as did a deletion mutant lacking the C-terminal hydrophobic portion. Two roles were suggested for HydA H305: this histidine may be important in electron transfer from the small subunit to cytochrome *b*, or it may act as the ligand to the cytochrome *b* heme (instead of the cytochrome *b* HydC H200, which is homologous to H208 of HoxZ in *A. vinelandii*) (Gross et al., 1998).

In hydrogenase oxidation of H_2, electrons are passed from the large subunit to the small subunit and then to an electron acceptor that is the conduit to the electron transport chain. In cytoplasmic hydrogenases, the acceptor is NAD^+, and in periplasmic hydrogenases the acceptor is a freely mobile cytochrome *c* (Bianco et al., 1992). In membrane-bound hydrogenases, the electron acceptor is an integral membrane cytochrome *b* (Dross et al.,1992; and Bernhard et al., 1997). Only in the last few years was this protein determined to be essential for physiologically relevant hydrogenase activity (Hidalgo et al, 1992; Menon et al., 1992; Cauvin et al., 1991; Sayavedra-Soto and Arp, 1992; Du et al., 1994; and Dross et al., 1992,). Since the purified heterodimer alone can catalyze hydrogenase reactions with artificial acceptors and donors (Arp, 1989), this integral membrane protein was not considered part of the structural enzyme, even though this membrane protein is in the same operon as the large and small subunits.

The earliest information about this third protein came from observations by Eisbrenner and Evans that hydrogenase activity in *Bradyrhizobium japonicum* was associated with a *b*-type cytochrome spectral signature (Arp, 1990). The next steps were genetic and physiological studies – sequencing and mutational analysis. The DNA sequence for this protein in *A. vinelandii*, called HoxZ, was determined by Menon et al. in 1990. They showed that *hoxZ* codes for a hydrophobic protein of 240 amino acids and 27.7 kDa. The protein has 53% nonpolar and 11% aromatic residues and four major hydrophobic domains of 20-26 amino acids. The hydrophobicity and even spacing of these domains implied that HoxZ was a membrane protein. Analyzing other hydrogenase sequences they found that HoxZ was 55% identical to a hydrogenase protein in *B. japonicum* and 51% identical to one in *E. coli*, and it aligned with membrane proteins in a similarity search of a protein database. They suggested that HoxZ may have a role in anchoring, proton transport, or as an electron carrier (Menon et al. 1990).

In addition to the *b*-type cytochrome signature on an absorbance spectrum, HoxZ and its homologues were thought to be *b*-type cytochromes based on hydrophobic transmembrane segments, potential histidine heme ligands, (Dross et al., 1992),

general sequence similarity between *R. leguminosarum* HupC and three other *b*-type cytochromes: cytochrome *b* of the respiratory complex III (the bc_1 complex), the photosynthetic b_6f complex (Hidalgo et al., 1992) and a cytochrome *b* from *Neurospora crassa* (Menon et al., 1992). These proteins were believed to transfer electrons to a quinone (Dross et al., 1992). The prediction about being a cytochrome *b* was correct (Gross et al., 1997), but the evidence for this and other basic aspects of the protein's structure and function, such as number of hemes, heme ligands, midpoint potential, membrane location of the catalytic subunits, and respiratory chain electron acceptor, came only after several more years.

From studies using deletions or insertions in HoxZ (Savavedra-Soto and Arp, 1992) and its homologues (HyaC in *E. coli*, Menon et al., 1991; HupM in *R. capsulatus*, Cauvin et al., 1991; HupC in *R. leguminosarum*, Hidalgo et al., 1992; HupC in *A. chroococcum*, Du et al., 1994, and HupC in *W. succinogenes*, Dross et al., 1992), researchers concluded that this membrane protein was essential for H_2-dependent reduction of the terminal electron acceptor in the electron transport chain, i.e., that Hox Z was part of the hydrogenase enzyme. Hydrogenase, when measured with artificial electron acceptors, was active. With the natural acceptor O_2, there was no H_2 oxidation activity in *R. capsulatus* (Cauvin et al., 1991); *R. leguminosarum* (Hidalgo et al., 1992) or *A. chroococcum* (Du et al., 1994), but slight activity was observed in *A. vinelandii* (Sayavedra-Soto and Arp, 1992).

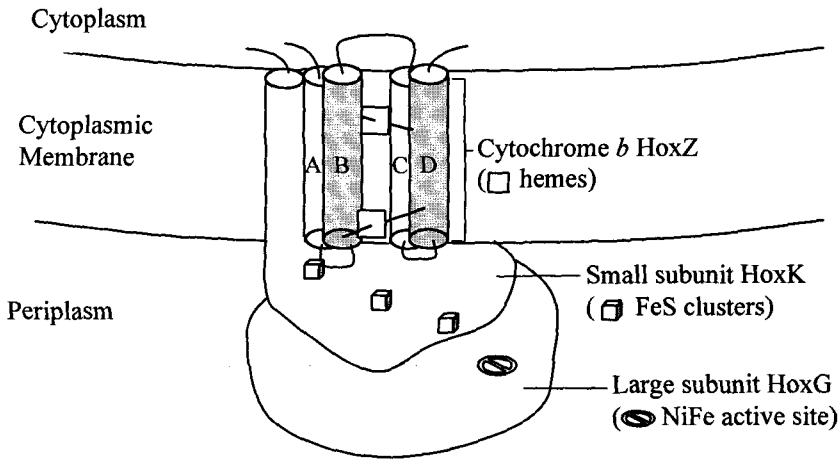
Although research had shown that HoxZ or its homologue was required for physiological activity of membrane-bound hydrogenases, the link between the heterodimer and the electron transport chain was not clear. In addition, location of the heterodimer on the cytoplasmic membrane was not known (was it cytoplasmic or periplasmic?), nor was the electron path from the heterodimer to the respiratory chain delineated (what was the direct acceptor?, and where did it come into the chain?). Using partial deletion mutants of HupC in *E. coli* and HoxZ in *A. vinelandii*, Menon et al. (1991) and Sayavedra-Soto and Arp (1992) concluded from the difference in activity between artificial and physiological electron acceptors that HoxZ was not involved in the production of an active heterodimer. Since the HoxZ deletion mutant

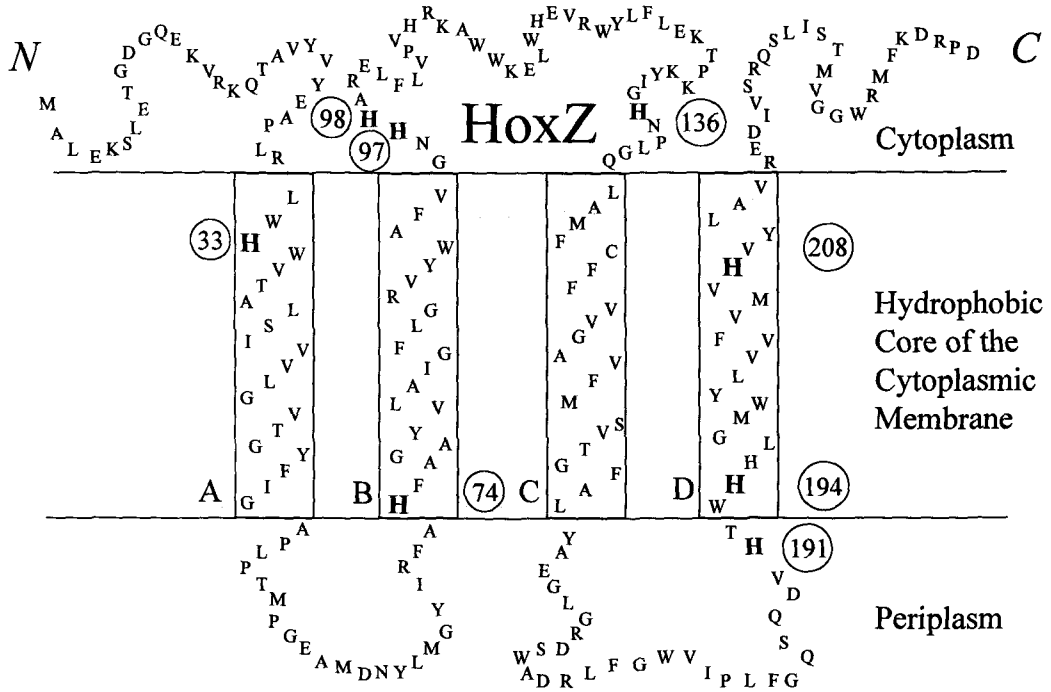
affected physiologically relevant hydrogenase activity, Sayavedra-Soto and Arp proposed four possible functions for this protein: 1) mediation of electron flow from hydrogenase to the electron transport chain, 2) attachment of hydrogenase to the membrane, 3) activation of hydrogenase, and 4) stabilization of hydrogenase. Mediation of electron flow was supported by the HoxZ$^-$ phenotype, the mutant had an active heterodimer but impaired physiological hydrogenase activity. They suggested an unspecified alternative pathway for the low activity observed. Another possibility mentioned was transfer to a nonspecific acceptor, such as a quinone, since hydrogenase showed flexibility in donating to various acceptors. The role of anchoring the heterodimer to the membrane was based on the sequence-derived structural information implying transmembrane regions and solubilization studies. Although detergent is required to remove the heterodimer from the membrane, it is not required thereafter to keep the heterodimer soluble. This result suggested that the heterodimer may bind to a membrane protein and that HoxZ and the heterodimer may form a tight complex. Activation of hydrogenase, a required step in preparing cell extracts, entailed the removal of oxygen and the presence of a reductant. Activation was proposed as a role for HoxZ since the spectrophotometric and amperometric assays with whole cells required an exogenous reductant. HoxZ may supply reductant from the cell to the heterodimer by reverse electron flow. The role of stabilization came from two types of observations. First, anaerobically prepared membranes had greater activity, as a per cent of whole cells, than aerobically prepared membranes of HoxZ$^-$ (16% vs. 0.1%) as compared to wild-type (72% vs. 46%). Secondly, membrane activity, as a per cent of whole cells, was less in HoxZ$^-$ (0.1%) than in wild-type (46%), and a greater proportion of activity occurred in the soluble fraction of HoxZ$^-$ (0.6%) than in wild-type (0.01%) (Sayavedra-Soto and Arp, 1992).

From *in vitro* reconstitution studies in *W. succinogenes* hydrogenase, a quinone analogue could not be reduced by the hydrogenase heterodimer, but could be reduced when HydC, the HoxZ homologue, was added to this system (Dross et al., 1992). They correctly assumed that the structural subunits are in a 1:1:1 proportion, but their SDS/PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) appears to have proportionately less of the cytochrome *b* than the heterodimer proteins. In their

analysis of protein amount and heme content, the amount of protein was overestimated and the heme content was underestimated. The protein overestimation is probably due to the assumption of a 1:1:1 ratio of the large, small, and cytochrome subunits, yet the amount of protein recovered was not in this ratio probably because integral membrane proteins are more difficult to solubilize than membrane-bound proteins. The heme(s) of cytochrome *b* are not covalently bound to the protein (Mathews , 1985) and can easily be lost upon isolation. One heme per enzyme was assumed, and the number of hemes assumed to be in the protein affects the calculation of heme content. Because of the protein ratio and single heme assumptions, they were unable to differentiate between half the amount of protein with two hemes and the full amount of protein with a single heme. With hydropathy profiles and an alignment of the amino acid sequences of HoxZ and six other homologues (two of them were only partial sequences), they designated four transmembrane regions, identified three fully conserved histidines, and suggested the first and last histidines as heme ligands to a single heme. Although Dross et al. (1992) indicated an analogy with cytochrome *b* of the cytochrome bc_1 complex, they concluded that their HoxZ homologue was a single heme-binding cytochrome *b*, based on Kröger's previous work (Kröger and Innerhofer, 1976).

A schematic of the HoxZ sequence in *A. vinelandii* drawn with the protein threaded through the cytoplasmic membrane (Fig. 1) showed that two histidines were on each side of the membrane and may be aligned to act as ligands for two hemes (Laura Meek, unpublished). A new alignment of the same sequences used by Dross et al. (1992), but with complete sequences of all proteins, showed four fully conserved histidines (Laura Meek, unpublished). The two partial sequences that Dross et al. (1992) used to identify fully conserved histidines were incorrect in the last several amino acids relative to complete sequences (specifically *Azotobacter chroococcum* (Du et al., 1994); and *Bradyrhizobium japonicum* (Van Soom et al., 1993)), which show that four histidines are fully conserved and five are partially conserved. While the current work on determining the four heme ligands in *A. vinelandii* HoxZ was in progress, three papers were published indicating the four conserved ligands. In trying to determine the ligands in a cytochrome *b* of nitrate reductase, Berks et al. (1995)



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  1 MALEKSLETG DGQEKVRKQT AVYVYEAPLR LWHWVTALSI VVLGVTGYFI GAPLPTMPGE
 61 AMDNYLMGYI RFAHFAAGYV LAIGFLGRVY WAFVGNHHAR ELFLVPVHRK AWWKELWHEV
121 RWYLFLEKTP KKYIGHNPLG QLAMFCFFVV GAVFMSVTGF ALYAEGLGRD SWADRLFGWV
181 IPLFGQSQDV HTWHHLGMWY LVVFVMVHVY LAVREDIVSR QSLISTMVGG WRMFKDDRPD
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Fig. 1. Model of hydrogenase in *A. vinelandii* HoxZ, and schematic and amino acid sequence of *A. vinelandii* HoxZ (Menon et al., 1990). Hydrogenase modeled after concepts by Hederstedt and Ohnishi (1992), Cammack (1995), and Berks et al. (1995).

analyzed potential histidine ligands of several *b*-type cytochromes and suggested that the hydrogenase cytochrome *b* had two hemes liganded by four conserved histidines and specified the putative ligands. These same histidines were proposed by Bernhard et al. (1997) when they published the redox potential of the two hemes. Gross et al. (1998) replaced three out of four of the histidines individually with alanine and methionine in HydC of *W. succinogenes*. The mutant strains could not use H_2 as a reductant source: when grown with formate or fumarate, the mutants did not transfer electrons to the two respective physiological terminal electron acceptors, fumarate or polysulphide. They concluded that these histidines were the ligands to two hemes, and that each of these three histidines was required for hydrogenase activity and quinone reactivity. From heme-*b* content and redox titration, they confirmed that this cytochrome had two *b*-type hemes. Substitution of three other histidines in HydC resulted in mutants that had wild-type activity. Two were not conserved and the third, which neighbors a ligand, is highly conserved.

The proposed ligands were near the membrane edge on opposite sides of the membrane (Berks et al. 1995; and Gross et al. 1998), and the hemes could function in the transport of electrons from the periplasmic to the cytoplasmic side (Bernhard et al, 1997). Like cytochrome *b* of Complex III in the respiratory chain, this would place a low potential heme at the periplasmic edge and a high potential heme near the cytoplasmic edge in order to transport electrons against the membrane gradient (Bernhard et al, 1997). In comparing redox titrations of wild-type and HoxZ membrane fragments of *A. eutrophus*, Bernhard et al. (1997) determined that the hemes in HoxZ have midpoint potentials of +10 mV and +166 mV.

In addition to specifying the E_m of the hemes, Bernhard et al. (1997) showed that HoxZ is not required for translocating the catalytic subunits to the periplasm, but does anchor the catalytic subunits to the periplasmic side of the plasma membrane, and is necessary for H_2-coupled respiration. In mutants requiring membrane-bound hydrogenase for growth, they showed that a deletion of HoxZ prevented growth on H_2, even though the enzyme was catalytically active, since it could reduce the artificial electron acceptor triphenyltetrazolium chloride using H_2 as a substrate. They

concluded that HoxZ is a critical link in the hydrogenase respiratory pathway, but was not required for H_2-dependent reduction of the redox dye.

Hemoproteins

Proteins that contain hemes are often categorized by function, such as electron transport or gas transport, and by prosthetic group. Cytochromes are heme-containing redox proteins that have electron transfer capability. Hemes are porphyrin (four pyrroles bridged by methenyl groups) prosthetic groups that bind an iron, which reversibly changes between Fe^{2+} and Fe^{3+} oxidation states (Mathews, 1985). Hemes differ by the substituents on the porphyrin ring, the ligands to the heme, and the type of bond between the heme and the protein. Cytochrome *b* has heme *b*, an iron-protoporphyrin IX, which has methyl and ethyl side groups particular to heme *b*, and two propionic acid groups, which are common to all hemes. Heme *b* has two histidine ligands to the iron and is not covalently bound via porphyrin thioether linkages to the protein, unlike cytochrome *c* (Thöny-Meyer, 1997; and Mathews, 1985). The absorption spectra of reduced cytochromes have three bands in the visible region called α, β, and γ. The α peak is characteristic of the reduced cytochrome and is used to identify *a*- (~600 nm), *b*- (~563 nm), and *c*- (~550 nm) type cytochromes (Thöny-Meyer, 1997). The environment of the heme group can affect the wavelength, and cytochromes within a group can have slightly different absorbances. The names of *b*-type cytochromes correspond to the wavelength of the α peak, such as b_{558} (Thöny-Meyer, 1997), or the potential, such as b_L (low) or b_H (high) (Gray et al., 1994). The midpoint potential is dependent on the structure of the apoprotein, the type of prosthetic group, the type of binding between the heme and the protein (McRee et al., 1994; Nastri et al., 1998; and Sorrell and Martin, 1989) and heme exposure (Stellwagen, 1978).

Many of the protein complexes that directly interact with quinones in the electron transport chain have integral membrane cytochrome *b* subunits at the protein complex-

quinone interface. Quinones are non-protein, lipid-soluble proton and electron carriers that interact with Complexes I, II, and III of the respiratory chain (Brock et al., 1994). Cytochrome *b*-quinone interactions occur in eubacteria, archaea, and eukaryotic mitochondria and chloroplasts. Aerobic respiratory systems have ubiquinones, and anaerobic systems have menaquinones. The *b*-type cytochromes transfer electrons to or from the quinone, or as in cytochrome *b* of the bc_1 complex, both to and from. Sequence and functional relationships among certain subsets of quinone-interacting cytochrome *b*'s have been noted: cytochrome bc_1 and cytochrome b_6f (Widger et al, 1984, and Hauska et al., 1988), succinate quinone reductase – fumarate reductase (Hagerhall et al., 1995), formate dehydrogenase – membrane bound nitrate reductase – membrane-bound [NiFe] uptake hydrogenase (Berks et al., 1995), and uptake hydrogenase – succinate: quinone reductase (Menon et al., 1992).

Because of its importance, sequence information, functionally important sites, and crystal structure, the cytochrome *b* of the bc_1 complex is useful as an archetypal cytochrome *b*. The sequences of cytochrome *b* of the bc_1 complex (complex III of the respiratory chain) have been the subject of extensive sequence alignments (over 800) (Degli Esposti et al., 1993). This alignment enabled the identification of partially and fully conserved residues, such as the histidine ligands. Structurally, this cytochrome *b* has eight α-helical membrane spanning segments, named A through H. The liganding histidines are on the parallel B and D helices. The bc_1 complex contains a *b*-type cytochrome, an [FeS], and a cytochrome *c*. Ubiquinone interacts with the *b*-type cytochrome via a modified Q-cycle mechanism. The *b*-type cytochrome, a single electron carrier, coordinates with the quinone, a two proton and two electron carrier, to transfer electrons down the respiratory pathway while transferring protons across the membrane, generating a proton gradient. For every two electrons donated by the quinone, one is recycled by the *b*-type cytochrome back to ubiquinone. The *b*-type hemes differ primarily in midpoint potential (low vs. high) and location (edge vs. center of the plasma membrane) and whether they donate to or accept electrons from the quinone. In bacteria, the high potential heme is on the cytoplasmic side of the membrane and the low potential heme is on the periplasmic side. Binding sites for the donation of electrons to the quinone (quinone reduction site: Q_i (in), Q_n (negative side)

and Q_c (cytoplasmic)) and for accepting electrons from the quinone (quinone oxidation site: Q_o (out), Q_p (positive side), and Q_z) have been suggested by mutation and inhibitor studies. Antimycin and hydroquinoline N-oxides (HQNO) inhibit at the Q_i site, and stigmatellins and myxothiazol inhibit at the Q_o site. Mutational resistance to inhibitors has been studied in various organisms and the amino acids and specific locations that confer resistance vary. However, Q_i-inhibitor resistant sites occur on the inside (cytoplasmic) loops of the protein and Q_o inhibitor resistant sites occur on the loops outside the cytoplasmic membrane (periplasmic side in bacteria). No specific motif is associated with the Q_i site, but the N-terminal segment as well as the cytoplasmic loops are thought to play a role. Residues in several loops outside the membrane and the conserved amino acid motif, PEWY, located on the EF loop, are critical for the Q_o site.

Other *b*-type cytochromes that interact with quinones are also parts of a protein complex and transfer electrons and anchor subunits (Hederstedt and Ohnishi, 1992). They have little sequence homology with the cytochrome *b* of the bc_1 complex and differ in the number of transmembrane segments, ligand location, and size of loops between the transmembrane segments. These proteins have four or five transmembrane segments that can be roughly aligned with the similar segments in the first portion of cytochrome *b* of the bc_1 complex, but none align as far as the PEWY site (Laura Meek, unpublished). Except for nitrate reductase, which has two ligands on helix B and two on helix E, all other enzymes have ligands on three or four helices, and therefore, their hemes cannot be aligned in parallel as they are in the bc_1 complex. Ligand location and size of the hydrophilic loops differ probably due to differences in functionality, for example, midpoint potential or requirements for quinone or subunit binding.

Two enzyme complexes with quinone-interacting *b*-type cytochromes are succinate:quinone reductase (SQR) and quinol:fumarate reductase (QFR). SQR catalyzes the oxidation of succinate and generates fumarate with the consequent reduction of quinone and occurs only in aerobically respiring cells. QFR, an enzyme in anaerobic cells, catalyzes the oxidation of quinol and generation of succinate from

fumarate. These enzymes are very similar and have similarities to hydrogenase in terms of structure and electron transport. Unlike hydrogenase, however, their subunits are bound to the inner cytoplasmic or mitochondrial membrane. The catalytic subunit and the subunit containing [FeS] clusters are membrane bound and anchored to one or two hydrophobic proteins that usually contain cytochrome *b* and transfer electrons to a quinone or from a quinol. The number of hemes in different SQR vary from zero to two, depending on the species (Hederstedt and Ohnishi, 1992). Studies with these enzymes suggest several important points: 1) the cytochrome *b* is an integral component of the enzyme (Hederstedt and Ohnishi, 1992), 2) apocytochrome *b* synthesis is independent of heme synthesis (Fridén and Hederstedt, 1990), 3) the apocytochrome *b* is integrated into the membrane (Fridénn and Hederstedt, 1990), 4) without heme, the catalytic subunits accumulate in the cytoplasm (Hederstedt and Andersson, 1986), 5) the cytochrome *b* forms part of the membrane binding site for the membrane-bound subunits (Fridén et al., 1987), 6) the heme in the cytochrome *b* is required to attain a conformation that enables the membrane-bound subunits to bind (Fridén et al., 1987), 7) the membrane-bound subunits without cytochrome *b* can reduce artificial electron acceptors but require the cytochrome to reduce the natural electron acceptor (ubiquinone) (Kita et al., 1989), and 8) the heme content, and likewise the ability to reduce the quinone, of the cytochrome *b* varies depending on the species (Hederstedt and Ohnishi, 1992).

In *B. subtilis* SQR, the first transmembrane segment of the cytochrome *b* can act as an export signal, and the apocytochrome *b* is inserted into the membrane before the heme is incorporated (Fridén and Hederstedt, 1990). The cytochrome *b* is exposed on both sides of the membrane (Fridén et al., 1987) and is believed to form part of the membrane binding site for the two membrane-bound subunits, since antigen for these proteins accumulated in the cytoplasm of a heme-deficient mutant. Addition of the appropriate heme precursor enabled SQR activity immediately, and the antigen was localized to the membrane (Hederstedt and Andersson, 1986). They suggested that incorporation of the heme resulted in a conformational change that either exposed binding sites for the two subunits or that the heme may interact directly with them. However, since neither of the subunits can bind individually to the cytochrome *b*, they

must bind together (Fridén and Hederstedt, 1990) and the [3Fe4S] of the small iron-sulfur protein subunit is required for binding to the membrane (Hederstedt and Ohnishi, 1992). In this SQR and in mammalian SQR, light-absorption and EPR spectra differ depending on whether the membrane-bound subunits are bound to the cytochrome *b* or not, indicating interaction between the heme and the membrane-bound subunits. The *b*-type cytochromes of *B. subtilis* SQR and *W. succinogenes* QFR (FrdC) have histidine ligands to two hemes on four different transmembrane segments (Hederstedt and Ohnishi, 1992), determining the orientation of the four helices (Hägerhäll et al., 1995). From EPR and MCD spectroscopic data, Fridén and Hederstedt concluded that the two histidines ligating each heme were oriented in a perpendicular position (Hägerhäll et al., 1995). When the cytochrome *b* of *B. subtilis* SQR was overexpressed in *E. coli* and purified, EPR signals and the near infrared MCD spectrum indicated that the imidazole planes were oriented nearly perpendicularly (Fridén et al., 1990). Partial deletion mutants of the two hydrophobic membrane subunits in *E. coli*, FrdC and D, could still bind the two membrane-bound subunits, although not as effectively as in wild-type, but could not transfer electrons to the physiological electron acceptor (Hederstedt and Ohnishi, 1992).

The heme in cytochrome *b* is similar to the heme in myoglobin (Voet and Voet, 1995). In cytochrome *b*, the two histidines bind a low-spin iron, but in myoglobin and hemoglobin, a single histidine acts as a ligand to a pentacoordinate heme, and the iron is high-spin. Cytochrome *b* is at best slowly autooxidizable and doesn't react with carbon monoxide or cyanide, whereas the globins function in gas transfer and readily react with CO, O_2, and CN^-. Although their spin and heme pockets differ; ligation, mutation, and local environment studies of myoglobin and hemoglobin may provide insights for cytochrome *b*.

Heme Ligands

Protein ligands to the heme prosthetic group vary by type of hemoprotein and function. For example, oxygen carrier proteins (myoglobin and hemoglobin) and peroxidases have a proximal histidine, oxidases use cysteine (cytochrome P-450 and nitric oxide synthase) and tyrosine (catalase), and electron transfer proteins (cytochromes) can have histidine, methionine or lysine ligands (DePillis et al, 1994, and Newmyer et al., 1996). In *b*-type cytochromes, ligation is hexacoordinate with bis-histidine ligation. The N3 of the imidazole side chain of histidine ligates the heme iron.

Site-specific Substitution of Heme Ligands to Non-ligands or Alternate Ligands

For several hemoproteins, such as cytochrome *c*, cytochrome *b*, peroxidases, nitric oxide synthase, guanylate cyclase, and myoglobin, the importance of a ligand in coordinating the cofactor was determined by site directed mutagenesis (Zhao et al., 1998). Substitutions in the ligand to the heme are expected to abolish function, either by altering the heme's oxidation-reduction potential or by changing the protein folding pattern (Hampsey et al., 1985; Sorrell and Martin, 1989; Mus-Veteau et al., 1992). Although replacements in ligation positions generally eliminate function, functional substitutions have been made, often with some loss of function (Sherman et al. 1968, Sorrel and Martin, 1989) or physical properties that can be differentiated from wild-type (Fríden and Hederstedt, 1990). Amino acid ligand replacement can provide two types of information: substitution to a non-liganding amino acid determines which residues are ligands, and substitution to an alternate ligand determines the relative contribution of the ligand to the protein structure and function.

Site-directed replacement of a conserved residue to an amino acid lacking a specific functional side group, with a consequent loss in protein function, provides the experimental confirmation that a particular residue is a ligand (McRee et al., 1994,

Decatur and Boxer, 1995; and Thöny-Meyer, 1997). Substitution of a residue into an α-helix of an integral membrane protein requires consideration of the local and general protein environment. The substituted residues should maintain helical stability and hydrophobicity. Two common non-ligand substitutions for histidines in hemoproteins are glycine and alanine, since their small size avoids creating steric hindrances and provides space for small molecules that may bind as exogenous ligands (Barrick, 1994). Glycine is a poor contributor to α-helical stability (Lyu et al., 1990; and O'Neil and DeGrado, 1990), and although it has a non-polar side chain, glycine is not particularly hydrophobic. The roles glycines play in an α-helix are providing a pocket for bulky cofactors or terminating a helix. Alanine, on the other hand, is a strong contributor to α-helical stability (Lyu et al., 1990; O'Neil and DeGrado, 1990; Padmanabhan et al. 1990) and is more hydrophobic than glycine. In addition, alanine is commonly found in α-helices, while glycine is not (Lyu et al., 1990). In cytochrome b_5, a microsomal protein with a soluble monoheme-containing core, replacement of a histidine ligand with alanine resulted in a protein that did not incorporate heme in vivo, and the hemoprotein could not be reconstituted by adding exogenous heme to purified apoprotein (Beck von Bodman et al., 1986).

In general, accepted substitutions for histidine are asparagine, glutamine, aspartic acid, glutamic acid, and arginine (primarily for isosteric reasons to minimize structural changes), and if maintenance of charge, as well as size is important, histidine could be interchanged with arginine or lysine (Plapp, 1995). When substituting an amino acid heme ligand, steric interference of large side chains and the charge should be considered. The charge in the local environment may differ from the expected physiological pH of the protein as a whole. A variety of altered hemoproteins have resulted when a conserved histidine heme ligand has been replaced, either naturally or a result of directed mutagenesis, to methionine, arginine or tyrosine.

A cytochrome histidine ligand replaced by methionine resulted in a pentacoordinate heme (Beck von Bodman et al., 1986; Dolla et al., 1994; Miles et al., 1993), a high spin heme (Sligar and Egeberg, 1987), an unstable protein (Dolla et al., 1994), a protein with a greatly altered redox potential (Sligar and Egeberg, 1987; Mus-

Veteau et al., 1992), altered electron exchange reaction rate constants (Dolla et al., 1994), or novel enzymatic activity (Sligar and Egeberg, 1987). Absorbance studies with flavocytochrome b_2 showed that only 5% of the heme sites were occupied when the histidine axial ligand was mutated to methionine (Miles et al, 1993).

A less common substitution is histidine to arginine. Sorrell and Martin (1989) created a cytochrome *c* histidine → arginine variant that had a slower rate of electron transfer, but did not determine whether the guanidyl group of the arginine was ligating the heme.

Tyrosine and histidine are natural ligands to the heme in nitrite reductase cytochrome cd_1 (Fülöp et al. 1995). Histidine → tyrosine ligand variants have been studied in hemoglobin, cytochrome *b* of the bc_1 complex, and SQR. In the hemoglobin research, histidine ligands were substituted in either the α or β subunits with differing results. The α subunit of Hb M Boston has a pentacoordinate heme with the heme iron being ligated to the phenolate oxygen of tyrosine, but not to the proximal histidine (Pulsinelli et al., 1973). In the β subunit of Hb M Saskatoon, the heme was ligated by both the distal tyrosine and the proximal histidine (Nagai et al, 1983, Nagai et al., 1989). His64→tyr variants of sperm whale and horse heart myoglobin have shown that iron binds to the hydroxyl group of the tyrosine (i.e., the heme iron binds to the oxygen of the tyrosine phenol group without displacing the hydrogen atom) (Pin et al. 1994; and Maurus et al., 1994).

In defining the ligands to the *b*-type cytochrome of the cytochrome bc_1 complex in *Rhodobacter sphaeroides*, histidines were altered to a variety of amino acids. The cytochrome *b* histidine ligands are H97 and 198 (low potential heme), and 111 and 212 (high potential heme). Histidines 111 and 198 were replaced with tyrosine and the high and low potential hemes were lost in both cases, as indicated by optical difference spectra. In addition, the cytochrome c_1 was not detectable either (Yun et al., 1991).

B. subtilis SQR contains an integral membrane *b*-type cytochrome with two hemes, both hexacoordinate. Mutants that had ligand histidine residues replaced with

tyrosine resulted in proteins with total or partial loss of heme (Hägerhäll et al., 1995). Fríden and Hederstedt (1990) replaced the six histidines in *B. subtilis* cytochrome b_{558}, the diheme anchor to SQR, individually to tyrosine. They chose tyrosine since the phenolate can act as a heme axial ligand, but the properties of a his- or tyr-pentacoordinate or his-tyr hexacoordinate heme are distinguishable from a bis-his coordinated heme. Only one of the ligand mutants with tyrosine substituting for histidine resulted in the loss of both hemes. Their his→tyr variants had at most half the relative amount of chromophore per mg protein as the wild-type, and in the three ligand mutants measured for protoheme content, the highest amount was only half of wild-type. As determined with antibody immunoblot analysis, the catalytic subunits localized to the cytoplasm, and SQR activity in the membranes was less than 3.3% of wild-type in these three ligand mutants. They concluded that replacement of the four ligands individually with tyrosine affected assembly of the enzyme. Replacement of one of the non-ligand histidines, H13, located near the membrane edge on the cytoplasmic side, affected function of the enzyme, but replacement of another on a hydrophilic loop on the periplasmic side did not affect enzyme assembly or function. These researchers assigned the two histidines located in the transmembrane segments on the periplasmic side as the ligands to the low potential heme and the two histidines close to H13 on the cytoplasmic side as the ligands to the high potential heme (Fríden and Hederstedt, 1990 and Hägerhäll et al., 1995).

Exogenous Ligands

An exogenous ligand is a non-protein bound molecule that can substitute *in trans* for the side chain of an amino acid residue in an active site. Proteins in which a histidine has been replaced with alanine or glycine have been chemically rescued with imidazoles, pyridines, phenols, furans,and other small molecules and their derivatives (DePillis et al, 1994; and den Blaauwen and Canters, 1993). Exogenous amines have reconstituted mutants lacking key lysine residues (Toney and Kirsch, 1989, and Harpel and Hartman, 1994). Free ligands provide environmental conditions that enable a

altered protein to function; however, the function may not be the same as wild-type in rate or type of reaction. In heme proteins, exogenous ligands may play several roles, such as maintaining protein conformation, providing the strength of binding necessary to pull the iron toward the plane of the heme, acting as a Lewis base electron donor to poise a redox potential (Barrick, 1994), and tethering the iron atom to affect coordination to the sixth ligand site (Newmyer et al, 1996).

While site-directed substitutions to alter key residues provide insight into the role an amino acid plays in a protein, particularly in terms of essentiality, exogenous ligands provide the opportunity to look at structure and function via localized changes. Intermolecular complementation provides the potential to look at the physicochemical properties of redox values, conformational stability, ligand-heme bond stability, and electron transfer kinetics.

Exogenous ligands have been studied with aspartate aminotransferase (Toney et al., 1989), subtilisin (Carter et al., 1991), trypsin (Perona et al., 1994), rhodopsin (Zhukovsjy et al., 1991), azurin (den Blaauwen et al., 1993) (DePillus et al., 1994), heme oxygenase (Wilks et al, 1995), ribulose-1,5-bisphosphate carboxylase/oxygenase (Lorimer, 1981; and Harpel and Hartman, 1994), horseradish peroxidase (Newmyer and Ortiz de Montellano, 1996; Newmyer et al, 1996), cytochrome *c* peroxidase (McRee et al, 1994), and myoglobin (DePillis et al, 1994; Barrick, 1994; Decatur and Boxer, 1995; Decatur et al, 1996). These examples of chemical rescue demonstrate the variety of residues and their possible surrogates in active sites. The reaction rates and structural differences of imidazole-reconstituted peroxidases and myoglobin are probably most pertinent to cytochrome *b*. In these studies, evidence of intermolecular complementarity is shown either by activity assays or structural measurements.

Peroxidases reduce peroxide to water (Newmyer et al., 1996) in a three reaction process in which the radical cation intermediate is either a porphyrin (as in horseradish peroxidase, HRP) or a protein (as in cytochrome *c* peroxidase, CCP) (Newmyer et al., 1996). HRP is a five-coordinate hemoprotein with H170 as the proximal iron ligand and H42 as the distal histidine, which promotes formation of the first intermediate by

acting as an acid-base catalyst (Newmyer and Ortiz de Montellano, 1996). These two histidines were individually replaced with alanine, and the rate of production of the first intermediate was reduced by a factor of ~10^5-10^6. Addition of imidazole or imidazole derivatives partially rescued the rate of production of the first intermediate (0.55% for H170A and 3.3% for H42A) (Newmyer and Ortiz de Montellano, 1996; Newmyer et al., 1996). In CCP, imidazole as an exogenous ligand to H175G resulted in a reactivation rate of ~5% (McRee et al., 1994).

Myoglobin is a five-coordinate heme protein with a proximal histidine ligand bound to the heme. The proximal ligand mutant of sperm whale myoglobin, H93G, can be chemically rescued by a variety of small organic ligands added in excess to the growth medium (DePillis et al, 1994). X-ray crystallography and ^{1}H NMR studies with imidazole as the exogenous ligand showed that the overall structure and function of H93G(Im) was similar to that ofwild-type myoglobin (Decatur and Boxer, 1995). X-ray crystallography demonstrated that imidazole was bound to the heme iron and that imidazole functioned as a proximal ligand in *trans* (Barrick, 1994). However, the conformation of imidazole differs from the imidazole side chain of the histidine ligand in wild-type.

In complemented myoglobin, the free imidazole ligand may attain a more favorable rotation or tilt than is possible with the covalently bound histidine. Decatur and Boxer (1995) suggested that this orientation may be due to the nonbonding and bonding interactions with the heme or to other stabilizing interactions in the protein pocket. Differences between H93G(Im) and wild-type occurred in bond distance between the iron and imidazole, iron atom displacement from the heme plane, tilt of the imidazole plane toward the heme plane, and rotation of imidazole around the nitrogen-iron bond (Barrick, 1994). In H93G(Im) the proximal ligand N-iron distance was 0.3Å closer than in wild-type, and the iron was nearly in the plane of the heme. The displacement of iron is only 0.03Å in H93G(Im) versus 0.23Å in wild-type, probably due to the pull of the histidine, which is not expected for imidazole (Barrick, 1994). The angle between the planes of imidazole and the heme were 75° in H93G(Im) and 87.5° in wild-type. This closer tilt may enable more favorable

interactions between the non-heme binding nitrogen of the imidazole and neighboring polar groups in the myoglobin. Rotation of free imidazole around the nitrogen-iron bond may minimize steric repulsion between the imidazole ring and the heme's pyrrole nitrogens. If the dihedral angle between the heme's NIV-Fe-NII plane and the imidazole plane is 0° or 90°, the imidazole's C2 and C4 atoms would eclipse the pyrrole nitrogens. In wild-type sperm whale myoglobin the angle is $\sim 10^{\circ}$ and in H93G(Im) it is $\sim 45^{\circ}$ (Barrick, 1994; and Decatur and Boxer, 1995). In studies with H93G(4-MeIm)CN, Decatur and Boxer (1995) concluded that the smaller size and different shape of imidazole as compared to histidine did not account for the difference in conformation.

Azotobacter vinelandii as a Model Organism

Members of the bacterial family *Azotobacteraceae* are aerobic, nitrogen-fixing, heterotrophs (organisms that get energy from the oxidation of organic compounds) (Brock et al., 1994) that stain Gram-negative. Cells are often found in pairs, and are rod-to-oval shaped, but the shape changes with growth conditions, and the sticky, extracellular slime promotes pairing and chaining. The organisms are strict aerobes, but can grow under microaerobic conditions. They can grow with fixed nitrogen, but can also fix atmospheric nitrogen when grown in nitrogen-free media with an appropriate carbon source (Buchanan and Gibbons, 1974). Fixation is most efficient under reduced oxygen pressure and low carbohydrate levels. Under nitrogen fixing conditions, molybdenum and iron are required (Buchanan and Gibbons, 1974).

Competence (the ability to bind and transport exogenous DNA) can be naturally induced in some bacteria by providing the proper growth conditions (Reusch and Sadoff, 1983). For *A. vinelandii*, optimal competence can be attained after a period of growth without added iron and molybdenum under oxygen-depleted, nitrogenase-repressed conditions (Page and von Tigerstrom, 1978; Page, 1982; and Page, 1985). Suitable temperatures, pH, carbon sources, and growth stage are also necessary (Page,

1982; and Page 1985). Competence is required for transformation (the genetic recombination of the bacterial chromosome with a fragment of homologous DNA) (Page and von Tigerstrom, 1978) and is initiated in *A. vinelandii* when dissolved oxygen in the medium is completely depleted (Page, 1982). Although *A. vinelandii* can become competent when fixing dinitrogen (Page, 1982), the addition of nitrogen enhances competence and results in a 10-fold increase in transformation frequency (Page and von Tigerstrom, 1978; and Doran et al., 1987). The effects of iron and molybdenum starvation, oxygen deprivation, and addition of fixed iron are associated with nitrogenase repression. Iron and molybdenum are structural components of nitrogenase, and iron is a component of several respiratory chain proteins. Competition for iron between nitrogenase and respiratory components may limit nitrogenase activity, and iron starvation decreases respiration (Page, 1982). Oxygen plays several critical metabolic roles since *A. vinelandii* is an aerobic organism, but nitrogenase is oxygen-sensitive. To protect nitrogenase from oxygen inhibition, *A. vinelandii* increases respiration (Dalton and Postgate, 1969; Yates, 1970; and Page, 1982). Although increased respiration occurs in iron-deficient nitrogen-fixing cells, respiratory control is not adequate to protect nitrogenase (Page, 1982). When cell growth stops due to decreased nitrogen availability under iron-limited, non-supplemented nitrogen conditions, dissolved oxygen content in the medium increases (Page, 1982). However, if nitrogenase is repressed by adding fixed nitrogen, growth is not restricted by nitrogen availability, and aerated cultures can attain the zero dissolved oxygen content necessary for competence (Page, 1982).

Competence can occur in the temperature range of 20-35° C, with an optimum at 30°C (Page and von Tigerstrom, 1979). For competence, pH can be between 6.0 and 8.0 (Page and von Tigerstrom, 1979), but for transformation, the optimum pH is 7.0-7.1 (Page and Sadoff, 1976). Suitable carbon sources are glucose, sucrose, glycerol and mannitol, but not acetate or hydroxybutyrate (Page and von Tigerstrom, 1978). Cells become competent at mid- to late exponential growth (26-30 hours growth on plates) and lose competence by the end of log phase (Page and Sadoff, 1976; and Page and von Tigerstrom, 1978). Transformation is correlated with poly-β-hydroxy-butyrate, a neutral lipid component of *A. vinelandii* cytoplasm and cell membranes

(Reusch and Sadoff, 1983). Poly-β-hydroxybutyrate affects membrane structure and likewise alters membrane barrier properties toward DNA (Reuch et al., 1987).

A. vinelandii possesses multiple nucleoids, and the number of nucleoids varies with growth stage (Maldonado et al. 1994). Experimental transformation in *A. vinelandii* is typically by cotransformation, in which two independent DNA molecules transform a cell (Bishop et al., 1977). One DNA molecule carries an antibiotic resistance marker, such as rifampicin, and the genetic marker on the second DNA molecule is mutated *A. vinelandii* DNA. Linearized plasmid DNA transforms cells with two to three times greater efficiency than covalently closed or open circular forms (Doran et al., 1987). Expression of rifampicin resistance occurs after three hours incubation in nonselective media and stabilizes after about 14 hours (Page and von Tigerstrom, 1979). A long period of nuclear segregation is required to produce genotypically homogeneous progeny with a stable phenotype (Page, 1985). As a consequence, only a fraction of the cells originally transformed carry the resistance marker and mutated gene (Page, 1985). Since the cells are often found in pairs or groups, multiple transfers before phenotypic screening aid in both genetic segregation and single cell isolation.

A. vinelandii is a good model organism for hydrogenase studies because it is a free-living, nitrogen-fixing bacterium that is well characterized and easily grown. Free-living, as compared to symbiotic, enables the organism to be readily cultured in the laboratory and avoids complications of multiple biological system interactions. *Azotobacter* studies can provide information on the association of nitrogenase and hydrogenase more easily than an obligate autotroph dependent on H_2 for growth. The basic biology and genetics of *A. vinelandii* are relatively well understood, and *A. vinelandii* has long been used as a model organism for nitrogen fixation. *A. vinelandii* has an uptake hydrogenase that primarily catalyzes in the oxidative direction (Przybyla et al., 1992). Working with *A. vinelandii's* single membrane-bound hydrogenase avoids the difficulties encountered with *E. coli's* four hydrogenases or *A. eutrophus'* two different forms of hydrogenase, a soluble and a membrane-bound form. For studies on the cytochrome *b* of hydrogenase, the *A. vinelandii* system is complicated

by the fact that there are three *b*-type cytochromes in the respiratory chain: in hydrogenase HoxZ, the bc_1 complex, and cytochrome *bd* oxidase. Hydrogenase donates electrons to the quinone and quinol donates to either the *bc*1 complex under low oxygen, non-nitrogen fixing conditions or to cytochrome *bd* oxidase, a terminal oxidase that functions under high oxygen, nitrogen fixing conditions (Bertsova et al., 1998). The contribution of each of these *b*-type cytochromes affects spectral analysis of membrane fractions, such as difference spectra. However, having multiple *b*-type cytochromes is a condition faced by most hydrogenase-containing bacteria, and some have three or four types, depending on the growth conditions.

Materials and Methods

Sequence Alignment and Phylogeny of Hydrogenase *b*-type Cytochromes

The sequence of *A. vinelandii* HoxZ from the National Center for Biotechnology Information (NCBI) GenBank accession number 23000 (Menon et al. (1990)) is shown in Figure 1. The designated transmembrane segments were most similar in terms of selected residues and length to those predicted by NCBI Entrez protein query for accession P23000 (*A. vinelandii* HoxZ). Sequences for eubacterial hydrogenase *b*-type cytochromes were retrieved from GenBank (Table 1) and aligned by hand using the GCG software program GDE (Wisconsin Package Version 10.0, Genetics Computer Group (GCG), Madison, WI) (Fig. 2). The phylogenetic tree of hydrogenase *b*-type cytochrome relationships (Fig. 3) was generated with *Aquifex aeolicus* as the outgroup using the GCG software PAUP. The tree was displayed with the software program TreeView (Page, 1996).

Isoelectric Point Calculation

The pI's (the pH where the protein has a net charge of zero) for wild-type and HoxZ mutants were calculated with ExPASy-ProtParam Tool (Wilkens et al., 1998).

Plasmids, Bacterial Strains, and Mutagenesis

The plasmids and bacterial strains used in this study are listed in Table 2. The nucleotide sequences for *hox* genes K, G, Z and M are from the NCBI GenBank database accession number L23970. Derivations of strains and expected phenotype are shown in Table 3.

Table 1. Accession numbers of hydrogenase *b*-type cytochromes.

Organism Name and Protein Name		NCBI GenBank Accession Number
Alcaligenes eutrophus	HoxZ	P31898
Aquifex aeolicus	HoxZ	AAC06857
Archaeoglobus fulgidus	HydC	AAB89865
Azotobacter chroococcum	HupZ	S53656
Azotobacter vinelandii	HoxZ	P23000
Bradyrhizbium japonicum	HupC	P21960
Escherichia coli	HyaC	P19929
Helicobacter pylori	HyaC	AAD06148
Oligotropha carboxidovorans	HoxZ	CAA72675
Pseudomonas hydrogenovora	HupC	BAA13224
Rhizobium leguminosarum	HupC	P27648
Rhodobacter capsulatus	HupM	P16145
Rhodobacter sphaeroides	HupC	CAA74589
Thiocapsa roseopersicina	HupC	AAA27411
Wolinella succinogenes	HydC	S22406

Table 2. Plasmids and bacterial strains

Plasmids and Strains	Relevant properties	Source or reference
Plasmids		
pAVhoxZ$^+$	*SmaI-SalI* fragment (1049 b) of *hoxGZM* containing *hoxZ* inserted into pBluescript II SK-	this study, subclone of SalI fragment inserted into pSelect (p302L) from L.A. Sayavedra-Soto*
pAVhoxZ$^-$	P302L with 231 b Kpn fragment deleted from *hoxZ*	from L.A. Sayavedra-Soto*
pDB303	*A. vinelandii* rif^r genomic DNA fragment in pUC8; Amp^r	from D.R. Dean**
pAVhoxZ H→A series	pAVhoxZ$^+$ with H→A individually in positions 33, 74, 98, 136, 174, 191, 194, and 194&208	this study
pAVhoxZ H→Y series	pAVhoxZ$^+$ with H→Y individually in positions 33, 74, 97, 194, and 208	this study
Strains		
A. vinelandii DJ	Rif^s *A. vinelandii* DJ (wild-type (Wt)), high transformation strain	from D.R. Dean*
A. vinelandii DJ R	Rif^r *A. vinelandii* DJ (WtR)	this study
A. vinelandii hoxKG$^-$S	Rif^s strain with nonfunctional *hoxKG* genes derived from *A. vinelandii* D.J.	from L.A. Sayavedra-Soto*
A. vinelandii hoxKG$^-$R	Rif^r hoxKG$^-$	this study
A. vinelandii hoxZ$^-$S	Rif^s strain with nonfunctional *hoxZ* gene, derived from *A. vinelandii* DJ transformed with pAVhoxZ$^-$	this study
A. vinelandii hoxZ$^-$R	Rif^r strain with nonfunctional *hoxZ* gene derived from *A. vinelandii* DJ transformed with pAVhoxZ$^-$ and pDB303	from L.A. Sayavedra-Soto*
A. vinelandii hoxZ H→A series	Rif^r *A.vinelandii* H→A individually in HoxZ positions 33, 74, 98, 136, 191, 194, and 194&208	this study
A. vinelandii hoxZ H→Y series	Rif^r *A.vinelandii* H→A individually in HoxZ positions 33, 74, 97, 194, and 208	this study
E. coli DH5α	Amp^s	Gibco BRL

* Sayavedra-Soto and Arp, 1992

** Virginia Polytechnic Institute and State University

Table 3. Derivation of strains and their expected H_2 oxidation phenotype.

Recipient strain	Wt (rif^S)	$HoxKG^-$ (rif^S)	Wt (rif^S)	$HoxZ^-$ (rif^S)
Donor DNA	Rif^r	Rif^r	Rif^r & purported ligand mutant	Rif^r & purported non-ligand mutant
Resulting strain	WtR	$HoxKG^-R$	H→A or Y	H→A or Y
Expected hydrogenase activity	+	–	–	+

Table 4. Oligonucleotide sequences used for mutating *hoxZ* DNA in plasmid pAVhoxZ^+.

Position and mutation	Oligonucleotides
H33A	5'> CGC CGC TGC GTC TCT GGG CAT GGG TCA CGG CGC TGT C <3' 5'> CCG TGA CCC ATG CCC AGA GAC GCA GCG GCG CCT CGT AG <3'
H33Y	5'> CGC CGC TGC GTC TCT GGT ATT GGG TCA CGG CGC TGT C <3' 5'> CCG TGA CCC AAT ACC AGA GAC GCA GCG GCG CCT CGT AG <3'
H74A	5'> TCC GCT TCG CCG CTT TCG CCG CCG GCT ACG TGC <3' 5'> GGC GGC GAA AGC GGC GAA GCG GAT GTA GCC CAT <3'
H74Y	5'> TCC GCT TCG CCT ATT TCG CCG CCG GCT ACG TGC <3' 5'> GGC GGC GAA ATA GGC GAA GCG GAT GTA GCC CAT <3'
H97Y	5'> GCC TTC GTC GGC AAC TAT CAC GCC CGC GAG CTG <3' 5'> GCG GGC GTG ATA GTT GCC GAC GAA GGC CCA GTA <3'
H98A	5'> TTC GTC GGC AAC CAC GCA GCC CGC GAG CTG TTC CTC <3' 5'> CTC GCG GGC TGC GTG GTT GCC GAC GAA GGC CCA GTA <3'
H136A	5'> AGT ACA TCG GCG CAA ACC CCC TGG <3' 5'> CAG GGG GTT TGC GCC GAT GTA CTT CTT CGG GGT <3'
H191A	5'> AGT CAG GAC GTG GCA ACC TGG CAC CAC CTG GGC ATG <3' 5'> GTG CCA GGT TGC CAC GTC CTG ACT CTG GCC GAA CAG <3'
H194A	5'> CAC CTG GGC CCA CCT GGG <3' 5'> TGC CCA GGT GGG CCC AGG <3'
H194Y	5'> TGC ACA CTT GGT ATC ACC TAG GCA TGT GGT AC <3' 5'> CCT AGG TGA TAC CAA GTG TGC ACG TCC TGG CT <3'
H208A	5'> TCG TCA TGG TGG CAG TCT ACC TGG CCG TG <3' 5'> AGG TAG ACT GCC ACC ATG ACG AAG ACG ACG AGG <3'
H208Y	5'> GTC TTC GTC ATG GTG TAT GTC TAC CTG GCC GT <3' 5'> TAG ACA TAC ACC ATG ACG AAG ACG ACG AGG <3'
HoxZ	5'> GCG ATG GCA CTG GAA AAA TCC CTG GAA AC <3' 5'> GGA GAT CAG CGA CTG CCG GGA AAC GA <3'

Oligonucleotides for the mutagenesis were designed using the software Oligo (ver. 3.0, Molecular Biology Insights, Plymouth, MN) and are listed in Table 4. The oligonucleotide-directed polymerase chain reaction (PCR) based methods of Merino et al. (1992) and Weiner et al. (1994) were used for site-directed mutagenesis of the plasmid template pAVHoxZ^{+}. The PCR product was then transformed into precompetent *E. coli* DH5α (Gibco BRL, Grand Island, NY), and the bacteria were plated on Luria Bertani (LB) Amp (60 mg/l) agar medium (Sambrook et al., 1989) and grown overnight. Single colonies were picked and used to inoculate LB Amp liquid medium and grown overnight. Plasmid preparations were made according to Lee and Rasheed (1990). Mutants were screened and verified by sequencing at the Central Services Lab of the Center for Gene Research and Biotechnology, OSU.

Transformation and Screening

Plasmids with *hoxZ* mutations expected to affect ligands were cotransformed with a gene conferring rifampicin (rif) resistance into a strain competent in H_2 oxidation, *A. vinelandii* DJ (Wt), with the expectation that the resulting strain would be rif-resistant and H_2-oxidation-deficient. Plasmids with *hoxZ* mutations expected to affect non-ligands were cotransformed into *A. vinelandii* HoxZ^{-}rif^{s} (a partial deletion mutant limited in H_2 oxidation capability and sensitive to rif), and the transformed strain was expected to have a rif-resistant H_2 oxidation-competent phenotype. To generate a rif-sensitive HoxZ^{-} strain, 1 μg of pHoxZ^{-} was transformed into competent *A. vinelandii* DJ without the aid of an antibiotic marker. Except for growing in Burk medium (Strandberg and Wilson, 1968) without rif, other aspects of transformation and screening were the same as for other mutants.

Bacteria were made competent by several transfers on iron- and molybdenum-deficient, 28mM ammonium acetate-supplemented Burk medium (Premakumar et al., 1994) under oxygen-limited conditions (Page and von Tigerstrom, 1978, Page and von Tigerstrom, 1979, Page and Sadoff, 1976). All HoxZ mutant plasmids were linearized

with the restriction enzyme SmaI (US Biochemical, Cleveland, OH, and Promega, Madison WI) to generate a blunt end cut in the DNA. 1 μg of linearized plasmids pHoxZ H33, 74, 194, 208 and 194,208→A or Y were individually cotransformed with 10 ng of the *EcoR*I (Promega, Madison, WI) 1.7-kb fragment of pDB303, which confers rif resistance, into rif^s *A. vinelandii* DJ (wt) by using the method of Premakumar et al. (1994). Linearized plasmids pHoxZ H97, 98, 136, and 191→A or Y were cotransformed similarly into rif^s *A. vinelandii* HoxZ$^-$.

In *E. coli*, resistance to rif is conferred by a mutation(s) in the β subunit of RNA polymerase (Jin and Gross, 1988). The gene conferring resistance in *A. vinelandii* is uncharacterized (Brigle et al., 1987), but is expected to be due to a mutation similar to that in *E. coli*. Strains growing in Burk+rif medium, even though resistant, are still affected by rif and grow more slowly than in Burk medium without antibiotic. To compare only *hox* mutants and not differences due to growth rate, all strains were made rif-resistant. A rif^r (rif-resistant) HoxZ$^-$ strain was available, and strains *A. vinelandii* DJ and *A. vinelandii* HoxKG$^-$ were made rif-resistant by transformation using the 1.7 kb EcoR1 fragment of pDB303.

After transformation, bacteria were transferred to rif-(20 mg/ml) augmented Burk medium to select for rif^r transformants. Since *A. vinelandii* has multiple chromosomes (Maldonado et al., 1994), single colonies were transferred three times on Burk+rif medium to allow for complete segregation (Premakumar et al., 1994). A phenotypic assay of H_2 oxidation ability similar to that of Sayavedra-Soto and Arp (1992) was used to screen for coincident transformation of the HoxZ mutants. Test tubes containing 2 ml Burk medium + rif (20 mg/ml) were inoculated from single colonies, and the bacteria were grown to an optical density (OD) at 600 nm of 0.4. Plastic caps on the test tubes were exchanged for butyl rubber stoppers and 4.46 μmol H_2 (high-purity commercial grade H_2 was purchased locally) was added with a 500 μl Hamilton syringe. Cultures were grown on a shaker table (150 rpm) for 16 hours at 30°C. For screening, 100 μl culture headspace was sampled and analyzed for the presence of H_2 using a Shimadzu GC-8A thermal conductivity detector (TCD) gas chromatograph

(GC) (200°C injection t, 120°C column t, 60mA current, and attenuation 4) with a Molecular Sieve 5A column.

Cultures with phenotypes differing from the background parental strain were selected as potential mutants. These cultures were transferred to Burk+rif agar medium, grown three days at 30°C, and a single colony was transferred to 2 ml Burk medium. This culture was grown on a shaker table overnight at 30°C and used for a genomic DNA preparation (Ausubel et al., 1993). A portion of the *hoxZ* gene was amplified using the polymerase chain reaction (PCR) (Ausubel et al., 1993), gel purified (Concert BRL Gibco Life Technologies Nucleic Acid Purification System), and sequenced to confirm the mutation.

Growth Conditions

Single colonies were picked to inoculate 2 ml Burk medium (pH 7.0) and the cultures were grown to late exponential phase and used as inoculum in either 5 ml Burk medium in a 25 ml flask for O_2-dependent H_2 oxidation assays, or in 50 ml Burk medium in a 250 ml flask for H_2-dependent MB reduction assays. To determine the OD_{600} at which H_2 oxidation was maximum, 5 ml cultures were grown to ODs_{600} between 0.5 to 1.2 on a shaker table at 30°C. All other cultures were grown to an OD_{600} of 0.8 to 1.0. For imidazole (Imd, Sigma, St. Louis, MO) grown cultures Imd (adjusted to pH 7.5 with HCl) was added to a final concentration of 10 mM (Barrick, 1994) at the time of inoculum addition. For 3-fluoropyridine (3FP, Sigma, St. Louis, MO) 3FP was dissolved in ethanol and added to a final concentration of 10 mM at the time of inoculum addition.

H_2 oxidation assays

Whole Cell O_2-dependent H_2 Oxidation: Physiological Electron Transfer Assay

This assay uses the physiological terminal electron acceptor, O_2, to assay for functionality of the hydrogenase trimer. The assay is based on the ability of electrons to flow from the catalytic heterodimer to oxygen, and the expectation that a dysfunctional component in the chain would result in decreased ability of hydrogenase to oxidize H_2. Preliminary experiments showed that cells resuspended in growth medium had H_2 oxidation activity, but cells resuspended in phosphate buffer did not, probably due to oxidation of the hydrogenase enzyme. Therefore, 3 mM sucrose, equivalent to a starvation level of carbon for *E. coli* (0.1% glucose) (Atlung et al., 1997), was added to the buffer with the expectation that this level would maintain respiration, enabling the enzyme to remain reduced, but not enable growth.

Suspension cultures (1.5 ml) were centrifuged 1 minute at 14,000 rpm, the growth medium was removed, and the pellet was resuspended in 50 mM phosphate buffer (pH 7.0) + 3 mM sucrose to protect against oxidative shutdown of hydrogenase. The resuspended bacteria were placed in a 10 ml serum vial capped with a rubber sleeve stopper, placed in a 30° water bath at a shaker rate of 200 cycles/min and allowed to equilibrate (5 minutes). At time 0, H_2 (4.46 μmol) was added to the vial with a 100 μl Hamilton syringe and a 200 μl sample of headspace sample was removed with a 500 μl Hamilton syringe and injected into the TCD GC. Vials were resampled after 15 and 30 minutes. To prevent gas leakage, vials were placed upside-down in the water bath after sampling. For assays at pH 9.0, the phosphate-sucrose buffer was titrated with 1 M NaOH from pH 7.0 to pH 9.0. For assays in which Imd, 3FP or chloramphenicol (Aldrich Chemical Company, Milwaulkie, WI) were added at time 0, chemicals were added with a 10μl Hamilton syringe to a final concentration of 10 mM, 10 mM, and 3 mM, respectively. Four individual replications of this assay for each strain were recorded. For the H194A and H194Y Imd wash assays, the strains were handled the

same as those grown with Imd, except that after replacing the growth medium with buffer, the vials were shaken on a shaker table for an hour at 30°C before assaying.

Whole Cell H_2-dependent MB Reduction: Heterodimer Functionality Assay

This assay uses the artificial electron acceptor methylene blue (MB, Sigma, St. Louis, MO) to assay for functionality of the hydrogenase catalytic heterodimer, thereby localizing the loss of physiological electron transfer to the substitutions in HoxZ. The assay is based on the coupling of H_2 oxidation directly to an electron acceptor, the reduction of which can be monitored spectroscopically. MB is marine blue when oxidized and clear when reduced. MB can accept electrons from hydrogenase, but can also be reduced by nonspecific endogenous cellular substrates, particularly in whole cells. Assaying with and without the addition of H_2 can account for the endogenous level of methylene blue reduction. Since oxygen can reoxidize MB, this assay must be carried out under anaerobic conditions (Arp, 1989).

Cultures of whole cells (40 ml) were centrifuged 10 minutes at 7,500 rpm and the growth medium was removed. The pellet was resuspended in 1 ml of 50 mM Tris buffer + 5 mM EDTA (pH 7.3) and placed in a 10 ml serum vial stoppered with a rubber sleeve cap. The vial was purged with N_2 (high-purity commercial grade N_2 was purchased locally) for seven minutes to remove O_2. A sample 7.5 ml cuvette (Beckman 2097) containing a total of 1 ml 50 mM Tris buffer + 5 mM EDTA (pH 7.3), including 150 μM MB as an electron acceptor, was stoppered with a sleeve cap and similarly flushed with N_2. H_2 (1 ml) was added with a 500 μl Hamilton syringe for assays with H_2. All reactions were started with the addition of 50 μl N_2-purged culture using a 100 μl Hamilton syringe. The decrease in absorbance at 690 nm was monitored with a Beckman DU®-70 spectrophotometer for 1 minute (690 nm, rate of 1200 readings per minute) and the greatest difference in absorbance in a 10 second time period was recorded. Four individual replications of this assay for each strain were recorded. Calculations were based on a molar extinction coefficient of 11.4 mM^{-}

$^{1}cm^{-1}$ at 690 nm. MSU Stat software (Montana State University, Bozeman, MT) was used to analyze data for both the O_2-dependent H_2 oxidation and H_2-dependent MB reduction assays.

Activity and Protein Assays

Protein content was determined for samples used in the activity assay, in which activity was compared to OD_{600} values between 0.5 to 1.2 using the O_2-dependent H_2 oxidation assay. For this assay, three cultures (5 ml) of the same OD were pooled and a 1.5 ml sample for the O_2-dependent H_2 oxidation assay and a 10 ml sample for the protein assay were used. The whole cell protein contents were determined by the microbiuret assay (Gornall et al., 1949) with bovine serum albumin as a protein standard. Three replicates of both assays were recorded.

Molecular Modeling of HoxZ

The transmembrane α-helices and heme ligation were modelled using the software InsightII (Ver. 2.2.0, Biosym Technologies, San Diego, CA). The α-helices, porphyrin rings and iron were generated from idealized parameters available in the biopolymer module of Insight. Three of the four helices contain ligands to the hemes. Therefore, this does not fix the helices positionally, as would ligands on four helices. The hypothetical position of the helices was based on normal histidine-heme iron bond distances (Barrick, 1994) and the helices being perpendicular to the cytoplasmic membrane. The planes of the imidazole side chains of the histidine ligands were assumed to be perpendicular to each other, and perpendicular to the hemes to minimize steric repulsions (Barrick, 1994; and Decatur and Boxer, 1995. Helices A, B, and D are positioned so that the ligands bind the heme irons with minimal changes of angles within the histidines. Helix C does not have a ligand and its position relative to the other three helices is assumed.

Results

Sequence Alignment and Phylogeny of Hydrogenase *b*-type Cytochromes

The alignment of 14 eubacterial hydrogenase *b*-type cytochromes (Fig. 2) shows that the four conserved histidines are found near the centers and ends of the transmembrane α-helical segments. One is near the center of helix A, one at the beginning of helix B, and two are in helix D, in agreement with Gross et al. (1997). The four non-conserved histidines selected for study are in the loop regions. Three of the four are in loop BC on the cytoplasmic side and the fourth is in loop CD on the periplasmic side of the plasma membrane. The phylogenetic tree (Fig. 3) indicates that the cytochrome *b* of *A. vinelandii* is most closely related to that of *A. chroococcum,* but the next closest relationship could not be determined since the bootstrap values were less than 50. Hence, the relationships are shown in Figure 3 as a polytomy. *A. vinelandii* HoxZ is most distantly related to the hydrogenase cytochrome *b* of *W. succinogenes.* Earlier phylogenies of the large and small subunits of hydrogenase (Wu and Mandrand, 1993) showed that the large subunit of *A. vinelandii* was most closely related to that of *A. chroococcum* and then to *Rhodocyclus gelatinosus* and *R. capsulatus*. The small subunit, which in general shows more heterogeneity, was most closely related to that of *A. chroococcum* and *R. capsulatus*.

Isoelectric Point of HoxZ Wild-type and Mutants

The calculated pI's for wild-type HoxZ and the mutants are: HoxZ (8.58), HoxZ$^-$ (8.86), HoxZ H→A (8.58), HH→AA (8.58), and H→Y (8.57). The isoelectric points of HoxZ and HoxZ$^-$ were calculated to update a previous report (Sayavedra-Soto and Arp, 1992). Isoelectric points for the single and double amino acid replacement indicate that the pI's for the mutants are similar to that of wild-type.

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                                                                         33
                                                                         ↓
Av ~~~~~~~~~~~~~~~~~~~~~MALEKSLETGDGQEKVRKQTAVYVYEAPLRLWHWVTALSIVVLGVTGYFIGAPLPTMP.G.EA...MD.NYLM
Ec ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~MQQKSDNVVSHYVFEAPVRIWHWLTVLCMAVLMVTGYFIGKPLPSVS.G.EATYL....FYM
Ac ~~~~~~~~~~~~~~~~~~~~~MALEKSLETGDGQEKVRKQTAVYVYEAPLRLWHWVTALSIVVLGVTGYFIGAPLPTMP.G.EA...MD.NYLM
Tc ~~~~MSRAASRLAPGAPTAEIRLTNIRDNAMNTSLPNVKQTAVYVYQAPLRAWHWINALSITILAITGYLIGSPLPTLP.G.EAS...D.HYVM
Rc MKGVSDERINAPVRGPDEIFEASRLTGDATREDLESIRRRTSVYVYEAPVRVWHWVNALAITILVVTGYFIASPLPSMQIG.EAT...DQ.FVM
Bj ~~~~~~~~~MMDAVAPASDAGPDLAAIAADASGERAVGR.PTVYVYEAPVRICHWVNAFSIIVLMVTGYLIGTPLPTVA.G.EAS...D.NFVM
Ae ~~~~~~~~~~~~~MSTKMQADRIADATGTDEGAVASGKSIKATYVYEAPVRLWHWVNALAIVVLAVTGFFIGSPPATRP.G.EASA....NFLM
Rl ~~~~~~~~~~~~~~~~~~~~MTIHETLAADAHGEKAIERQ.SVYVYEAPVRIWHWINAFSILTLALTGYFIGSPLPSVP.G.EASA....NFLM
Ph ~~~~~~~~~~~~~~~MTTPVPAGHGDQSGADRMAAPQRRSRLMYVYQAPVRIWHWINAAAIVVLAVTGYFIASPIATRP.G.EASA....NFMM
Oc ~~~~~~~~~~~~~~MSDITNMRHEGVLETESHELVGGSRQTSVYVYEAPVRLWHWINAIAILVLALTGFFIGRPLPTMP.G.EASA....NFLM
Rs MDTEIVTIHVPNPNAPTPLEASRKTGTARPEDIESIFYK.TSVYVYEVPVRLWHWLNAAMIFLLIGTGYLIARPLPTMEMG.EAT...DQ.FVF
Aa ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~MFKRIFIFSASLRIWHWINFLTIMVLFFTGLYIGTPYFTTSSGYEATYAYDKNITM
Hp ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~MDKMNKVVLHKEYSGFVRFFHWVRALSIFALIATGFYIAYPFLQ.P.NS.SFY.KGVYLLQ
Ws ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~MENRANTEGTFERELEFTALTRWFHWIRAIAIFVLIVTGFYIAYPFLTPIKNSEPT.....NFMY

      74
      ↓                     97,98                                        136
Av GYIRFAHFAAGYVLAIGFLGRVYWAFVGNHHARELFLVPVHRKAWWKELWHE.VRWYLFLEKTPKKYIGHNPLGQLAMFCFFVVGAVFMSVTGFAL
Ec GYIRLIHFSAGMVFTVVLLMRIYWAFVGNRYSRELFIVPVWRKSWWQGVWYE.IRWYLFLAKRPSADIGHNPIAQAAMFGYFLM.SVFMIITGFAL
Ac GYIRFAHFAAGYVLAIGFLGRVYWAFVGNHHARELFLVPVHRKAWWKELWHE.VRWYLFLEKVPKKYIGHNPLGQLAMFCFFVIGAVFMSVTGFAL
Tc GYIRFAHFAAAYVFVIAFIFRIYWAFVGNRYSHQLFTLPFWRASFWHELIHE.VRWYAFKETEPAKYIGHNPLAHLFMVVIITVGGLVMIVTGFAL
Rc GYIRFAHFAAGVVMSVAFFGRIYWAFVGNRHAWQMFYIPIFNKRYWKEFVFE.LRWYFFLEEEPKKYIGHNPLAHAAMFTFITLGITFMMITGWAL
Bj GYIRFAHFAAGQVLAVFFLTRILWAFVGNHHSRQIFYIPVHRKQFWKEVLHE.IRWYAFLEREPKMYVGHNPLAQTAMFTGFTLFVAFMIVTGFAL
Ae GYIRFAHFVAAYIFAIGMLGRIYWATAGNHHSRELFSVPVFTRAYWQEVIS.MLRWYAFLSARPSRYVGHNPLARFAMFFIFFLSSVFMILTGFAM
Rl GYIRFIHFAAGQLLAVFLILRVYWAFVGNVHARQIFYVPFWSGRFWKEWLHEVG.WYTFLVRQPKKYVGHNPLPQFTMFLMFTLPLLFMAITGFAL
Ph GYIRFAHFSAAYIFTIGMLGRVYWAFVGNKHARELFHVPIFTRAYWSDLTS.ILRWYGFISKRPNRYTGHNPLARVAMYIIFVLRSIYMILTGFAL
Oc GYIRFTHFTAGYILAIGFLFRFYWSLVGNHHAKQLFRLPLTSRHCWSEIWFE.LRWYLFIEKRPKKYAGHNPLAQIAMFLFITIGTMFMILTGFAL
Rs GYIRFAHFAAGQVLTVAFAARILWAFFGNVHSRQMFKLPVWNLRWIRELIYE.LKWYLFLVPEPRKYVGHNPLAHAAMFFFITLGMSFMIVTGFAL
Aa GFIRFLHFSAGYVLLFSFLFRIFIAFF.RKGDR.LF.IPKFWTIEYWEGIKETLKEYLLIGSRKPMIR..NPLAR.TAYFFVYLMIFFMIATGFGM
Hp AYVRSFHVMFGFLLISALIFRTYL.FFTKESLME..RK.SFSQLLSPKAWIDQMKAYFLISGKPHTKGAYNPI.QLVAYSTLIVLIVLMSLSGMVL
Ws ALARSWHQIFGFALIAVTIFRVYLFIFDKGCRVE..RA.SFWDLINPLTWFRQLRNYMLLGPHPHLKGVYNPV.QLAAYMGLMVLILLISVTGIIL
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Fig. 2. Alignment of membrane-bound hydrogenase *b*-type cytochromes. Bars denote transmembrane segments, arrows indicate conserved histines.

Fig. 2. Alignment of membrane-bound hydrogenase *b*-type cytochromes, continued. Bars denote transmembrane segments, arrows indicate conserved histines.

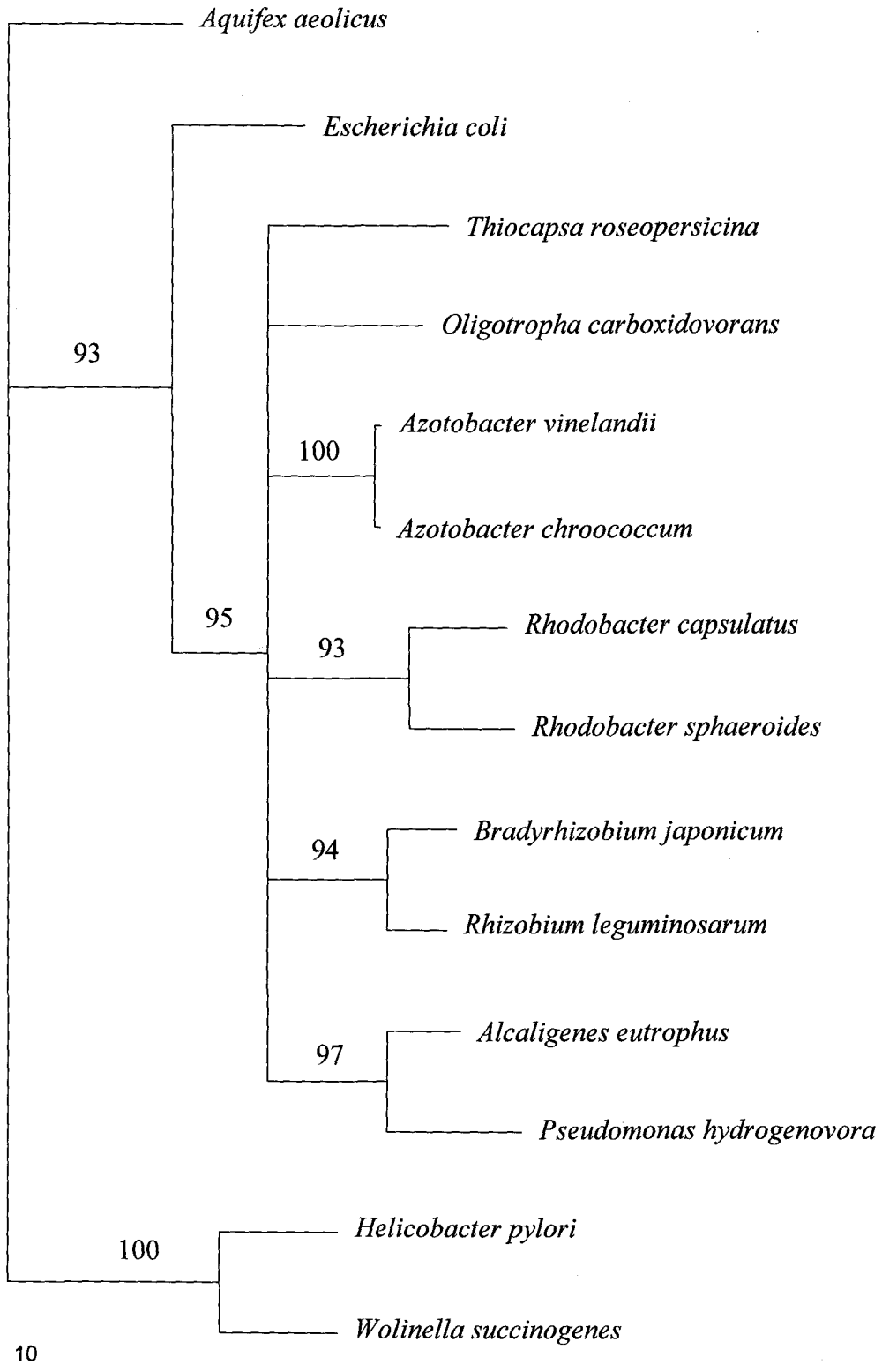

Fig. 3. Phylogeny of membrane-bound [NiFe] hydrogenase *b*-type cytochromes.

Wild-type Activity Assay and Protein Determination

Bacterial growth can be followed by measuring the OD of the culture. In order to determine when to harvest *A. vinelandii* for assays, the OD and protein amounts were compared to H_2 oxidation activity. Protein amounts generally increase through mid-stationary phase, but enzyme activity often peaks during some part of the growth process. Whole cell hydrogenase activity determined with O_2 as the terminal electron acceptor showed that physiologically relevant H_2 oxidation was greatest in the OD_{600} range of 0.8-1.0 (Figure 4). On this basis, *A. vinelandii* cultures for all other assays were harvested at ODs of 0.8 through 1.0, and protein levels in this range could be used to calculate activity per mg protein (150 μg/ml was selected).

Hydrogen Oxidation Assays

Whole Cell O_2-dependent H_2 Oxidation: Physiological Electron Transfer Assay

To determine whether hydrogenase is functional in physiological electron transport in various *A. vinelandii* mutants and Wt, H_2 oxidation with O_2 as the terminal electron acceptor was assayed by measuring the decrease in H_2 over a 30 min period (Table 5). Substitutions of the amino acids acting as ligands in hydrogenase cytochrome *b* were expected to affect hydrogenase trimer activity. Wild-type *A. vinelandii* DJ (Wt) and rif-resistant *A. vinelandii* DJ (WtR) served as positive controls. Hox KG^-(KG^-R) (a partial deletion mutant of the catalytic heterodimer) served as a negative control. HoxZ^- (Z^-R) (a partial deletion mutant of hydrogenase cytochrome *b*) served as a control for the loss of a functional HoxZ (Figure 5, Table 6). Both Wt and WtR oxidized 1.1 μmol H_2 in 30 minutes (26% of the initial H_2 added) and were not significantly different. Neither KG^-R nor Z^-R was significantly different from the buffer-only control that had no cells, indicating that neither had H_2 oxidation activity.

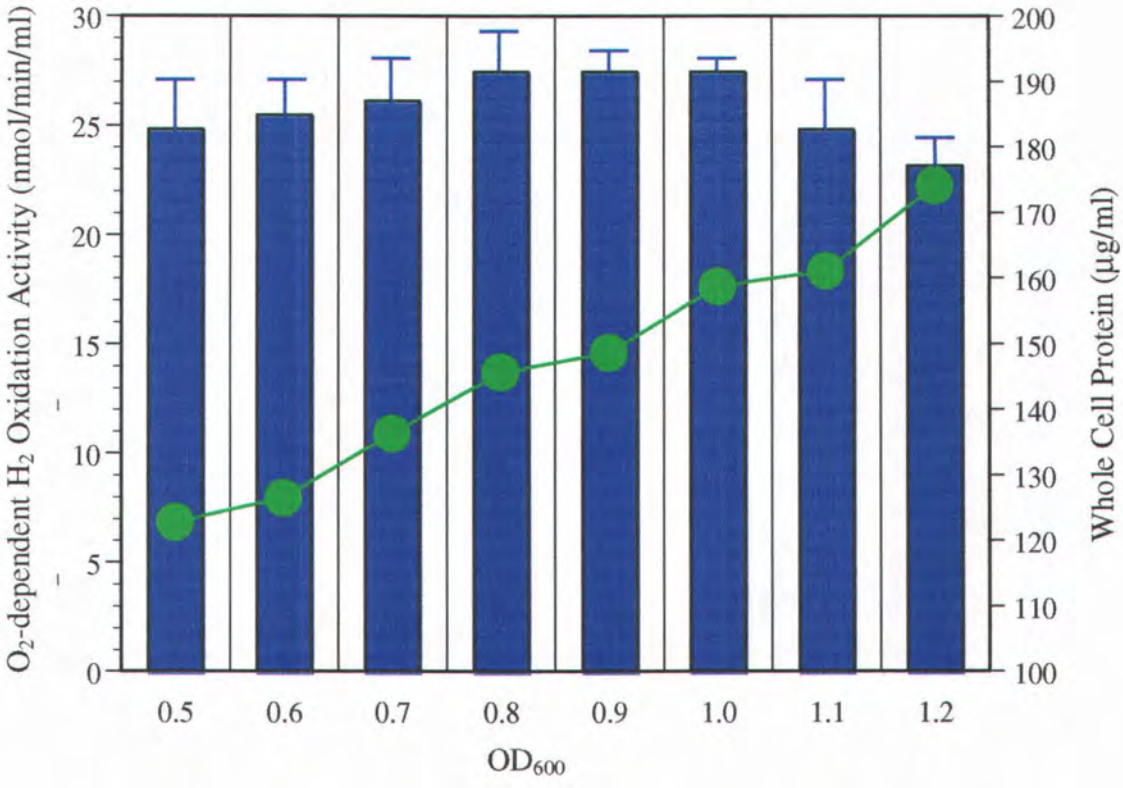

Fig. 4. Wild-type *A. vinelandii* hydrogenase activity assay. H_2 oxidation activity ■, protein ●.

Table 5. Comparison of strains in O_2-dependent H_2 oxidation.

Strain and treatment*	Mean nmol H_2 oxidized/min/ml and Std Err		% Wt
Controls			
Wt	25.3 ghij	1.0	100
Wt(Imd)	26.5 ij	1.4	105
WtR	25.8 hij	1.1	102
WtR(Imd)	24.6 fghi	1.1	97
KG⁻R	0.3 abc	0.6	1
KG⁻R(Imd)	0.5 abc	0.5	2
Z⁻R	0.5 abc	0.3	2
Z⁻R(Imd)	0.8 abc	0.8	3
Buffer Control	0.6 abc	0.6	2
Ligands			
H33A	0.5 abc	0.5	2
H33A(Imd)	2.2 c	1.1	9
H33Y	0.7 abc	0.5	3
H33Y(Imd)	0.2 abc	0.2	1
H74A	0.2 abc	0.2	1
H74A(Imd)	0.5 abc	0.3	2
H74Y	1.5 abc	0.9	6
H74Y(Imd)	2.0 bc	0.9	8
H194A	0.7 ab	0.2	3
H194A(Imd)	25.5 hij	0.8	101
H194Y	5.7 d	0.8	23
H194Y(Imd)	23.3 fg	1.0	92
H208A	- 0.2 ab	0.5	-1
H208A(Imd)	1.7 abc	0.7	7
H208Y	0.2 abc	0.2	1
H208Y(Imd)	0.0 ab	0.4	0
H194,208A	- 0.2 a	0.2	-1
H194,208A(Imd)	0.7 abc	0.8	3
Non-ligands			
H97Y	27.3 j	0.6	108
H97Y(Imd)	23.1 fg	0.6	91
H98A	22.6 f	0.8	89
H98A(Imd)	23.6 fgh	0.6	93
H136A	6.4 de	0.3	25
H136A(Imd)	8.2 e	1.5	32
H191A	24.6 fghi	1.1	97
H191A(Imd)	23.8 fgh	1.7	94
$LSD_{.05}$	2.2.		

*Imidazole (10 mM) was added to the growth medium. Cultures (1.5 ml) at OD_{600} 0.8-1.1 were resuspended in 50 mM phosphate buffer (pH 7.0) + 3 mM sucrose. Four individual replications of each strain were assayed.

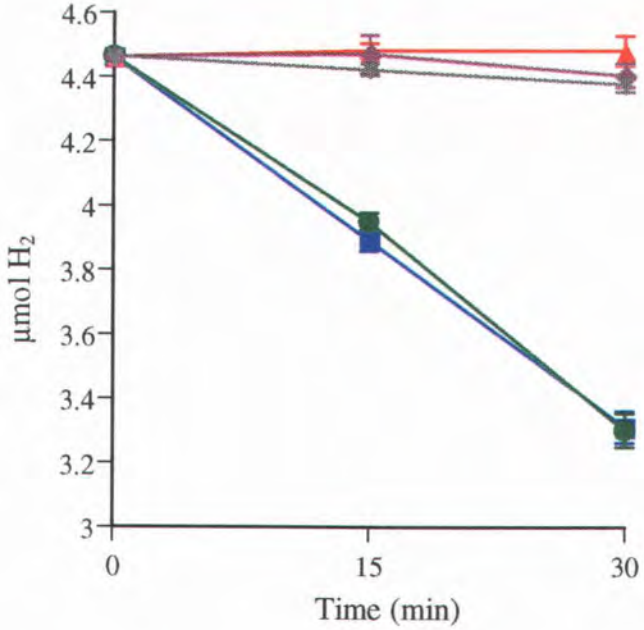

Fig. 5. O_2-dependent H_2 oxidation of wild-type and deletion mutant controls. Wt ■,WtR ●, HoxKG⁻R▲, HoxZ⁻R◆, and buffer-only control▼.

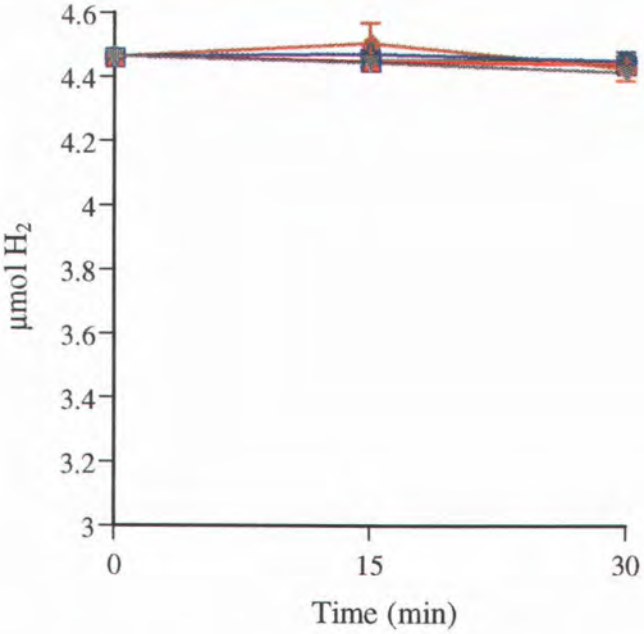

Fig. 6. O_2-dependent H_2 oxidation of HoxZ His → Ala ligand mutants. H33A■, H74A● , H194A▲, H208A◆, H194, 208A✧, and buffer-only control▼.

Table 6. Comparison of controls in O_2-dependent H_2 oxidation.

Strain and treatment*	Mean nmol H_2 oxidized/min
Wt	1.15 b
WtR	1.11 b
KG⁻R	0.02 a
Z⁻R	0.05 a
Control (no cells)	0.08 a
$LSD_{.05}$	0.14

*Cultures (1.5 ml) at OD_{600} 0.8-1.1 were resuspended in 50 mM phosphate buffer (pH 7.0) + 3 mM sucrose. Four individual replications of each strain were assayed.

Table 7. O_2-dependent H_2 oxidation of heme ligand alanine mutants.

Strain and treatment*	Mean nmol H_2 oxidized/min
H33A	0.02 a
H74A	0.01 a
H194A	0.02 a
H208A	0.03 a
H194,208A	0.01 a
Control (no cells)	0.05 a
$LSD_{.05}$	0.06

*Cultures (1.5 ml) at OD_{600} 0.8-1.1 were resuspended in 50 mM phosphate buffer (pH 7.0) + 3 mM sucrose. Four individual replications of each strain were assayed.

Table 8. O_2-dependent H_2 oxidation of non-ligand mutants.

Strain and treatment*	Mean nmol H_2 oxidized/min
H97Y	1.18 c
H98A	1.14 c
H136A	0.28 b
H191A	1.10 c
Control (no cells)	0.07 a
$LSD_{.05}$	0.16

*Cultures (1.5 ml) at OD_{600} 0.8-1.1 were resuspended in 50 mM phosphate buffer (pH 7.0) + 3 mM sucrose. Four individual replications of each strain were assayed.

No H_2 oxidation was detected for the mutants with substitutions in putative ligand positions 33, 74, 194 and 208 (Fig. 6, Table 7). None of these His → Ala mutants are functional in physiological electron transport from hydrogenase to O_2. These positions are the four conserved histidines in aligned sequences of hydrogenase *b*-type cytochromes, and are therefore likely to be the ligands to the two hemes in HoxZ.

There are 11 histidines in HoxZ. Two histidines in the center portion of the BC loop were not targeted for substitution. Mutants in nine positions could be obtained in *E. coli*, but not all rif^r transformants into *A. vinelandii* gave HoxZ mutants when screened. Histidines that were not fully conserved were not expected to be ligands to the hemes. Four of these histidines in loop regions near the membrane edges were successfully replaced in *A. vinelandii* with alanine or tyrosine. H_2 oxidation assays of putative non-ligand mutants (Fig. 7, Table 8) indicated that all four had H_2 oxidation activity and that three of the four mutants (with replacements in positions 97, 98, and 191) had activity similar to wild-type. Residues 97 and 98 are on the cytoplasmic side of the plasma membrane, and 191 is on the periplasmic side. Residue 136 is on the cytoplasmic side of the membrane. Mutant H136A had approximately one-third of the activity of wild-type. The decreased ability of H136A to oxidize H_2 implies that the histidine in position 136 serves an important function.

Assays with the His → Tyr replacements in the ligand positions (Fig. 8, Table 9) shows that, except for H194Y, tyrosine in these positions gives the same result as the His → Ala mutants. Mutant H194Y appears to have some H_2 oxidation activity (consumption in 30 minutes of ~5% of the initial H_2 added and 23% of Wt activity) and is significantly different from both wild-type and the buffer-only control. The typical range for the pK_a of the phenolic side chain of tyrosine in proteins is 9.5-10.5 (Mathews and van Holde, 1996), and the assay buffer pH is 7.0. *A. vinelandii* can oxidize H_2 over a wide range of pH (7-10), and H194Y was assayed to determine if the H_2 oxidation rate would be greater at a higher pH. Preliminary assays indicated that pH 9.0 caused the greatest H_2 oxidation of the pH values above 7.0. However

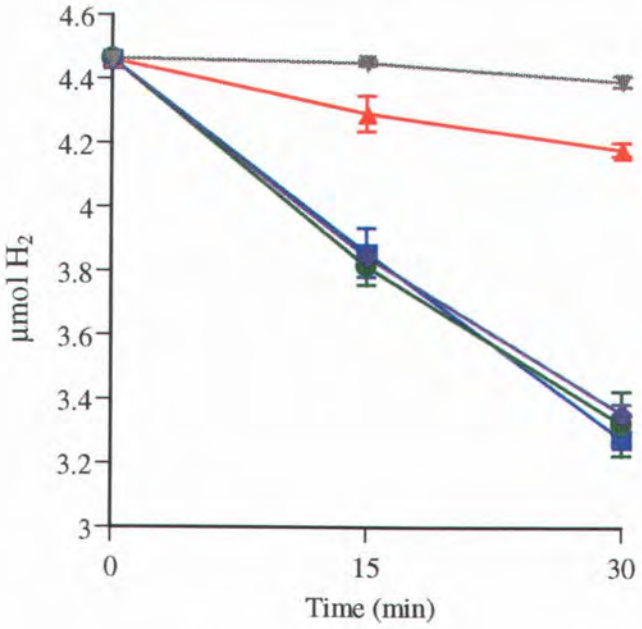

Fig. 7. O_2-dependent H_2 oxidation of HoxZ His → Ala non-ligand mutants. H97Y■, H98A●, H136A▲, 191A◆, and buffer-only control▼.

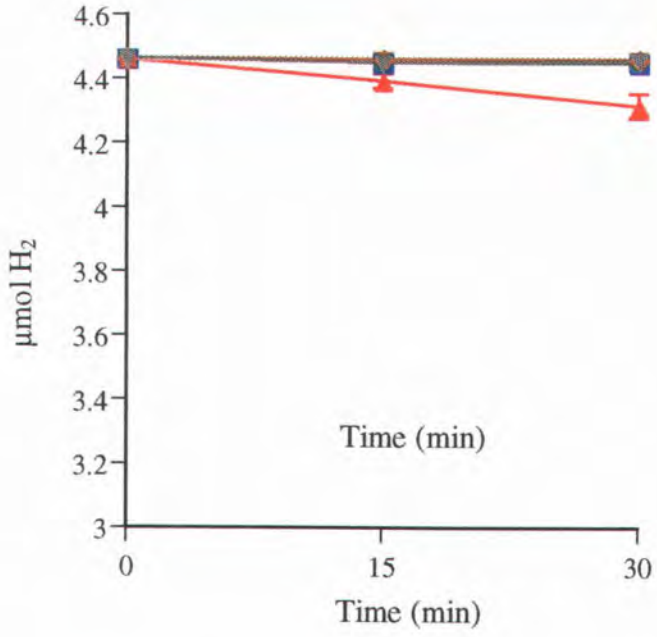

Fig. 8. O_2-dependent H_2 oxidation of HoxZ His → Tyr ligand mutants. H33Y■, H74Y●, H194Y▲, 208Y◆, and buffer-only control▼.

Table 9. O_2-dependent H_2 oxidation of heme ligand tyrosine mutants.

Strain and treatment*	Mean nmol H_2 oxidized/min
H33Y	0.01 a
H74Y	0.01 a
H194Y	0.26 b
H208Y	0.00 a
Control (no cells)	0.02 a
$LSD_{.05}$	0.06

*Cultures (1.5 ml) at OD_{600} 0.8-1.1 were resuspended in 50 mM phosphate buffer (pH 7.0) + 3 mM sucrose. Four individual replications of each strain were assayed.

Table 10. Effect of pH on O_2-dependent H_2 oxidation of H194Y.

Strain and treatment*	Mean nmol H_2 oxidized/min
H194Y at pH 7.0	0.29 b
H194Y at pH 9.0	0.30 b
Control (no cells)	0.05 a
$LSD_{.05}$	0.12

*Cultures (1.5 ml) at OD_{600} 0.8-1.1 were resuspended in 50 mM phosphate buffer (pH 7.0) + 3 mM sucrose. Four individual replications of each strain were assayed.

Table 11. Effect of Imd on Wt and WtR in O_2-dependent H_2 oxidation.

Strain and treatment*	Mean nmol H_2 oxidized/min
Wt	1.17 b
WtR	1.21 b
Wt(Imd)	1.19 b
WtR(Imd)	1.13 b
Control (no cells)	0.08 a
$LSD_{.05}$	0.12

*Cultures (1.5 ml) at OD_{600} 0.8-1.1 were resuspended in 50 mM phosphate buffer (*p*H 7.0) + 3 mM sucrose. Imidazole (10 mM) was added to the growth media. Four individual replications of each strain were assayed.

in comparing four replications of each pH, there was no significant difference between H194Y assayed at pH 7.0 and pH 9.0, but both were significantly different from the buffer-only control (Table 10).

Imidazole with the Whole Cell O_2-dependent H_2 Oxidation: Physiological Electron Transfer Assay

Imidazole has the ability to act as an exogenous ligand to heme in proteins where a natural ligand has been replaced. To determine if this small molecule could act as a free ligand in physiologically relevant H_2 oxidation, a saturating concentration of Imd was added to the growth medium. To ensure that the addition of Imd did not have unintended effects, the control Wt and deletion mutant strains were grown with a saturating concentration of Imd and assayed. There was no significant difference in H_2 oxidation between Wt, WtR, Wt(Imd), and WtR(Imd) (Fig. 9, Table 11). KG^- R(Imd) and Z^-R(Imd) were not significantly different from the buffer-only control (Fig. 10, Table 12). Imidazole concentration assays and time of addition experiments were performed to ascertain their influence on Wt hydrogenase activity. H_2 oxidation of Wt grown with Imd concentrations from 1 mM to 10 mM were not significantly different from Wt (Fig. 11, Table 13). Wt with 10 mM Imd added at the beginning of the assay along with the H_2, was not significantly different from Wt or Wt grown with Imd. The addition of Imd and chloramphenicol, to inhibit protein synthesis, did not affect the ability of Wt to oxidize H_2 (Figure 12, Table 14). Overall, Imd did not affect the ability of Wt or the deletion mutants to oxidize H_2.

Addition of 10 mM Imd to the growth medium of the His → Ala ligand mutants did not increase H_2 oxidation activity, except in H194A, which recovered activity to a level comparable to Wt (Fig13), indicating that Imd apparently acts as a free ligand in this position. H_2 oxidation activities of H33A(Imd), H74A(Imd), H208A(Imd), and H194, 208A(Imd) were not significantly different from the buffer-only control (Table 15). Over the range of 1-10 mM, H_2 oxidation of H194A(Imd) and Wt(Imd) were not

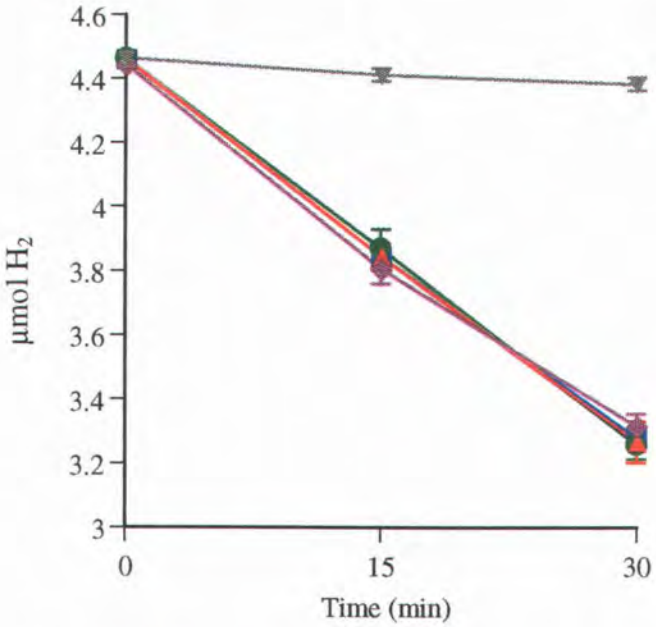

Fig. 9. The effect of Imd addition to the growth medium on O_2-dependent H_2 oxidation of Wt. Wt■, WtR●, Wt(Imd)▲, WtR(Imd)◆, and buffer-only control▼.

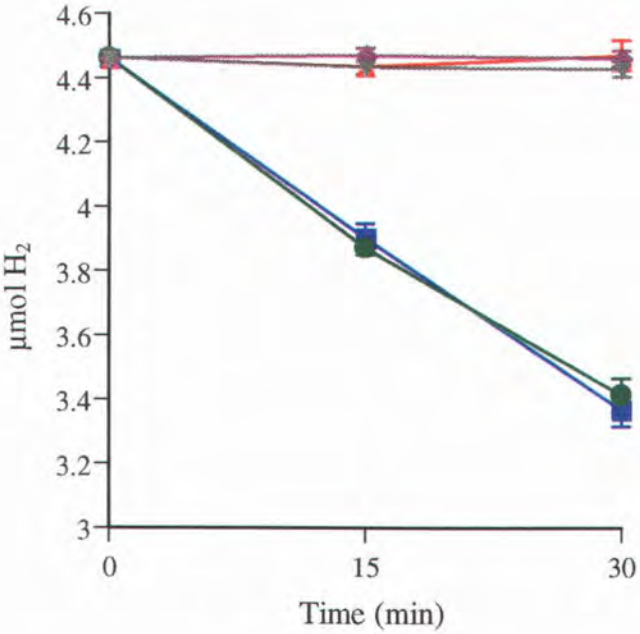

Fig. 10. The effect of Imd addition to the growth medium on O_2-dependent H_2 oxidation of controls. Wt(Imd)■, WtR(Imd)●, HoxKG$^-$R(Imd)▲, HoxZ$^-$R(Imd)◆, and buffer-only control▼.

Table 12. Effect of Imd addition to the growth medium on controls in O_2-dependent H_2 oxidation.

Strain and treatment*	Mean nmol H_2 oxidized/min
Wt(Imd)	1.09 b
WtR(Imd)	1.05 b
KG$^-$R(Imd)	-0.01 a
Z$^-$R(Imd)	0.00 a
Control (no cells)	0.03 a
$LSD_{.05}$	0.12

*Cultures (1.5 ml) at OD_{600} 0.8-1.1 were resuspended in 50 mM phosphate buffer (pH 7.0) + 3 mM sucrose. Imidazole (10 mM) was added to the growth media. Four individual replications of each strain were assayed.

Table 13. Effect of Imd concentration added to the growth medium on Wt in O_2-dependent H_2 oxidation.

Strain and treatment*	Mean nmol H_2 oxidized/min
Wt	1.03 b
Wt grown in 1 mM Imd	1.03 b
Wt grown in 5 mM Imd	0.93 b
Wt grown in 10 mM Imd	0.97 b
Control (no cells)	0.02 a
$LSD_{.05}$	0.14

*Cultures (1.5 ml) at OD_{600} 0.8-1.1 were resuspended in 50 mM phosphate buffer (pH 7.0) + 3 mM sucrose. Four individual replications of each strain were assayed.

Table 14. Effect of Imd added to the reaction mixture on Wt in O_2-dependent H_2 oxidation.

Strain and treatment*	Mean nmol H_2 oxidized/min
Wt	1.15 b
Wt (10 mM Imd added at time 0)	1.06 b
Wt (10 mM Imd and 3 mM Chloramphenicol added at time 0)	1.04 b
Wt (grown in 10 mM Imd)	1.07 b
Control (no cells)	0.05 a
$LSD_{.05}$	0.11

*Cultures (1.5 ml) at OD_{600} 0.8-1.1 were resuspended in 50 mM phosphate buffer (pH 7.0) + 3 mM sucrose. Four individual replications of each strain were assayed.

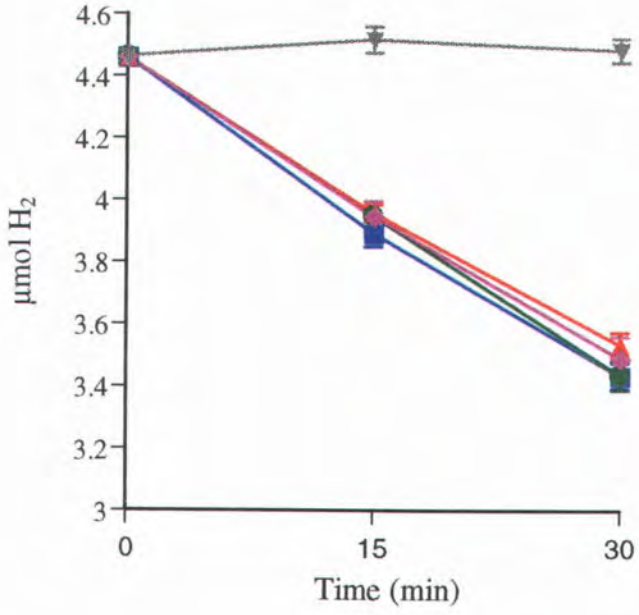

Fig. 11. The effect of Imd concentration added to the growth medium on O_2-dependent H_2 oxidation of wild-type. Wt■, Wt + 1 mM Imd●, Wt+ 5 mM Imd▲ ,and Wt + 10 mM Imd◆, and buffer-only control▼.

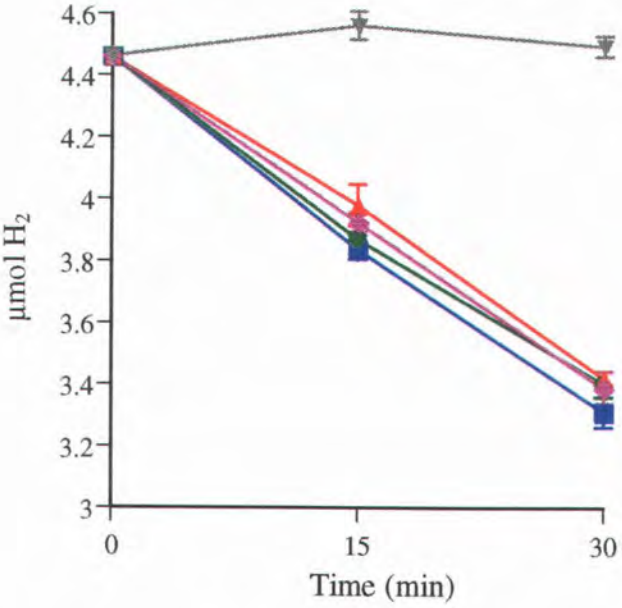

Fig. 12. The effect of Imd addition to the reaction mixture (added at time 0) on O_2-dependent H_2 oxidation of wild-type. Wt■, Wt+Imd at time 0●, Wt+ Imd and Chloramphenicol added at time 0▲, Wt grown in ImdR(Imd) ◆, and buffer-only control▼.

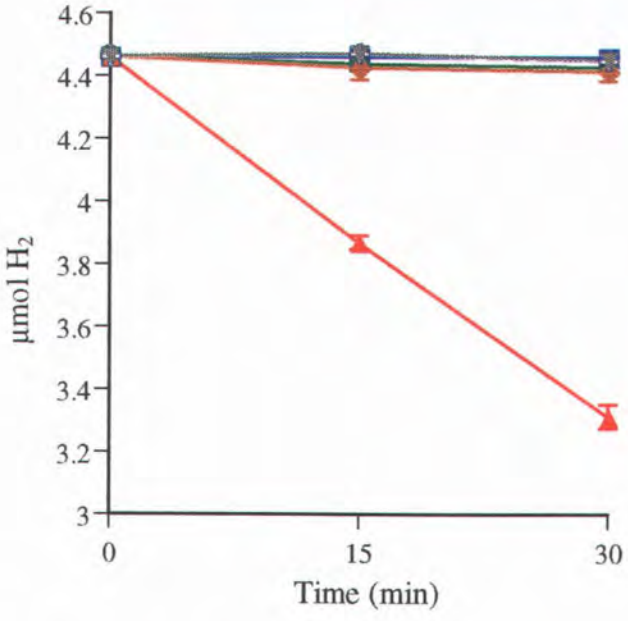

Fig. 13. The effect of Imd addition to the growth medium on O_2-dependent H_2 oxidation of HoxZ ligand mutants. H33A(Imd) ■ , H74A(Imd) ● , H194A(Imd) ▲ , H208A(Imd) ◆ , H194,208A (Imd) ✧, and buffer-only control ▼.

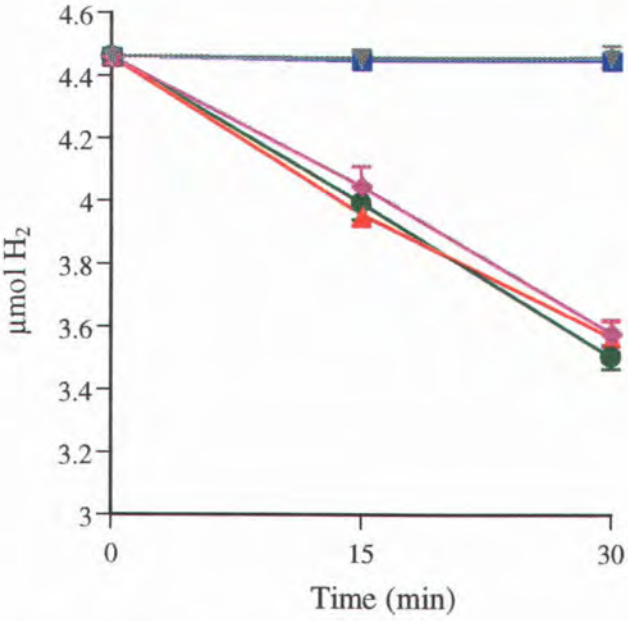

Fig. 14. The effect of Imd concentrations added to the growth medium on O_2-dependent H_2 oxidation of HoxZ H194A. H194A ■, H194A + 1 mM Imd ●, H194A+ 5 mM Imd ▲ , and H1194A+ 10 mM Imd ◆, and buffer-only control ▼ .

Significantly different from Wt (Fig. 14, Table 16). When Imd was added at the start of the assay, there was an increase in H_2 oxidation activity over time, but not to the same rate as H194A grown in Imd (Fig 15, Table 17). If chloramphenicol is added simultaneously, H_2 oxidation also increased over time and at a rate not significantly different from that without chl. These results indicate that new protein synthesis is not required for Imd to become incorporated and serve as a ligand in *trans*.

The His → Tyr ligand mutants with Imd followed a similar pattern to the His → Ala ligand mutants in that only H194Y had activity when grown in Imd (Table 5). A comparison of both mutants grown in Imd shows that there is no significant difference among Wt, H194A(Imd) an H194Y(Imd) (Figure 16, Table 18). The presence of the larger phenol side chain in the heme pocket does not appear to hinder the ligation of the free ligand. Imidazole does not appear to wash out if cultures grown in Imd are shaken in Imd-free buffer for an hour before assaying (Table 19).

The non-ligand HoxZ mutants were also grown with Imd and assayed for O_2-dependent H_2 oxidation (Table 5). The addition of Imd to the growth medium of the non-ligand mutants did not result in increased activity. Only H136A had less than Wt activity in the O_2-dependent H_2 oxidation assay, but H136A did not recover H_2 oxidizing capability upon the addition of Imd.

Another small molecule commonly used for exogenous ligand assays to replace histidine is pyridine. 3-Fluoropyridine did not appear to act as a free ligand in HoxZ H194A (Table 20), and concentrations higher than 10 mM appeared toxic to cells. No additional assays with this molecule or other potential exogenous ligands were done.

Whole Cell H_2-dependent MB Reduction: Heterodimer Functionality Assay

The artificial electron acceptor methylene blue (MB) can bind directly to the heterodimer (Przybyla et al., 1992) and may have other non-specific binding locations

Table 15. Effect of Imd addition to the growth medium on heme ligand mutants in O_2-dependent H_2 oxidation.

Strain and treatment*	**Mean nmol H_2 oxidized/min**
H33A(Imd)	-0.00 a
H74A(Imd)	0.03 a
H194A(Imd)	1.15 b
H208A(Imd)	0.04 a
H194,208A(Imd)	0.01 a
Control (no cells)	0.01 a
$LSD_{.05}$	0.08

*Cultures (1.5 ml) at OD_{600} 0.8-1.1 were resuspended in 50 mM phosphate buffer (pH 7.0) + 3 mM sucrose. Imidazole (10 mM) was added to the growth medium. Four individual replications of each strain were assayed.

Table 16. Effect of Imd concentration added to the growth medium on HoxZ H194A in O_2-dependent H_2 oxidation.

Strain and treatment*	**Mean nmol H_2 oxidized/min**
H194A	0.01 a
H194A grown in 1 mM Imd	0.96 b
H194A grown in 5 mM Imd	0.89 b
H194A grown in 10 mM Imd	0.88 b
Control (no cells)	0.02 a
$LSD_{.05}$	0.12

*Cultures (1.5 ml) at OD_{600} 0.8-1.1 were resuspended in 50 mM phosphate buffer (pH 7.0) + 3 mM sucrose. Four individual replications of each strain were assayed.

Table 17. Effect of Imd addition to the reaction mixture on HoxZ H194A in O_2-dependent H_2 oxidation.

Strain and treatment*	**(nmol H_2 oxidized/min)**
H194A	-0.01 a
H194A (10 mM Imd added at time 0)	0.38 b
H194A (10 mM Imd and 3 mM chloramphenicol added at time 0)	0.29 b
H194A (grown in Imd)	0.88 c
Control (no cells)	0.04 a
$LSD_{.05}$	0.12

*Cultures (1.5 ml) at OD_{600} 0.8-1.1 were resuspended in 50 mM phosphate buffer (pH 7.0) + 3 mM sucrose. Four individual replications of each strain were assayed.

Table 18. Comparison of Wt with H194A(Imd) and H194Y(Imd) in O_2-dependent H_2 oxidation

Strain and treatment*	**Mean nmol H_2 oxidized/min**
Wt	1.07 b
H194A(Imd)	1.05 b
H194Y(Imd)	1.05 b
Control (no cells)	0.03 a
$LSD_{.05}$	0.13

*Cultures (1.5 ml) at OD_{600} 0.8-1.1 were resuspended in 50 mM phosphate buffer (pH 7.0) + 3 mM sucrose. Imidazole (10 mM) was added to the growth media. Four individual replications of each strain were assayed.

Table 19. Effect of washing on Imd-grown H194 mutants in O_2-dependent H_2 oxidation.

Strain and treatment	**Mean nmol H_2 oxidized/min**
H194A(Imd) washed	0.88 b
H194A(Imd)	0.88 b
H194Y(Imd) washed	0.76 b
H194Y(Imd)	0.85 b
Control (no cells)	0.02 a
$LSD_{.05}$	0.32

*Cultures (1.5 ml) at OD_{600} 0.8-1.1 were resuspended in 50 mM phosphate buffer (pH 7.0) + 3 mM sucrose. Imidazole (10 mM) was added to the growth medium. Washed was shaken in buffer one hour before assaying. Four individual replications of each strain were assayed.

Table 20. Effect of 3-fluoropyridine on HoxZ H194A and Wt in O_2-dependent H_2 oxidation.

Strain and treatment*	**(nmol H_2 oxidized/min)**
H194A	-0.03 a
H194A (grown in 10 mM 3FP)	-0.07 a
Wt	1.07 b
Wt (grown in 10 mM 3FP)	1.07 b
Control (no cells)	-0.03 a
$LSD_{.05}$	0.13

*Cultures (1.5 ml) at OD_{600} 0.8-1.1 were resuspended in 50 mM phosphate buffer (pH 7.0) + 3 mM sucrose. Four individual replications of each strain were assayed.

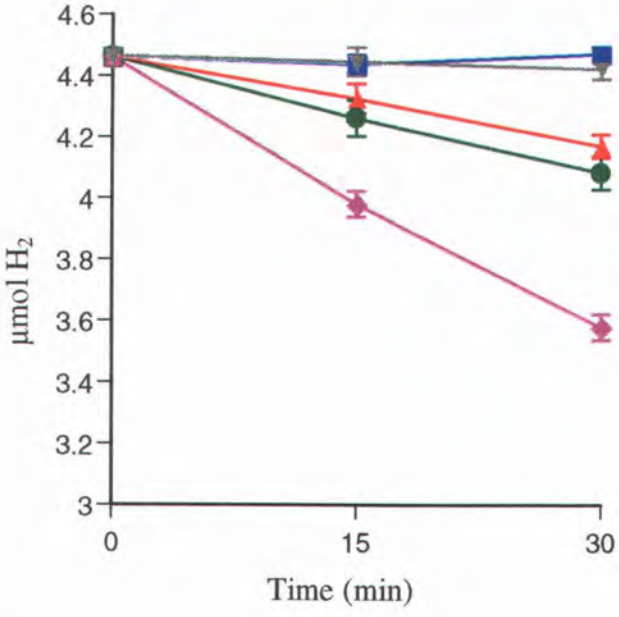

Fig. 15. The effect of Imd added to the reaction mixture (time 0) on O_2-dependent, H_2 oxidation of H194A. H194A ■, H194A + Imd at time 0 ●, H194A + Imd and Chloramphenicol added at time 0 ▲, H194A grown in ImdR(Imd) ◆, and buffer-only control ▼.

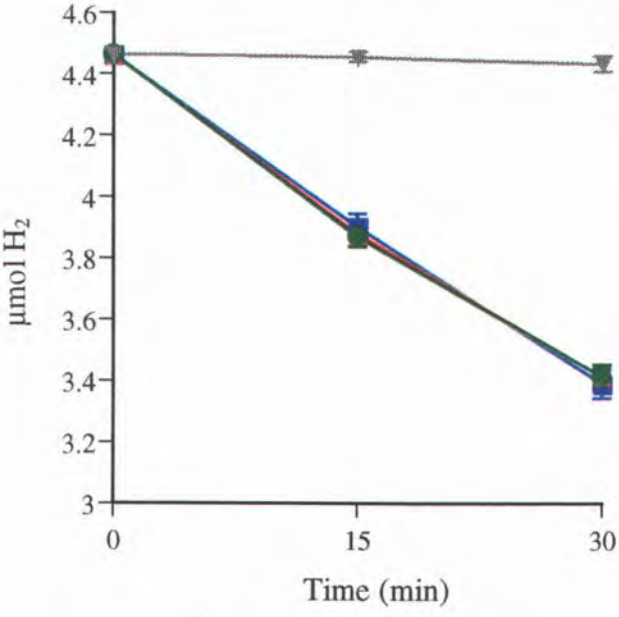

Fig. 16. Comparison of O_2-dependent H_2 oxidation of wild-type with HoxZ H194A + Imd and H194Y + Imd. Wt ■, H194A(Imd) ▲, H194Y(Imd) ●, and buffer-only control ▼.

along the electron transport chain and elsewhere in the cell. MB reduction is a useful tool to determine the activity of the hydrogenase heterodimer, regardless of the functionality of the respiratory chain. Since MB can also be nonspecifically reduced, experiments were conducted in the absence and presence of H_2 (Table 21), to account for the level of endogenous cellular reduction. In addition, assays were conducted on strains grown in the absence and presence of Imd. The control strains of Wt and the partial deletion mutants show that without H_2 all three strains have similar levels of endogenous reduction of MB (Fig. 17). The endogenous level of MB reduction was approximately 32% of total Wt MB reduction when H_2 was added. Endogenous levels for all MB assays were similar. However, with H_2, the controls showed three distinct levels of activity relative to the endogenous level of MB reduction: none, partial and full. H_2 did not stimulate reduction of MB by the KG deletion mutant, which is consistent with no hydrogenase heterodimer activity. HoxZ$^-$ can reduce MB, indicating a functional hydrogenase heterodimer, but MB reduction was 70% of the Wt level. The lack of a complete HoxZ affected the ability of the heterodimer to reduce MB. The MB reduction of Wt was considered to be full activity. Control strains grown with Imd were not significantly different from the control strains grown without Imd.

In assays with H_2, ligand mutants varied with position (Fig. 18, Fig. 19), but were similar between alanine and tyrosine variants at the same position. H33A/Y had full activity, regardless of the presence of Imd. Mutants of position 74 had only partial activity, whether Imd was present or not. Positions H194A/Y and H208A/Y individually had partial activity without Imd and full activity with Imd. The double ligand mutant H194, 208A with Imd showed substantial increase in activity but did not regain full activity.

With or without Imd, three of the four non-ligand mutants had at least full Wt activity. With H_2, but with or without Imd, H136A had significantly greater MB reduction (>20%) than Wt and was the only strain with significantly greater than Wt activity. H97Y was unusual in that it had less than Wt activity when assayed without Imd, but had activity comparable to Wt when grown with Imd (Fig. 20).

Table 21. Comparison of endogenous and H_2-dependent MB reduction activity of strains.

Strain and treatment	Endogenous MB reduction: Mean nmol MB reduced/min, Std Err and % Wt			H_2-dependent MB reduction: Mean nmol MB reduced/min, Std Err and % Wt		
Controls						
Wt	15.5 ghijklmnopqr	4.1	100	48.0 hijkl	1.4	100
Wt(Imd)	10.6 abcdef	2.2	69	44.7 ghij	0.8	93
WtR	13.3 cdefghijklmno	1.3	86	44.1 ghi	3.2	92
WtR(Imd)	13.0 cdefghij	0.6	84	44.9 ghij	3.1	94
KG⁻R	9.6 abc	0.8	62	13.1 a	2.5	27
KG⁻R(Imd)	13.1 cdefghijkl	1.9	85	11.8 a	1.5	25
Z⁻R	17.5 jklmnopqrs	1.6	113	33.6 ef	2.6	70
Z⁻R(Imd)	12.4 bcdefghi	2.6	80	33.6 ef	2.6	70
Ligands						
H33A	10.1 abcde	1.3	65	42.6 gh	2.2	89
H33A(Imd)	15.9 ghijklmnopqr	0.5	103	46.8 ghijkl	1.0	98
H33Y	20.8 s	0.8	134	49.2 ijkl	2.8	102
H33Y(Imd)	13.3 cdefghijklmn	1.2	86	44.6 ghij	1.1	93
H74A	10.0 abcd	1.3	65	23.6 bc	1.2	49
H74A(Imd)	13.6 cdefghijklmnop	0.8	88	27.9 cd	1.6	58
H74Y	11.7 bcdefg	2.9	75	29.2 cdef	1.3	61
H74Y(Imd)	15.6 ghijklmnopqr	1.8	101	30.5 def	2.8	63
H194A	10.6 abcdef	0.8	68	21.5 b	1.2	45
H194A(Imd)	13.0 cdefghijk	1.8	84	48.9 ijkl	2.0	102
H194Y	14.9 efghijklmnopqr	1.7	96	31.0 def	2.1	65
H194Y(Imd)	18.9 rs	0.9	122	50.8 kl	2.0	106
H208A	11.9 bcdefgh	1.7	77	28.2 cde	2.7	59
H208A(Imd)	14.6 defghijklmnopqr	1.9	94	45.7 ghijk	1.7	95
H208Y	15.1 fghijklmnopqr	1.3	97	34.4 f	1.4	72
H208Y(Imd)	11.8 bcdefgh	0.7	76	41.3 g	1.4	86
H194,208A	7.9 ab	1.0	51	15.7 a	0.2	33
H194,208A(Imd)	11.3 bcdefg	0.9	73	32.5 def	1.6	68
Non-ligands						
H97Y	7.4 a	0.6	48	32.5 edf	2.6	68
H97Y(Imd)	12.4 bcdefghi	0.8	80	42.6 gh	1.8	89
H98A	14.6 defghijklmnopqr	2.6	94	46.6 ghijkl	1.7	97
H98A(Imd)	16.6 hijklmnopqrs	1.2	107	49.7 jkl	0.9	104
H136A	17.1 ijklmnopqrs	2.1	110	57.7 m	2.1	120
H136A(Imd)	17.9 lmnopqrs	1.8	116	59.6 m	2.3	124
H191A	13.2 cdefghijklm	1.7	85	48.7 ijkl	2.0	101
H191A(Imd)	14.0 cdefghijklmnopq	2.7	90	51.4 l	1.0	107
$LSD_{.05}$	4.8			5.6.		

*Cultures (40 ml) at OD_{600} 0.8-1.1 were resuspended in 1 ml Tris buffer + 5 mM EDTA (pH 7.3) and purged with N_2. For Imd, 10 mM Imd was added to the growth media. For H_2-dependent, 1 ml H_2 was added to assay. Four individual replications of each strain were assayed.

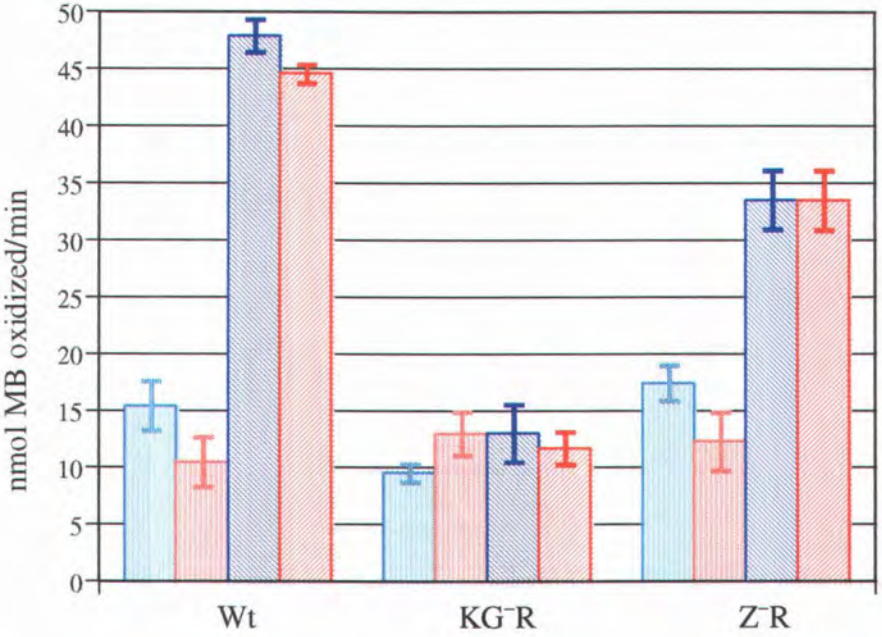

Fig. 17. H_2-dependent, MB reduction of controls. $-H_2$ -Imd, $-H_2$ +Imd, $+H_2$ -Imd, $+H_2$+Imd.

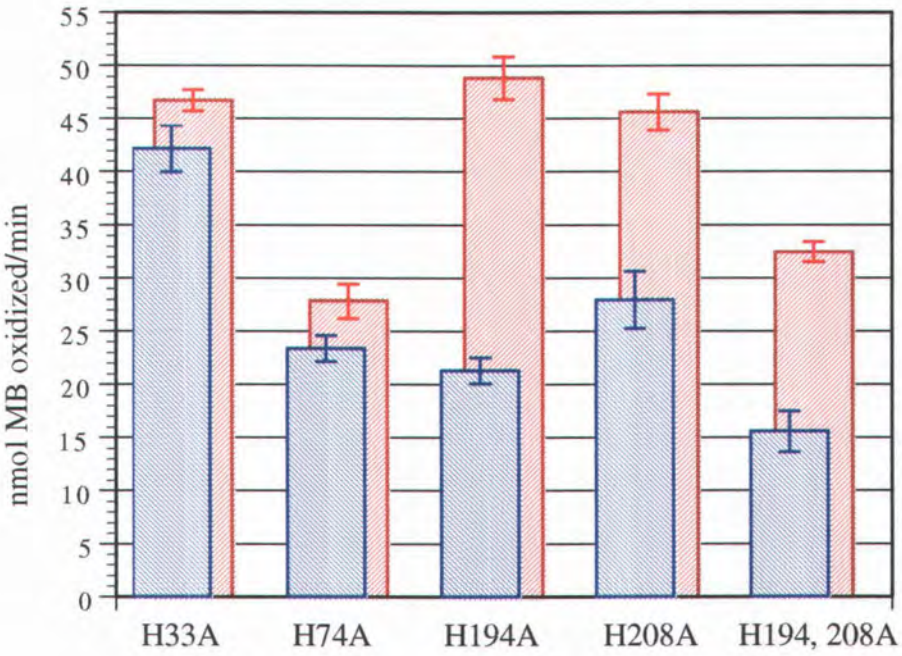

Fig. 18. H_2-dependent MB reduction of ligand mutants. $+H_2$ -Imd, $+H_2$+Imd.

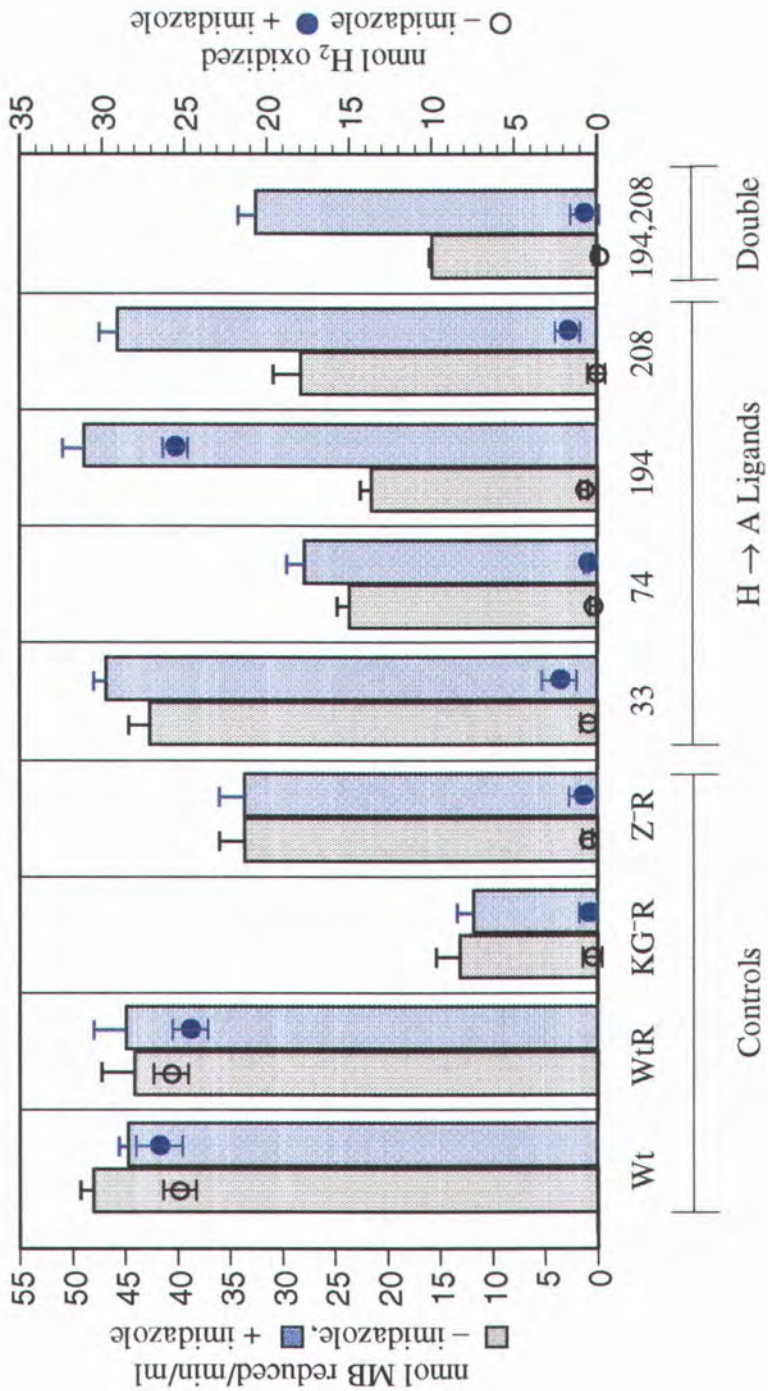

Fig. 19. Effect of Imd on H_2-dependent MB reduction and O_2-dependent H_2 oxidation. MB reduction: □ – imidazole, ■ + imidazole; H_2 oxidation: ○ – imidazole, ● + imidazole. Bars denote standard error; only top half of column error is shown.

The MB reduction results indicate that the heterodimers in the HoxZ partial deletion mutant and point mutants were catalytically active, and that the lack of activity in the O_2-linked H_2 oxidation assay was localized to HoxZ. Since the rates of reduction in HoxZ$^-$ and three of the ligand mutants were not comparable to Wt, this indicates that the heme ligands in HoxZ are required to enable full catalytic capability of the hydrogenase heterodimer. Unlike their roles in electron transport, in which each ligand was required for hydrogenase function, the four ligands do not play equivalent roles in their effect on heterodimer activity as shown by MB reduction (Fig.19). Depending on the position, Imd differs in its ability to substitute for ligands, and fulfill their functional or structural role (Fig.19).

Molecular Modeling of HoxZ

The model of *A. vinelandii* HoxZ (Fig. 20) shows a possible positioning of the four helices, histidine ligands and two hemes. Since the ligands are on three helices, the relative positions of the helices are not fixed and the angle between them may vary from this snapshot. The histidines can bind the hemes without undue strain when the helices are positioned perpendicular to the membrane. The imidazole side chains of the histidines bound to the same ligand can be placed approximately perpendicular to each other, also without strain.

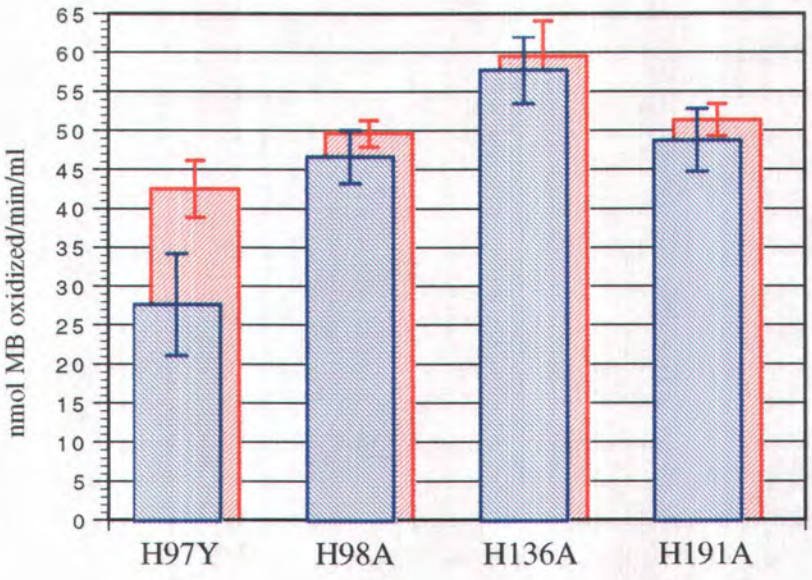

Fig. 20. H_2-dependent, MB reduction of non-ligand mutants. $+H_2$ -Imd ▨ , $+H_2$+Imd ▨.

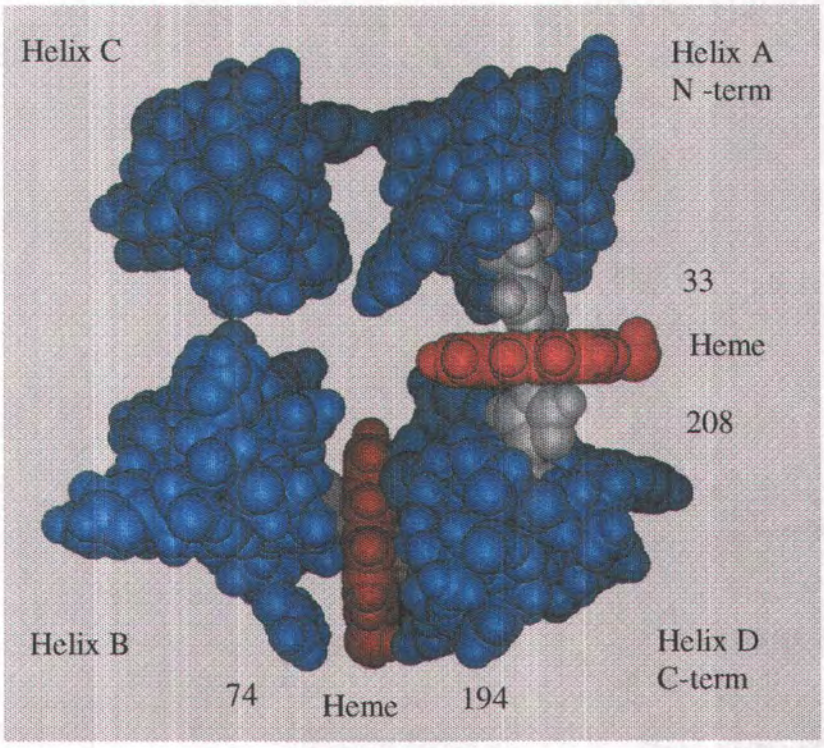

Fig. 21. Molecular modeling of HoxZ heme ligation, view from the cytoplasmic side down the transmembrane α-helices.

Discussion

The goals of this research were to determine the ligands to the heme(s) in HoxZ, characterize the nature of the HoxZ heme ligands, and define the importance of the HoxZ heme ligands to hydrogenase in *A. vinelandii*. Though it may not be unusual for a residue or a protein to have multiple roles, it is often difficult to separate their functional and structural contributions to the protein or enzyme. Site-directed replacement of critical residues, such as heme ligands, combined with chemical rescue provides an opportunity to explore the nature of the ligands and the protein, and separate, at least, the functional/structural contributions from the purely structural ones.

This work identified the four histidines in HoxZ that are the ligands to two hemes in the cytochrome *b* of *A. vinelandii* hydrogenase. By examining the chemical tolerance of histidine sites for residue substitution and small molecule complementation, reactions in hydrogenase assays indicate that each of the four heme ligand sites in *A. vinelandii* HoxZ is unique, and one non-ligand histidine is important to hydrogenase in physiological electron transport. The functionality of one ligand site can be partially restored by residue replacement and fully by addition of an exogenous ligand. Chemical rescue can restore structural integrity in two ligand sites, and the structural integrity of HoxZ affects hydrogenase heterodimer activity. Overall, the HoxZ heme ligands are required functionally and structurally for full hydrogenase activity.

Sequence Alignment and Phylogeny of Hydrogenase *b*-type Cytochromes

The sequence alignment of hydrogenase *b*-type cytochromes shows that several residues are completely conserved, most importantly the histidines serving as axial ligands to the hemes. This alignment of hydrogenase *b*-type cytochromes contains a

diverse group of bacteria: aerobes, anaerobes, nitrogen fixers, photosynthesizers, autotrophs and heterotrophs. The similarities seen in biochemical assays of distantly related hydrogenase *b*-type cytochromes imply a similar structural and functional importance to hydrogenase in all these proteins. Not unexpectedly, *A. vinelandii* HoxZ is most closely related to the hydrogenase *b*-type cytochrome of *A. chroococcum,* similar to the relationships seen in the phylogenies of the large and small subunits of hydrogenase (Wu and Mandrand, 1992).

Identification of Ligands by Physiologically Relevant Hydrogenase Activity

In metalloproteins, ligands to the metals are often determined by substitution of the residue by site-directed replacement with consequent loss of activity. The ligands in HoxZ were identified by replacing eight histidines in or near the membrane individually with alanine or tyrosine. Alanine cannot ligate the heme, and changes in electron transfer activity requiring this *b*-type cytochrome were expected to be useful in distinguishing the ligands and non-ligands. HoxZ in *A. vinelandii* has a pI of 8.58. The pI's of the single and double alanine replacements are 8.58, the single tyrosine replacement is 8.57, and HoxZ$^-$ is 8.86.

In *A. vinelandii* wild-type, the maximum hydrogenase activity is at a growth stage commensurate with an OD_{600} of 0.8 through 1.0 (Fig.4). The limits of the O_2-dependent H_2 oxidation assay were defined by assaying the controls Wt, WtR, HoxKG$^-$R and HoxZ$^-$R (Fig. 5). Wt and WtR oxidized 167 nmol H_2/(min x mg), and this indicates the maximum activity by *A. vinelandii* hydrogenase. Like HoxKG$^-$R, HoxZ$^-$R had no H_2 oxidation activity. No activity with the partially deleted HoxZ is in agreement with insertional or deletional inactivation of the HoxZ homologues in *R. capsulatus* (Cauvin et al., 1991), *A. vinelandii* (Menon et al., 1992), and *R. leguminosarum* (Hidalgo et al., 1992).

Like the partial deletion mutants, strains with replacements of the four fully conserved HoxZ histidines lost H_2 oxidation activity when assayed for physiological electron transport, whereas the other other H→A mutants all had hydrogenase activity. In *A vinelandii*, the four histidines 33, 74, 194, and 208 are essential for hydrogenase activity and are proposed as the ligands to the two hemes in *A. vinelandii* HoxZ.

In HydC, the hydrogenase cytochrome *b* of *W. succinogenes*, Gross et al. (1998) altered six histidines, including three of the four fully conserved histidines, to alanine and methionine. Replacements in the three fully conserved sites resulted in the loss of hydrogenase quinone reactivity, while the other three mutants with replacements had wild-type activity. With either alanine or methionine replacement of the conserved histidines, when H_2 was added to these mutants, their ability to reduce the quinone analogue was very low or absent. Their ability to transfer electrons from H_2 to fumarate (1% of wild-type) or polysulphide (3.6% of wild-type) was less than 0.05 U mg^{-1} cell protein. The quinone could not be reduced by the hydrogenase cytochrome *b*, but was reduced by formate, and fumarate could oxidize the quinol, indicating that fumarate reduction was functional, and that loss of activity was localized to the hydrogenase. The loss of hydrogenase activity was not due to lack of HydC apoprotein, nor the inability of the heterodimer to bind to the membrane, since the *W. succinogenes* HydC ligand mutants had amounts of HydC protein similar to wild-type, as determined by Western blot analysis, and the heterodimer was present in the membrane fractions (Gross et al., 1998). The three histidines they replaced with alanine are homologous to *A. vinelandii* 33, 74, and 194. They were unable to replace the histidine homologous to *A. vinelandii* 208, but suggested the same four histidines as ligands to the hemes for the hydrogenase cytochrome *b* (Gross et al., 1998). The results in *A. vinelandii* HoxZ support the conclusion that these four fully conserved histidines are ligands to two hemes, and provide evidence that loss of any of these four ligands results in the complete loss of hydrogenase activity.

Physiologically Relevant Hydrogenase Activity of Non-ligand Mutants

In *A. vinelandii* four partially conserved histidines in the loops near the edges of the membrane were substituted with alanine or tyrosine. Three of these, H97Y, H98A, and H191A, had full wild-type O_2-dependent H_2 oxidation activity. Although the non-ligand histidines that Gross et al. (1998) altered were in different positions, all those mutants, including substitution of the fifth partially conserved histidine homologous to HoxZ H195, showed wild-type activity.

One of the *A. vinelandii* HoxZ non-ligand mutants, H136A, had one-third the O_2-dependent H_2 oxidation activity of wild-type. This result implies that the histidine in this position plays a role in the structure or function of HoxZ. H136 is in the BC loop region on the cytoplasmic side of the plasma membrane near the hydrophobic core of the membrane. This location places the residue in close proximity to the high potential heme, opposite the side where the heterodimer binds. Besides ligation, other possible roles suggested for conserved histidines in *b*-type cytochromes are functions in quinol binding and modulating heme redox potential (Berks et al, 1996).

A variety of residues have been indicated in the inhibition of quinone reduction in the cytochrome *b* of the bc_1 complex. H136 in *A. vinelandii* HoxZ, H217 of the bc_1 complex (*R. capsulatus* numbering), and H13 of *B. subtilis* succinate:quinone reductase (SQR) are all histidines located on the cytoplasmic side of the plasma membrane. Substitutions to each of these non-ligand histidines affects activity. In the bc_1 complex, quinone is reduced by the cytochrome near the hydrophobic core on the cytoplasmic side of the membrane. Inhibition sites for Q_i (quinone reduction) are in the cytoplasmic loops near the membrane. A variety of residues have been indicated in the inhibition of quinone reduction. H217, a histidine located on helix D near the cytoplasmic membrane, has been shown to affect electron transport. The residue in position 217 affects the redox properties of the quinone in the Q_i site, and Gray et al. (1994) suggested that H217 forms part of the binding pocket of the Q_i site. H217R has a Q_i site semiquinone stability constant 10 times greater than wild-type, while

H217D/L have stability constants much lower than wild-type. No specific motif has been identified for the Q_i site, but it is likely, at least within a group of homologous proteins, that conserved residues might make up the quinone binding pocket.

In the *B. subtilis* SQR cytochrome *b*, individual substitution of five histidines affected activity (Hägerhäll et al., 1995). Four of the mutants were defective in anchoring the flavoprotein and iron-sulfur protein heterodimer and were assumed to be the ligands to two hemes. The fifth substituted protein had half the activity of wild-type and functioned as an anchor, and its substituted histidine was, therefore, assumed to play no role in heme ligation. The residue, H13, is on the cytoplasmic side of the membrane where the heterodimer binds. The fifth replacement, H13Y, affected the high potential heme, as shown by EPR, and Hägerhäll et al. (1995) concluded that this was due to a structural change in the local environment of the heme, b_H.

Tyrosine as a Substitute Heme Ligand for Histidine in HoxZ

The ligand histidines were replaced with tyrosine to explore the tolerance of the ligand sites for amino acid residue substitution. Though slightly larger, tyrosine can be considered isosteric with histidine, and has a different pK_a: 10.1 for tyrosine, 6.0 for histidine. A tyrosine residue is capable of binding heme. Tyrosine is the natural ligand in catalase (Newmyer et al., 1996) and the cytochrome d_1 of nitrite reductase cytochrome cd_1 (Fülöp et al., 1995) and in the natural methemoglobin mutants (Pin et al. 1994).

Three of the four H→Y *A. vinelandii* HoxZ ligand mutants (replacements in positions 33, 74 and 208) had the same loss of hydrogenase activity as the strains with H→A ligand replacements and confirm that the histidines in these positions are the ligands to the hemes. However, the H194Y mutant had ~20% of wild-type activity, demonstrating that tyrosine partially substitutes as a ligand for histidine in this position.

The neighboring histidine, H195, could be the reason that tyrosine is a functional substitution in position 194. Other possibilities for tyrosine functioning as a ligand in this position are steric constraint in the ligand site or the electronic contribution of aromatics. Regardless of the reason, the other ligand sites are not effective in providing the appropriate features to allow a functional substitution.

It is interesting that position 194 ligates the low potential heme and is on the periplasmic side, where HoxZ serves as a membrane anchor to the heterodimer. To function in electron transport, the heme must have an appropriate redox potential. The low activity shows that electron transport is not optimal, and the lowered methylene blue heterodimer activity with H194Y supports this conclusion. The larger size of the tyrosine residue may prevent optimal conformation and helix bundling. Perhaps the reason for the lowered activity is that H194Y may cause a structural problem in HoxZ leading to a sub-optimal anchoring of the heterodimer or electron flow from the heterodimer.

Heme binding to tyrosine appears to be pH dependent and aided by the local heme pocket environment (Pin et al., 1994, and Nagai et al. 1989). In the sperm whale (SW) myoglobin E7 mutant H64Y, Pin et al. (1994) found that tyrosine is bound to the heme iron by the phenolate oxygen of tyrosine without displacing the hydrogen atom of the phenolate. H64Y(E7) SW myoglobin has a heme iron electronic structure similar to hemoglobin M Boston and M Saskatoon, two examples of naturally occuring alterations in hemoglobin (Hb M), which have tyrosine substitutions at the distal histidine site. The pK_a of tyrosine in a protein usually ranges from 9 to 12, but in M Saskatoon and M Boston the pK_a's are much lower (5.2 and 4.8). Nagai et al. (1989) reasoned that coordination to the heme would stabilize a deprotonated tyrosine if the tyrosine were in a strong ionic environment on the distal side.

Gross et al. (1998) replaced the hydrogenase cytochrome *b* ligands with methionine. None of the resulting mutants had hydrogenase activity. Methionine is a ligand, along with histidine, to the heme in cytochrome *c*, which usually has a higher redox potential than the cytochrome *b* of hydrogenase or the *bc*1 complex. Their

experiments did not indicate whether the loss of activity was due to lack of binding or to a dysfunctional redox potential.

Imidazole Reconstitutes Physiologically Relevant Hydrogenase Activity

Exogenous ligands to the heme in proteins can provide environmental conditions that enable the heme to function, although the functions may be altered from the native state. The ligand may provide the strength of binding required to pull the iron toward the plane of the heme, act as a Lewis acid electron donor to poise a redox potential, and/or serve to maintain protein conformation (Kevin Gable, personal communication, and Barrick, 1994). Imidazole was used in these experiments to explore the tolerance of the ligand sites for exogenous ligands.

Imidazole does not affect the wild-type O_2-dependent H_2 oxidation activity (Fig. 9, 11, and 12) and the partial deletion mutants do not gain activity in the presence of imidazole (Fig. 10). In three of the four ligand mutants, the addition of imidazole had no effect. However, addition of Imd restored full O_2-dependent H_2 activity to H194A (which has no activity without Imd), and to H194Y (which has partial activity without Imd) (Fig. 13, 16). This chemical rescue is not concentration dependent within the range of 1-10 mM (Fig. 14) and is not growth dependent (Fig. 15).

Incorporation of the free ligand can be uncoupled from protein synthesis, indicating that imidazole can gain immediate access to the heme cavity, and can act as a ligand to the heme in existing protein without new protein synthesis being required. The immediate incorporation of imidazole and subsequent H_2 oxidation implies that the apoprotein HoxZ may already be inserted into the membrane and only requires the ligation of the heme to be functional in physiological electron transport. Alanine in histidine's position was not expected to affect imidazole binding, but apparently tyrosine in the heme pocket does not prevent imidazole from binding to heme either.

The free ligand imidazole apparently fulfills the structure and function of a ligand in HoxZ electron transport to the same capacity as histidine.

The H194A samples that were grown with imidazole, centrifuged, the medium replaced with buffer, and shaken for an hour, retained the same H_2 oxidation activity as samples assayed immediately. Washing does not appear to reduce the *in trans* substitution of imidazole in HoxZ. Either the ligation is stable enough that the exogenous ligand is not washed out or the Imd remaining in the cell from the saturated Imd solution is adequate to replenish any free ligand that is lost. In guanylate cyclase, Zhao et al. (1998) prepared for an exchange of exogenous ligands by removing excess imidazole and not adding imidazole in the last purification step. Upon analysis, they found that 80% of their protein still had heme bound.

One of the roles suggested for HoxZ is electron transport from the heterodimer to the electron transport chain. Imidazole added as an exogenous ligand to H194A/Y stimulated electron transport. Since imidazole is not tethered to the protein like the histidine, it is unlikely that it would have the same effect as the histidine in pulling the iron toward the heme plane, and it seems more likely that the effect of imidazole is on the redox potential of the heme, enabling it to function in electron transport, or in forming a conduit for electron transfer from the heterodimer. The other histidine ligands may have different local environments that do not aid in positioning or stabilizing a substitution or addition of a free ligand, or are not able to provide the appropriate redox potential for the heme. The position of H194 is fixed since H208 is on the same helix, whereas H33 and H74 are the only ligands on helices A and B, respectively. Without a histidine ligand to align the helix with the heme, the altered amino acid (and heme pocket) may not be positioned to face the heme. If helix D is positioned appropriately by H194, and an imidazole is ligated in the H208A position, the local environment may not provide an appropriate redox potential for the high potential heme.

Imidazole has been used with other heme protein variants to serve as an exogenous ligand and rescue heme binding (Barrick, 1994; Newmyer et al., 1996;

Newmyer and Ortiz de Montellano, 1996; McRee et al., 1994; Wilks et al., 1995; Zhao et al., 1998). In each of these systems, heme in the native protein is five-coordinate with histidine serving as the proximal ligand. Imidazole (5-25 mM) also bound to the heme as the proximal ligand in proteins where the histidine was replaced with another residue.

With sperm whale myoglobin H93G, Barrick (1994) demonstrated that imidazole structurally and functionally substituted for the histidine ligand. Though there were slight differences between the orientation of the imidazole to the heme as compared to wild-type, crystal structures showed that imidazole bound to the heme iron, and absorption spectra with O_2, CO_2, and CN^- provided evidence that H93G(Imd) functioned like wild-type. In studies with horseradish peroxidase H170A, Newmyer et al. (1996) showed that the mutant alone did not generate reaction intermediates, but with 25 mM imidazole was catalytically active at 0.5% the rate of wild-type. Crystal structures of cytochrome *c* peroxidase H175G showed a bisaquo heme, whereas the addition of the free ligand resulted in the binding of imidazole to heme iron (McRee et al., 1994). Enzyme activity with the mutant was 1.6% of wild-type, and with H175G(Imd) was 4.5% of wild-type. In a soluble version of heme oxygenase, Wilks et al. (1995) generated a H25A mutant, which resulted in a catalytically inactive protein. Unlike the H25A mutant, H25A(Imd) has absorption and resonance Raman spectra that closely resembled wild-type. Soluble guanylate cyclase H105 (Zhao et al., 1998) was identified as the heme ligand when the addition of 10 mM Imd rescued heme binding in H105G as shown by absorption spectroscopy. These studies used purified soluble enzymes and probed for imidazole replacement of a single heme ligand.

In contrast, imidazole replacements in the HoxZ six-coordinate system were carried out *in vivo* in an integral membrane protein, and multiple ligand sites were probed. The 194 site in HoxZ is unique in allowing imidazole to act as a ligand functionally, but the rationale for this is not clear. It cannot be due solely to being on helix D, a helix with two ligands, since H208A(Im) is not active in physiological hydrogenase assays. Two features set site 194 apart.

First, 194, the only one that allowed tyrosine and imidazole replacement, is the only site with a neighboring histidine. In some proteins, such as *A. vinelandii* hydrogenase HoxK (Sayavedra-Soto and Arp, 1993), ferredoxin I FdxA (Martin et al, 1990), and nitrogenase molybdenum-iron protein NifD and NifK (Li et al., 1990), a neighboring cysteine residue substituted for a missing ligand. In HoxZ, H194 is neighbored by H195, and is the only ligand next to a charged residue. H195 is highly conserved, but whether H195 plays a role, such as stabilizing the ligand, is not known. Neither H195, nor its counterpart, H187, in *W. succinogenes*, can substitute for the ligand, as seen by complete lack of activity of the ligand mutants using the physiological acceptor in both species (Gross et al., 1998). The *W. succinogenes* HydC mutant H187A had wild-type activity, indicating no measurable effect resulting from this mutation in a neighboring residue. So this residue does not play a critical role in wild-type, but perhaps this neighboring residue or a residue on an adjoining helix orients and stabilizes the imidazole or imidazole side chain of histidine.

Studies with cytochrome *c* peroxidase found that substitution of the proximal histidine ligand to glutamate or glutamine gave enzymes with activity similar to wild-type. This flexibility may have been dependent on the aid of the hydrogen bond from aspartate. Replacement of this supporting aspartate, which normally forms a H-bond with the histidine, resulted in 1000-fold decreased activity (Newmyer et al., 1996). H195 may provide a strong ionic environment by acting as a proton acceptor for the tyrosine and imidazole. An analogous situation is provided by photosystem II and a histidine that, from modeling studies, appeared to point into a cavity containing the redox-active tyrosine D. Since the tyrosinate proton of D (Y160) was thought to be hydrogen bonded to a neighboring basic amino acid, Kim et al. (1997) used the mutant H189L complemented with imidazole to show that H189 functions as a proton acceptor for tyrosine D.

Tethering the heme to the protein backbone was found to be critical to catalysis in the two peroxidases, perhaps to disfavor heme binding to a distal residue (Newmyer et al. 1996), or to limit movement of iron into the heme plane (McRee et al. 1994).

Newmyer et al. (1996) suggested that the hydrogen bond between the ligand histidine and an aspartate plays a role in positioning the ligand and increasing its imidazolate character. Since the H194 site in *A. vinelandii* can be chemically rescued by imidazole to full wild-type activity, as measured by H_2 oxidation activity with O_2 as the electron acceptor, it appears that tethering to the protein backbone is not necessary in this position. The difference between the peroxidase systems and HoxZ may be due to the difference in coordination, normally five vs. six coordinate.

A second feature that sets H194 apart is the residue in the –3 position. The heme porphyrin has two propionic acid side chains that face to the outside of the membrane. In diheme proteins, usually three of the four propionic acids form salt bridges with a positively charged residue in the third position in the helix preceding or following the ligand (N$\pm$3). Residue 194 is the only ligand to have histidine (H191) in a position to bind the propionic acid. The other positions have arginine (H33 and H74) or alanine (H208) in the third position. The replacement of residue H191 to alanine in *A. vinelandii* resulted in a wild-type phenotype so this position is not essential for wild-type activity. However, in H194A(Imd), the histidine in position 191 may influence the electronic structure of the porphyrin.

The addition of imidazole to the non-ligand mutants H97Y, H98A, H136A, and H191A did not increase the O_2-dependent hydrogenase activity of these mutants. H97Y, H98A and H191A had full wild-type activity, and imidazole did not increase nor decrease this activity. H136A, with or without imidazole, has about one-third the activity of wild-type; imidazole does not chemically rescue the replaced residue in this position.

3-Fluoropyridine (3FP) acted as an exogenous ligand with myoglobin and bound to heme in position of H93G (Decatur et al. 1996). However, 3FP did not serve as a ligand in position 194 in HoxZ.

Ligand Positions on the Helices Determine Heme-Heme Orientations

In cytochrome *b* of the bc_1 complex, two hemes are ligated by histidines on helices B and D, and the histidines on each helix are 13 residues apart. The *b* cytochrome is proposed to extend beyond the edge of the membrane on one side, placing the high potential heme near the middle of the membrane and the low one near the other edge. Because the four histidines are on two parallel α-helices, the hemes are also expected to be positioned edge-on in parallel.

In other quinone-interacting proteins, the histidines are found on two, three or four α-helices. In nitrate reductase, the ligands are on helices B and E, but the hemes are predicted to not be parallel (Berks et al., 1995). Those proteins with ligands on three or four helices cannot have the hemes positioned edge-on in parallel. Except for the bc_1 complex, quinone-interacting proteins are structurally similar in terms of size of transmembrane helices, protein length, and residue composition, regardless of whether they reduce the quinone or oxidize the quinol. The histidines of formate dehydrogenase are located on three helices (Berks et al., 1995) and the cytochrome *b*'s of fumarate reductase of *W. succinogenes* (Körtner et al., 1990, Gross et al., 1998) and succinate:menaquinone reductase of *B. subtilis* (Fridén and Hederstedt, 1990) have ligands on four helices. In these proteins, the ligands to hemes serve to bundle the helices together as well as dictating conformation of the helices. The loss of a ligand may also imply the loss of relative positioning of the helices.

In hydrogenase cytochrome *b*, one axial ligand to each of the two hemes is on helix D. The second axial ligand to one heme is located on helix A and the second axial ligand to the other heme is on helix B. If the helices are positioned essentially perpendicular to the membrane, then it is unlikely that a neighboring residue on helix D could substitute for a ligand as long as the axial ligand partners are on separate helices. An α-helix with a single ligand may not be constrained in rotation, but an α-helix with two ligands would be constrained by the unsubstituted residue. Because of the position on the α-helix, substitution on helix D by a neighboring residue would

either pack the helices too tightly together or place the hemes on opposite sides of helix D (Fig. 19).

Functionality of the Hydrogenase Heterodimers of HoxZ Mutants

To localize the loss of O_2-dependent H_2 oxidation activity to the substitutions in HoxZ, hydrogenase activity in the mutants was determined with an assay (H_2-dependent MB reduction) requiring only functional heterodimers. Methylene blue can accept electrons from hydrogenase, and upon reduction, changes color from blue to clear. This change in spectroscopic absorbance can be quantitatively measured, and must be assayed anaerobically to prevent oxygen from reoxidizing the methylene blue. In whole cells, methylene blue can be reduced non-specifically, and the endogenous reduction was determined by assaying without adding H_2 (Arp, 1989).

Without H_2, all strains had similar, but low levels (~32% of wild-type with H_2) of endogenous activity, and this was comparable to HoxKG$^-$ with or without H_2 (Fig. 17). In all other strains except HoxKG$^-$, when H_2 was added, reduction of methylene blue was immediate and more rapid than in the absence of H_2, which indicates that the heterodimer is functional (Fig. 17 and 18). This work was unable to confirm that reverse electron transport through HoxZ is necessary for reduction of the catalytic site (Sayavedra-Soto and Arp, 1992), since the heterodimers were sufficiently reduced from the flushing of nitrogen gas and removal of oxygen to reduce MB without a lag period.

With H_2, the rates of MB reduction in the control strains fell into one of three classes. The MB reduction rate in HoxKG$^-$ was similar in the presence and absence of H_2 and is consistent with no hydrogenase activity. The wild-type reaction can be considered as the combination of the endogenous and full hydrogenase reaction. HoxZ$^-$ reduced MB more rapidly in the presence than in the absence of H_2, indicating that the heterodimer is catalytically active. However, the rate of MB reduction for HoxZ$^-$ was significantly lower than the wild-type value.

The quantitative aspect of this MB assay is important in evaluating the role of HoxZ in the hydrogenase activity of the heterodimer. In this assay with H_2, the *A. vinelandii* HoxZ mutant had 70% of wild-type activity. This activity is comparable to the 50-80% of wild-type activity reported for three-day-old cells of this strain reduced with $Na_2S_2O_4$ and assayed with the hydrogen electrode (Sayavedra-Soto and Arp, 1992).

With *A. vinelandii* HoxZ, all H→A mutants showed H_2-dependent MB reduction, i.e., all had functional hydrogenase heterodimers. Alanine and tyrosine in the same position gave similar reactions, but different positions reacted differently. H33A/Y had full wild-type activity. Since this position is on the cytoplasmic side, it may not affect heterodimer binding and may not be necessary for providing the correct anchor position for the heterodimer. Like HoxZ$^-$, which had partial activity, H74A, H194A and H208A had less than wild-type activity. This result indicates that HoxZ influences the ability of heterodimer to catalyze the reduction of MB at full catalytic rate. Apparently the H33 site is not critical to heterodimer activity, whereas its high potential heme partner, H208, is, as are H74 and H194, the ligands to the low potential heme on the periplasmic side.

From the O_2-dependent H_2 oxidation experiments, all four of these histidines were shown to play a role in physiological electron transport. Since MB can bind directly to the heterodimer, HoxZ's role in electron transport from the heterodimer in the H_2-dependent MB reduction assay is uncertain. Perhaps these three HoxZ ligands, H74, 194 and 208, are fulfilling solely a structural role in anchoring the heterodimer correctly, or perhaps they are influencing alternative and generally more efficient pathways of electron transfer to MB. The requirements for electron transfer are not precise: the electron transfer rate depends on the distance between the electron donor and acceptor (Boxer, 1990). Ferber and Maier (1993) have demonstrated in liposomes of *B. japonicum* that ubiquinone analogs can accept electrons from the heterodimer, indicating that transfer can occur under non-optimum conditions, although the rate may not be as high as with a fully functional cytochrome *b*.

Three non-ligand mutants had at least wild-type activity in the MB reduction assay, and one position, 136, had ~20% greater than wild-type activity. If changing H136→A opened a methylene blue sink, this would account for the increased activity. H136A had partial activity in the physiologically relevant O_2-dependent H_2 oxidation assay, indicating that H136 plays an important, though not essential, role in H_2-dependent electron transport. The residues immediately around 136 are partially conserved G135 and the fully conserved N137 and P138. H136 in the loop between the second and third helices near the cytoplasmic membrane and the high potential heme, a location similar to various residues important to the Q_i site in the bc_1 complex and quinol:fumarate reductase (QFR). His82 and Glu29 in QFR have been suggested from mutant studies to be involved in protonation and deprotonation reactions similar to Glu212 in the photosynthetic reaction center (Hederstedt and Ohnishi, 1992).

Hydrogenase cytochrome *b* mutants have been tested for heterodimer activity in various ways. A deletion of a homologue to HoxZ, HupC in *Pseudomonas hydrogenovora*, had 47% (U mg protein^{-1}) of wild-type activity when assayed with methylene blue (Ohtsuki et al., 1997). In *P. hydrogenovora*, H_2 can be used as the sole source of energy, but HupC$^-$ did not grow on H_2. Although Ohtsuki et al. (1997) concluded that HupC was a component of H_2-specific electron transport, they did not differentiate between heterodimer and trimer activity. Since HupC$^-$ had hydrogenase activity in the MB assay, they dismissed loss of hydrogenase activity as the reason for the lack of autotrophic growth with H_2. Amperometric measurement of H_2-dependent O_2 consumption of the glucose-grown HupC$^-$ was only 3% of Wt. These data reflect the data for *A. vinelandii*: the cytochrome *b* deletion mutant has no activity with the physiological terminal electron acceptor and has partial heterodimer activity as compared to wild-type.

Bernard et al. (1996) tested the *A. eutrophus* heterodimer in two ways. First, they separated the soluble and membrane fractions for MB assays since *A. eutrophus* has both a soluble and a membrane-bound hydrogenase. Membrane fractions of the large subunit deletion mutant and HoxZ$^-$ had no H_2-dependent MB-reducing capability as

compared to wild-type. Second, they assayed for heterodimer activity in gels stained under H_2-saturated conditions with the two artificial electron acceptors, PMS (phenazine methosulfate) and nitroblue tetrazolium, which would indicate if the membrane-bound hydrogenase had PMS-reducing capability. The heterodimer in the HoxZ mutant had PMS-reducing activity, and from this qualitative test, they concluded that HoxZ played no role in heterodimer activity. Menon et al. (1991) suggested that the discrepancy between the two types of assays with artificial electron acceptors could be due to different assay conditions. Preliminary experiments in this *A. vinelandii* research offer a potential explanation. In spectroscopy assays with MB, whole cells of all strains had some activity, but membrane preparations had little residual MB-reducing activity (at most <15% of whole cells).

The *W. succinogenes* mutants (three cytochrome *b* ligand variants and two HydA small subunit histidine replacements) did not reduce the cytochrome *b* hemes nor a quinone analogue and did not transfer electrons to the terminal electron acceptor (Gross et al., 1998). In benzylviologen reducing assays of cytochrome *b* variants, the soluble fraction had 10% or less activity of the membrane fraction, indicating that the heterodimer was attached to the membrane. The heterodimers of cytochrome *b* mutants could use H_2 as a substrate to reduce benzylviologen, and the mutants' abilities were at least 50% of the wild-type strain. Using isoelectric focusing to obtain heterodimer and trimer preparations, Gross et al. found that the heterodimer alone had 75% of trimer benzylviologen reducing activity. The three non-ligand histidines replaced in this cytochrome *b* had wild-type activity in both quinone and benzylviologen reduction.

Imidazole Addition Effects in the H_2-dependent MB Reduction Assay

Addition of imidazole did not affect the *A. vinelandii* wild-type, HoxKG$^-$R, and HoxZ$^-$R controls, nor H33A/Y or H74A/Y. In these two HoxZ positions not affected by the addition of imidazole, one mutant has full activity, regardless of imidazole, and

one cannot recover activity even when grown with imidazole. Both of these positions are in the helices with a single ligand.

When mutants with replacements in the other two ligand positions and the double ligand replacement H194,208A were grown with imidazole, the ability to reduce MB with H_2 was substantially increased. Imidazole reconstituted wild-type activity in the mutants 194A/Y and 208A/Y. Both positions are in Helix D, and with single mutations, the helix may be held in the proper position by the unaltered ligand. The double mutant did not have activity in O_2-dependent H_2 oxidation and did not regain full H_2-dependent MB reduction. Without either protein ligand, perhaps helix D was not fixed in position, and full activity with imidazole would require a three way intermolecular reaction instead of just two, like the single positions (Kevin Gable, personal communication).

The non-ligand mutant H97Y had less than wild-type activity in the MB reduction assay and, when grown with imidazole, MB reduction was similar to wild-type. This mutant had very low endogenous MB reduction activity, but this does not fully account for the low H_2-dependent reduction. Perhaps the tyrosine blocked a MB binding site and the addition of imidazole enabled the mutant to overcome the block. Further research, perhaps with a point substitution to alanine in this position, may help clarify this.

Imidazole rescue of hydrogenase activities of replaced ligands provided a useful tool for comparing the roles of structure and function of the heme ligands. Imidazole rescues physiologically relevant activity in position 194, i.e., imidazole can fulfill functional and structural roles in this position. Since imidazole cannot rescue electron transfer activity in H208A/Y(Imd), then the contribution of imidazole in the heterodimer assay is unlikely to be functional, but rather structural. Imidazole likely fulfills a structural role, that of binding the heme, in position H194A/Y(Imd) also, and this is supported by the partial recovery of MB reduction activity in H194,208A(Imd).

Heme synthesis mutants in cytochrome *b* of SQR in *E. coli* indicate the importance of heme in the assembly of the *b*-type cytochrome. In heme synthesis mutants, the catalytic heterodimer accumulated in the cytoplasm. When 5-aminolevulinic acid was present in the culture, heme synthesis could continue and the heterodimer was found in the membrane portion. Nakamura et al. (1996) concluded that heme is important in the functional assembly of this complex. In SQR of *B. subtilis*, individual replacement of four histidines in the transmembrane segments affected heme ligation and assembly of the enzyme, and they concluded that heme is required for the assembly of the enzyme (Hederstedt and Ohnishi, 1992). Since the membrane bound apocytochrome cannot firmly or functionally bind the catalytic subunits, Fridén et al. (1987) suggested that this cytochrome *b* has different conformations depending on whether or not the heme is bound.

Several lines of evidence indicate that the presence or absence of the hydrogenase cytochrome *b* results in a conformational difference in the heterodimer. First, in *E. coli*, enzyme assays were performed following non-denaturing polyacrylamide gel electrophoresis (associated subunits, such as the heterodimer, electrophoresed as a complex instead of single proteins). An *E. coli* deletion mutant of HyaC (a HoxZ homolog) showed three forms of hydrogenase heterodimer, whereas wild-type had only a single band (Menon et al., 1991). The multiple forms were membrane bound indicating that HyaC was not the sole anchor for the large and small subunits. They suggested that without HyaC, multiple forms may occur because the heterodimer had increased enzyme susceptibility to proteolysis or occurred because of differential folding of the heterodimer.

Second, in *A. eutrophus,* the heterodimer was localized by hydrogenase activity via an in-gel nitroblue tetrazolium reduction with H_2 and PMS present (Bernhard, 1996). As in the *E. coli* assay, soluble proteins and solubilized membrane proteins had been electrophoresed on a native gel, allowing subunits to run as a complex. In the HoxZ deletion mutant, the majority of the activity appeared in the soluble fraction, unlike wild-type. In both the soluble fraction and the solubilized membrane fraction, the activity was in a lower position on the gel than wild-type, indicating that the

heterodimer of HoxZ$^-$ is in a different conformation than the heterodimer in wild-type (Bernhard et al., 1996).

Third, in HoxZ of *A. vinelandii*, ligand binding in positions 74, 194 and 208 are apparently structurally important for full heterodimer activity, and this implies that binding the hemes in HoxZ results in a conformationally complete HoxZ, which affects the heterodimer. A conformational change in HoxZ point mutants, via imidazole, enables the heterodimer to bind in a conformation that allows these two subunits to regain full activity.

Inherent protein conformation appears critical for *b*-type cytochromes. Although cytochromes *c* and c_1 can be purified so that their midpoint potentials, inhibitor sensitivity, stability, and spectral properties are the same as they are in a membrane, the integral membrane *b*-type cytochromes have quite different properties when isolated as compared to being in a membrane. This observation implies that protein-protein and protein-lipid interactions affect the native conformation of *b* cytochromes, and that the loss of these interactions influences their inhibitor, redox, and spectral characteristics, as well as their ability to retain the heme and the heme site geometry. The cytochrome *b* heme site geometry is strained, due at least in part, to polypeptide imposed constraints. The ligand geometry at the heme iron is octahedral, and with axial ligation, the imidazole planes are perpendicularly oriented. Even when bis-histidine ligation is retained during isolation, relaxation of protein-induced constraints results in changes in ligand geometry (the octahedral geometry becomes more rhombic in character) (Salerno et al., 1986). It seems likely that the converse must also have an effect: elimination or change in a ligand to the heme may relieve strain and could affect conformation of the polypeptide.

Three positions are critical for proper HoxZ conformation, i.e. full H_2-dependent MB reduction activity, and the H→A results are supported by H→Y results. HoxZ is not the sole anchor for the heterodimer, but is required for full heterodimer activity. Without this hydrogenase cytochrome *b*, a substantial amount of the heterodimer is found in the soluble fraction membrane fraction (Bernhard et al, 1996), and the

heterodimer found in the membrane fraction is in multiple forms (Menon et al., 1991). If the large and small subunits are not post-translationally modified until they are membrane bound (Menon et al., 1991, and Fu and Maier, 1993), perhaps this requires more than just binding by the C-terminal portion of the small subunit. The presence of HoxZ provides a structural base for a mature and conformationally different heterodimer than in the absence of a complete HoxZ. This base allows binding of a fully active heterodimer, which is better attached to the membrane than in the absence of the HoxZ anchor.

HoxZ in *A. vinelandii* was suggested to stabilize hydrogenase in cell extracts since aerobically prepared wild-type cell extracts had much greater activity than HoxZ extracts, and anaerobic preparation increased the activity (Sayavedra-Soto and Arp, 1992). Stability of hydrogenase has been tested in various ways in different organisms. In the current work, preliminary experiments with membranes showed that H_2-dependent MB reduction activity was comparable between wild-type and HoxZ, but since both had only a very small percentage of the activity of whole cells, only whole cell studies were continued. In H_2-dependent MB reduction assays with whole cells, imidazole made up the difference in activity between H194A and wild-type. Heme binding, rather than stability, explains the difference in activity. When imidazole was added to the H194A at the start of the O_2-dependent, H_2 oxidation assay, the heterodimer was immediately capable of hydrogenase activity without new protein synthesis. Cauvin et al. (1991), working with an insertion mutant in HupM of *R. capsulatus*, found that hydrogenase activity was quickly lost upon cell breakage. However, if H_2 was present, regardless of the presence of O_2, hydrogenase activity could be maintained. In *P. hydrogenovora*, Ohtsuki et al. (1997) compared the stability of the HupC mutant with wild-type by incubating cell extracts at 30°C or bubbling them with O_2, and then measuring hydrogenase activity (heterodimer MB reduction). The activity was similar to that remaining in the wild-type strain and they concluded that HupC was not involved in the stabilization of hydrogenase.

From HoxZ$^-$ studies in *A. eutrophus,* Bernhard et al. (1996) results supported the proposed roles of coupling hydrogenase to the respiratory chain and anchoring the

heterodimer in the membrane, but not with reductive activation of the hydrogenase enzyme nor stabilization of the enzyme. In their studies, HoxZ$^-$ could not sustain lithoautotrophic growth of *A. eutrophus* using H_2 as an energy source, which is compatible with HoxZ$^-$ as a conduit to the electron transport chain. The role of HoxZ$^-$ as an anchor for the heterodimer was underscored by the majority of the PMS activity being found in the soluble fraction of the cells.

The current HoxZ studies in *A. vinelandii* indicate that HoxZ is required structurally and functionally for physiological electron transfer, and is needed structurally for full catalytic capability of heterodimer. These results support the HoxZ roles suggested by Sayavedra-Soto and Arp (1992) of mediating electron flow from hydrogenase to the respiratory chain and attaching the heterodimer to the membrane.

Conclusions

The four heme ligands in *A. vinelandii* HoxZ are H33, H74, H194, and H208. With site-directed substitution and imidazole replacement, each ligand can be individually identified by a combination of H_2 oxidation assays (O_2-dependent H_2 oxidation and H_2-dependent MB reduction). This report is the first use of imidazole complementation in an integral membrane protein, and the only report of multiple ligand replacements in a heme protein. The four ligands are essential for electron transport from the hydrogenase heterodimer to the respiratory chain, and the non-heme ligand H136 plays a role in O_2-dependent H_2 oxidation. Both ligands on the periplasmic side of the plasma membrane, those nearest the heterodimer, and one of the ligands on the cytoplasmic side, are required for optimal heterodimer catalytic activity. Thus, in the hydrogenase of *A. vinelandii*, the ligands in the *b*-type cytochrome HoxZ are required structurally and functionally for optimal H_2 oxidation activity.

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