AN ABSTRACT OF THE THESIS OF

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Blood vessel injury was found to release intracellular pools of protein D-aspartyl/L-isoaspartyl carboxyl methyltransferase (PIMT) into the extracellular milieu, where it became trapped. Trapped PIMT was able to utilize radiolabeled S-adenosyl-L-methionine (AdoMet) introduced into the circulation to methylate blood vessel proteins containing altered aspartyl residues specifically at the site of injury. *In vitro* studies more fully characterized this endogenous PIMT activity in thoracic aorta and inferior vena cava. At least 50% of the PIMT activity released during injury, was resistant to non-ionic detergent extraction, suggesting that the enzyme activity can become trapped within or behind the extracellular matrix (ECM). Analysis of inferior vena cava, found that 90% of the altered aspartyl residues in blood vessels are inaccessible to methylation by intracellular PIMT under physiological conditions. Subfractionation of inferior vena cava on the basis of solubility found that at least 40% of the altered aspartyl containing proteins in blood vessels are insoluble in non-ionic detergent

containing buffers and are highly resistant to extraction by protein denaturants.

Analysis of peptides revealed that the majority of the altered aspartyl groups in blood vessels are located extracellularly. Digestion of these extracellular matrix proteins with cyanogen bromide (CNBr), followed by methylation with (PIMT), found that about 60% of the altered aspartyl residues in the ECM are solubilized by this treatment. The presence of hydroxyproline in amino acid hydrosolates of this fraction and acidic pH gel electrophoresis of methylated peptides, allowed the identification of collagen as the major PIMT substrate in the CNBr-soluble material. CNBr peptides derived from both type I and type III collagen were found to methylated. It is estimated that one centimeter of blood vessel contains on the order of 5×10^{14} altered aspartyl residues involving 1% to 5% of the total extracellular protein.

Protein Methylation at Sites of Blood Vessel Injury

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Dean of Graduate School

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Darin J. Weber, Author

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Introduction

1.1 Introduction

1.1.1 Why blood vessels are interesting

The leading cause of mortality in adults of Western countries are pathologies associated with the vascular system, including coronary artery disease (Goldberg 1982; Luscher and Noll 1994; Gary and Clarke 1995), hypertension (Todorovich-Hunter et al. 1988; Schultze et al. 1994), diabetes (Reiser et al. 1992; Tanno et al. 1993) and atherosclerosis (Pasquinelli et al. 1989; O' Brien et al. 1994). In recognition of this, over the last several decades a much greater understanding of the physiology, cell biology and biochemistry of the vascular system has been pursued. Blood vessels are highly organized tissues, consisting of several cell types whose homeostasis is dependent not only on external cues in the bloodstream, but also on internal cues communicated between the various cells in this tissue (Luscher et al. 1993). It is disruptions in the normal homeostatic, proliferative and anti-proliferative cues, due to undefined injury events, that are thought give rise to many of these pathologies. This thesis describes how blood vessel injury can lead to enzymatic methylation of altered aspartyl residues at sites of injury. How altered aspartyl residues in blood vessel proteins can form and subsequently be detected by enzymatic methylation is described below.

1.1.2 Blood vessel function and structure

In the vascular system there are two main types of blood vessels; arteries and veins. Arteries deliver oxygen and nutrients to the tissues, and veins carry away waste products and carbon dioxide from these same tissues. Though the two main types of blood vessels differ in function, structurally, they both can be divided into three distinct layers; intima, media and adventitia (Anthony and Thibodeau 1979) (Figure 3.1 of Chapter 3).

1.1.2.1 Intima

The intima consists of the lumen of the blood vessel through which blood flows. The major constituents of this layer are endothelial cells and the basal lamina upon which these cells grow. Endothelial cells form a continuous monolayer throughout the vascular system and act as a permeability layer against blood solutes, possess important synthetic-metabolic-secretory functions and maintain a non-thrombogenic surface for proper blood flow (Gimbrone 1980; Goldberg 1982). The basal lamina consists of a thin mat of filamentous proteins, proteoglycans and glycoproteins upon which the endothelial cells grow (Alberts and al 1994).

1.1.2.2 Media

This is the most complex structural layer of a blood vessel. It contains two main cell types; large amounts of smooth muscle cells and small amounts of fibroblasts. These cells are embedded in an extracellular matrix of proteins and

glycoaminoglycans. Smooth muscle cells are under the control of the sympathetic nervous system and are responsible for controlling the degree of constriction or dilation of the blood vessel. They, along with fibroblasts, also secrete most of the extracellular matrix components, primarily collagen, elastin and various glycoaminoglycans (Carey 1991). Veins contain greater amounts of collagen fibers while arteries contain more elastic fibers. Recent studies have found that in the major arteries and veins virtually no turnover of elastin occurs (Campbell et al. 1991; Johnson et al. 1993) and that vascular collagen has a half life of at least several years (Robert and Labat-Robert 1995).

1.1.2.3 Adventitia

This outermost layer is composed of a loose meshwork of connective tissue and fibroblasts. Capillaries and nerve cells which feed and innervate the media are found in this layer. The adventitia is less well defined than the other two layers primarily because it often resembles the tissue of the organ through which the blood vessel is passing. Its function is basically supportive and protective (Rothe 1980).

1.1.3 Altered Aspartyl Residues

Since major blood vessels contain significant populations of metabolically stable cells and proteins, it is likely that alterations in the aspartyl and asparaginyl residues of proteins in this tissue will be present. Alterations at these amino acid residues are known to arise spontaneously and accumulate in

a time dependent manner (Bada 1984). These altered aspartyl residues are formed as a result of a reaction initiated by attack of the amide nitrogen of the C-terminally neighboring amino acid upon the side chain carbonyl carbon, of either aspartyl or asparaginyl residues. In the case of asparagine, this attack also causes deamidation. The result of this attack is the formation of an unstable succinimide intermediate, which may hydrolyze to yield either L-aspartate or L-isoaspartate (Geiger and Clarke 1987; Stephenson and Clarke 1989).(Figure 1.1). Alternatively, the succinimide may also epimerize to the D-configuration, yielding D-aspartate and D-isoaspartate upon hydrolysis. However, the major product of succinimide hydrolysis is the L-isoaspartyl form. Aspartyl and asparaginyl residues, account for nearly 10% of amino acids in a typical protein, so the multiple products formed as a result of this reaction are thought to account for a large part of the micro-heterogeneity observed within proteins (Aswad 1995). The accumulation of altered aspartyl residues in proteins is a relatively slow process, typically affecting between 0.1% to 5% of the proteins in a given protein population (Johnson and Aswad 1990; Chazin and Kossiakoff 1995). Factors such as primary sequence, pH, temperature and importantly, the metabolic stability of a protein, are known to affect the rate and extent of altered aspartyl accumulation in proteins (Johnson and Aswad 1990; Ota and Clarke 1990). Reports on the effects that altered aspartyl residues have upon the activity of proteins range from complete loss to no effect (Johnson and Aswad 1990; Teshima et al. 1995).

Figure 1.1 Formation of altered aspartyl residues. L-aspartyl and L-asparaginyl residues cyclize to an L-succinimide, losing water and ammonia, respectively. Epimerization at the α-carbon results in a mixture of L-succinimide and D-succinimide. Hydrolysis of the succinimides at either the α- or β-carbonyl groups results ultimately in a mixture of L-aspartyl, D-aspartyl, L-isoaspartyl and D-isoaspartyl residues.

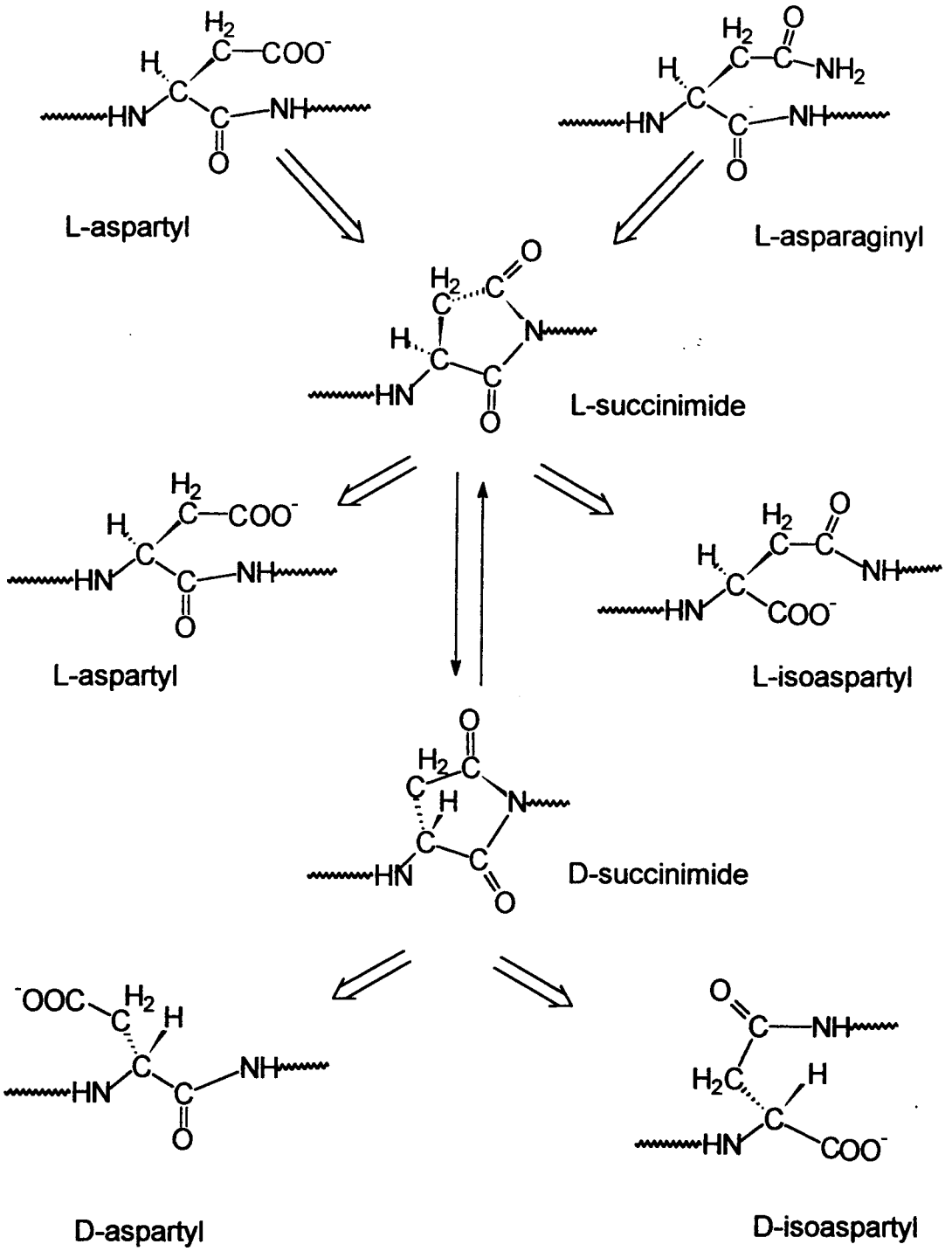
L-aspartyl
L-asparaginyl
L-succinimide
L-aspartyl
L-isoaspartyl
D-succinimide
D-aspartyl
D-isoaspartyl

Figure 1.1

In light of the metabolic stability of blood vessels and the potentially deleterious effects of altered aspartyl accumulation in proteins, a means to assay for altered aspartyl content in the vascular system was sought.

1.1.4 Protein D-aspartyl/L-isoaspartyl carboxyl methyltransferase (PIMT)

In 1982, an enzyme now called protein D-aspartyl/L-isoaspartyl carboxyl methyltransferase (E.C. 2.1.1.77) (PIMT), was shown by McFadden and Clarke to be capable of methylating altered aspartyl residues (D-aspartyl) in membrane proteins of erythrocytes and it was proposed that this reaction could lead to the "repair" of these altered residues back to their normal L-configuration (McFadden and Clarke 1982). Clarke and coworkers and Aswad and coworkers, subsequently showed that PIMT can methylate L-isoaspartyl residues in peptides which is followed by a non-enzymatic de-methylation step that leads directly to the conversion of the altered aspartyl residue to the normal L-configuration (O'Conner and Clarke 1983; Geiger and Clarke 1987; Johnson et al. 1987; Johnson et al. 1987; McFadden and Clarke 1987). PIMT catalyzes this reaction by transferring a methyl group from S-adenosyl-L-methionine to the free carboxyl group of the altered aspartyl residue. This carboxyl methyl ester is unstable at physiological pH and temperature and soon hydrolyzes, yielding methanol. This leads to the formation of a succinimide intermediate, which being unstable, can hydrolyze to the normal L-aspartyl configuration approximately 25% of the time (O'Conner and Clarke 1983; Johnson and Aswad 1985; Murray and Clarke 1986; Ota and Clarke 1990) (Figure 1.2). Thus, several

rounds of enzymatic methylation, followed by non-enzymatic de-methylation are required to restore most of the L-isoaspartyl residues in a population to the normal L-configuration. However, in the case of altered aspartyl residues that arise from asparagine, repair is not complete, since only L-aspartyl is generated following methylation.

Evidence of a widespread, physiological repair function for PIMT, is supported by the finding that almost every protein examined, can serve as a substrate for PIMT and the fact that PIMT, a cytosolic enzyme is widely distributed in nature and has been found in virtually all tissues assayed (Aswad 1995). More importantly, incubation of PIMT with AdoMet and proteins known to contain altered aspartyl groups has been shown to lead to the regeneration of normal aspartyl residues and restoration of activity in some enzymes (Johnson et al. 1987; Johnson, Murray et al. 1987; Lowenson and Clarke 1992; Brennan and Clarke 1995).

The broad substrate specificity of PIMT, implies that to varying degrees, most proteins contain altered aspartyl residues. It also suggests that this enzyme along with AdoMet, could serve as a molecular probe for identifying altered aspartyl residues in the vascular system and may have potential as a therapeutic in restoring these alterations to the normal L-configuration.

Figure 1.2 Methylation of altered aspartyl by PIMT leads to formation of normal aspartyl residues in protein. PIMT incorporates a methyl group from S-adenosyl-L-methionine into an ester linkage either with the α-carboxyl of L-isoaspartyl residues or the β-carboxyl of D-aspartyl residues. Enzymatically formed methyl esters are subject to rapid non-enzymatic displacement by nucleophilic attack by the amide nitrogen of the adjacent, C-terminal amino acid. This results in succinimide formation which can spontaneously epimerize and hydrolysis to form four products; L-asp, D-asp, L-isoasp and D-isoasp. Both D-asp and L-isoasp can be re-methylated by PIMT and cycled through the pathway illustrated.

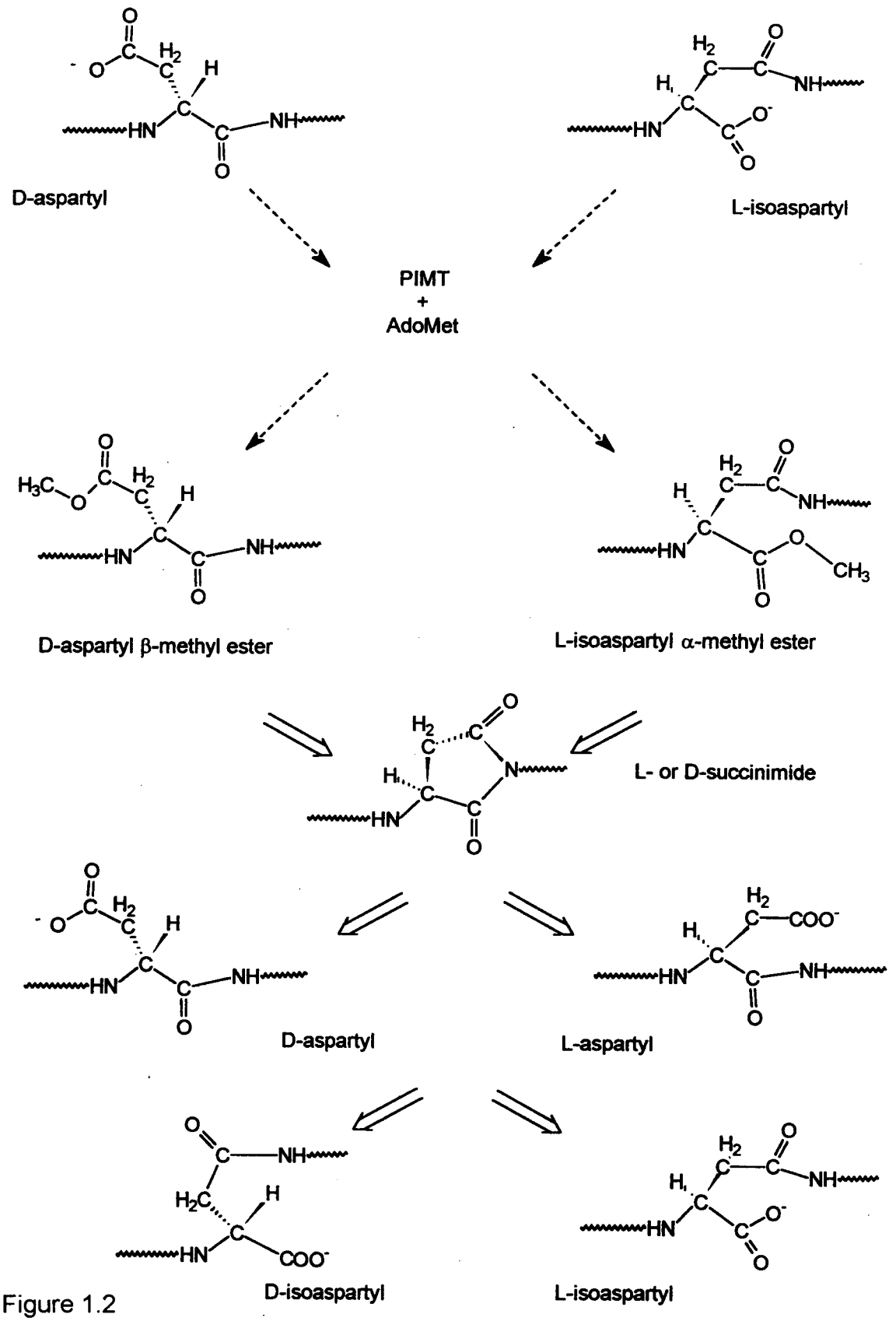
D-aspartyl
L-isoaspartyl
PIMT
+
AdoMet
D-aspartyl β-methyl ester
L-isoaspartyl α-methyl ester
L- or D-succinimide
D-aspartyl
L-aspartyl
D-isoaspartyl
L-isoaspartyl

Figure 1.2

1.1.5 Blood vessels, altered aspartyl residues and PIMT

In the initial stages of this thesis, *in vitro* perfusion of rat veins with radiolabeled AdoMet, revealed that even in the absence of exogenously added PIMT, methylation of blood vessel proteins occurs. In Chapter 2, experimental results are presented identifying PIMT as the enzyme responsible for the observed methylation. It is further shown that intracellular stores of PIMT are released as a result of blood vessel injury. This released PIMT becomes trapped in the extracellular matrix and is able to methylate blood vessel proteins from luminally administered AdoMet, both *in vitro* and *in vivo*. Chapter 3 follows up this work by focusing on the substrates that are methylated by PIMT as a result of blood vessel injury. It is shown that the extracellular matrix contains the majority of altered aspartyl residues in this tissue. Finally, Chapter 4 identifies collagen as the major methyl accepting PIMT substrate in the extracellular matrix of blood vessels.

In summary, this thesis describes the discovery that blood vessel injury causes a general release of intracellular enzymes into the extracellular matrix, where they become entrapped and capable of reacting with substrates delivered by the blood stream. As a specific example, this thesis shows how trapped PIMT can utilize luminally supplied S-adenosyl-L-methionine to methylate proteins in the extracellular matrix, the major repository of altered aspartyl residues in this tissue.

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Chapter 2

Detection and Characterization of an Isoaspartyl Methyltransferase which becomes Trapped in the Extracellular Space during Blood Vessel Injury.

Darin J. Weber and Philip N. McFadden

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2.1. Abstract

Injury to rat blood vessels *in vivo* was found to release intracellular pools of protein D-aspartyl/L-isoaspartyl carboxyl methyltransferase (PIMT) into the extracellular milieu, where it became trapped. This trapped cohort of PIMT was able to utilize radiolabeled S-adenosyl-L-methionine (AdoMet) introduced into the circulation to methylate blood vessel proteins containing altered aspartyl residues. Methylated substrates were detected only at the specific site of injury. Detailed, *in vitro* studies more fully characterized this endogenous PIMT activity in thoracic aorta and inferior vena cava. Methylation kinetics, immunoblotting and the lability of methylated substrates at mild alkaline pH, were used to demonstrate that both types of blood vessel contain an endogenous protein D-aspartyl/L- isoaspartyl carboxyl methyltransferase (PIMT) . At least 50% of the PIMT activity was resistant to non-ionic detergent extraction, suggesting that the enzyme activity can become trapped within or behind the extracellular matrix (ECM). Quantities of lactate dehydrogenase (LDH), another soluble enzyme of presumed intracellular origin, was found to be similarly trapped in the extracellular space of blood vessels.

2.2. Introduction

Formation of altered aspartyl residues is one of the most common types of spontaneous changes occurring in the polypeptide backbone of proteins (Takemoto 1995). These altered aspartyl groups arise from spontaneous racemization of L-aspartyl residues as well as from spontaneous deamidation

and isomerization of L-asparaginyl residues (Clarke 1985). Protein D-aspartyl /L-isoaspartyl carboxyl methyltransferase (PIMT), an enzyme widely distributed in nature, has been shown to be capable of methylating these altered aspartyl residues (McFadden and Clarke 1982) . This unique post translational modification can restore abnormal D-aspartyl or L-isoaspartyl residues back to the normal L-aspartyl form (Clarke 1985). PIMT uses a methyl group donated by S-adenosyl- L-methionine (AdoMet) to specifically identify altered aspartyl in proteins by methylating the altered polypeptide residue through an ester linkage on the free carboxyl group. Early in our efforts to detect altered aspartyl groups in blood vessels, we were surprised to find that perfusion of veins with radiolabeled S-adenosyl- L-methionine, in the absence of added PIMT, resulted in detectable levels of methylated proteins in the vascular wall, suggesting that blood vessels contain an endogenous PIMT which can utilize S-adenosylmethionine delivered from the luminal side of the blood vessel. In this report we describe how injury to blood vessels leads to the trapping of this methyltransferase within the meshwork of extracellular proteins of the vessel wall.

2.3. Experimental Procedures

2.3.1 Experimental Animals

Healthy, female Sprague-Dawley rats of approximately 300 grams and three months of age were obtained through Oregon State University Lab Animal

Resources. The animals were maintained in accordance to university guidelines for animal care and were fed a standard rat chow diet (Purina) and water ad libitum.

2.3.2 Excision of blood vessels

Rats were killed by asphyxiation with CO_2. Three centimeter sections of inferior vena cava were removed from each rat following a thoraco-abdominal incision in the right side of the chest. Ten minutes were required to completely excise the vein upon initiation of the procedure. Each blood vessel was immediately perfused with RPMI 1640 cell culture medium (Gibco-BRL) pH 7.4 under low pressure to wash the lumen free of all blood cells and plasma proteins. Veins were maintained at ice cold temperature in the above cell culture medium until the vessels from the other rats used in each experiment were obtained. Eight one centimeter vessel segments were obtained from four rats in one hour. The loose adventitial connective tissue, which contained capillaries, was carefully separated from the rest of the blood vessel and discarded. Thoracic aortas were obtained in a similar manner. In some experiments vessels were extracted with detergents, as described in the Figure legends, prior to carrying out radiometric methylation assays (described below).

2.3.3 Blood Vessel Perfusion

One centimeter segments of blood vessel were affixed to plastic tissue culture dishes (Falcon) using a 1% solution of low-melt agarose (Ultra-Pure,

United States Biochemical) prepared in RPMI 1640 at 37° C. The agarose served as a gas permeable support to immobilize the vessel and keep it hydrated it in nutrient medium. The ends of the vessel were clamped to plastic 200 μL pipet tips (Fisher Scientific) as inlets and outlets, to ensure that only the vessel lumen was bathed during perfusion. Perfusion was done with the radiometric assay mixture described below.

2.3.4 Radiometric Activity Assay

Unless otherwise noted, the assay mixture consisted of 7.6 μM S-adenosyl-L-[*methyl*-^{3}H] methionine (New England Nuclear) at a specific radioactivity of 2.5 Ci/mmol prepared in RPMI 1640 pH 7.4. Each assay was carried out at 37 °C for 20 minutes, or as noted in the figure legends. In experiments employing exogenously added isoaspartyl methyltransferase, a total of 0.6 units of protein D-aspartyl/L-isoaspartyl carboxyl methyltransferase (EC 2.1.1.77) purified from rabbit erythrocytes (Gilbert et al. 1988) was added to the assay mixture. For experiments testing the ability of blood vessel extracts (described below) to methylate isoaspartyl containing substrates, one milligram of ovalbumin (Sigma, A-5503) was added to the assay mixture. At the conclusion of the reaction period, the blood vessel and its luminal contents were immediately mixed with an equal volume of 20% trichloroacetic acid (TCA), giving a final concentration of 10% TCA. Vein segments from the perfusion experiments described above were subsequently transferred to 0.5 ml polypropylene tubes containing 10% TCA. All samples were washed twice

with 200 µl, of 10% TCA to remove any unincorporated S-adenosyl-L-[*methyl*-^{3}H]methionine (^{3}H-AdoMet).

2.3.5 Quantification of Volatile Radioactivity

Following the radiometric assay just described, TCA washed substrates were treated with 0.2 M sodium hydroxide for twenty minutes at room temperature, in tightly capped polypropylene tubes. Following base treatment, the alkaline samples were re-acidified to pH 2.0 by adding 20 µl of 3M citric acid. Aliquots (80µl) of each sample, in duplicate, were transferred to one dram glass vials (Kimble) which were then placed inside a 20 ml polypropylene scintillation vial (Fisher) containing 4.0 ml of scintillation fluid (Scintiverse E, Fisher) and tightly capped. The [^{3}H]-methanol formed via ester hydrolysis was allowed to volatilize and partition into scintillation fluid from the inner vial of the tightly capped scintillation vial for 24 hours. Radioactivity trapped in the scintillation fluid was measured to an accuracy of +/- 5% using a Beckman LS 6000 scintillation counter. Volatilization efficiencies (typically 50%) were obtained by an internal standard of [^{14}C]-methanol.

2.3.6 Endogenous Activity Extracts

One centimeter vein and artery segments were finely homogenized in liquid N_2 using mortar and pestle. The homogenate was resuspended by vortexing in 50 µL of phosphate buffered saline (PBS), containing 1% (v/v) 2-mercaptoethanol (Sigma, St. Louis, Mo.), pH 7.4. The soluble material was

then collected following centrifugation at 10,000 x g for five minutes at 4° C. This extract contained at least 95% of the endogenous enzyme activity detected in blood vessels and was used without further purification.

2.3.7 Cellular Localization of Endogenous Enzyme Activity

One centimeter segments of veins and arteries were enzymatically dissociated to obtain separate populations of endothelial and smooth muscle cells (Chameley-Campbell et al. 1979; Campbell et al. 1989). Briefly, endothelial cells were obtained from blood vessel segments by perfusion with 0.1% collagenase type I (Worthington) for fifteen minutes at 37° C in RPMI, pH 7.4, followed by perfusion with 0.1% Trypsin (Worthington) for an additional fifteen minutes under the same conditions. Smooth muscle cells were then obtained by cutting up endothelial stripped blood vessels into several pieces followed by incubation in RPMI pH 7.4 containing 0.05% elastase and 0.1% collagenase (Worthington) for two hours at 37° C. The cells obtained from each treatment were collected by centrifugation at 1,000 x g for one minute at room temperature. The pellets were washed twice with 1.0 ml of PBS and centrifuged as before to remove digestive enzymes. Finally, the cell pellets were resuspended in 100 μL of PBS and frozen in liquid N_2 to lyse the cells. After thawing on ice, the lysates were clarified by centrifugation at 10,000 x g for ten minutes at 4° C. The supernatants derived from both the endothelial cells and the smooth muscle cells were assayed for enzymatic activity as described under section 2.3.4.

2.3.8 pH Lability of Methylated Vein Substrates

Following the radiometric activity assay described previously, the loss of volatile radioactivity from methylated blood vessel substrates was monitored as a function of pH. The following buffers were prepared: 0.1M Na^+ citrate; pH 4.0, 0.1M Na^+ citrate; pH 5.0, 0.1M Na^+ citrate; pH 6.0, 0.1M NaH_2PO_4; pH 7.0, 0.1M Tris-HCl; pH 8.0, 0.1M NaH_2PO_4; pH 9.0, NaH_3BO_3 ;pH 10, and 0.1M NaOH. Each vein segment was immersed in 200 ul of the appropriate buffer for twenty minutes at 25° C. At the end of the incubation, an equal volume of 20% TCA was added for a final concentration of 10% TCA. Any volatilized radioactivity was detected by placing aliquots of the 10% TCA into an inner vial within a tightly capped scintillation vial containing 4.0 mls of scintillation fluid and allowing volatile radioactivity to diffuse from the inner vial into the scintillant. To detect radioactivity remaining associated with each vein segment after the pH treatment, the veins were washed twice with 10% TCA and then 0.2M NaOH was added to release any remaining volatile radiolabel, which was quantified as previously described. Methylation of one milligram ovalbumin with purified rabbit PIMT was used to directly compare the lability of isoaspartyl methyl esters with the lability of blood vessel substrates methylated by the endogenous enzyme activity.

2.3.9 Immunoblotting

One centimeter segments of vein were each extracted with 100µl of 1% Triton X-100 (v/v) in PBS for ten minutes on ice with occasional vortexing. The detergent soluble fractions (TX-100 soluble) were retained and the extracted vein and artery were each washed twice with 200µl of PBS to remove excess detergent. The detergent insoluble vein and artery were each powdered in liquid N_2 and any enzyme released by this treatment was recovered by extraction in 100µl of PBS (TX-100 insoluble). Additionally, a one centimeter untreated vein and artery segments were each homogenized in liquid N_2 using a mortar and pestle and the released enzyme activity was recovered by extracting in 100µl of 1% TX-100 (v/v) in PBS. The extracts prepared from these homogenates were considered to contain all of the PIMT activity (Total) in each blood vessel type. Ten microliters of each extract was assayed for enzymatic activity using the radiometric assay described above. The remainder was lyophilized in a centrifugal concentrator (Savant Speed Vac). Each sample was then electrophoresed on a 12% polyacrylamide gel (Laemmli 1970). The resolved proteins were transferred to nitrocellulose and probed with rabbit antisera developed against a zebrafish (*Branchio danio)* PIMT fusion protein (Lindquist et al. 1996) . Immunoreactive bands were visualized with bromochloroindolyl/nitro blue tetrazolium (BCIP/NBT) reaction after probing the blot with an alkaline phosphatase conjugated secondary antibody as described (Harlow and Lane 1988).

2.3.10 Cell Viability Assay

A modified assay procedure for lactate dehydrogenase (LDH) (Decker and Lohmann-Matthes 1988) was used to assess the extent of cell lysis and enzyme entrapment occurring in various vein preparations. After each treatment (described in the Figure legends), lumens of one centimeter vein segments were perfused with 300nM of NADH (Sigma) and 300nM of pyruvate (Sigma) in 50μl of Dulbecco's Modified Eagle Medium pH 7.4 without sodium pyruvate or phenol red (Life Technologies). The assay was carried out for four minutes at 25° C and then stopped by recovering the perfusate and bringing it to a volume of 1.0 ml in PBS containing 16mM oxamate (Sigma), an inhibitor of LDH. The LDH dependent decrease in absorbance at 340nm was measured within one hour in a Hewlett-Packard HP8452A Diode Array spectrophotometer. Percent lysis was calculated relative to the maximal amount of LDH activity detected in aqueous extracts of veins powdered in liquid N_2.

2.3.11 *In Vivo* Blood Vessel Injury

Rats were anaesthetized with sodium pentobarbital (50 mg/kg) by interperitoneal injection. Once a deep state of anesthesia was obtained (15 minutes), a small incision, two centimeters in diameter, was made in the left side of the chest. This allowed access to the inferior vena cava, thoracic aorta and heart. Hemostatic forceps (14 cm, crile-style) were inserted through the incision and each blood vessel clamped for five seconds, using the second locking

position on the forceps. The inferior vena cava was clamped approximately one centimeter below it's junction with the heart. The thoracic aorta was clamped approximately 0.5 centimeters below the heart (halfway between heart and diaphragm muscle). Immediately after unclamping of blood vessels, 55μCi of [^{3}H-methyl]-AdoMet in 50μl of saline was injected into the left ventricle of the heart through a 26 gauge syringe needle. Following an incubation period of ten minutes, the rat was asphyxiated with CO_2 . Each injured blood vessel was excised (1.0 cm for aorta and 2.6 cm for vein) and luminally perfused with saline to remove all blood. Blood vessels were then cut into 0.2 segments and individually immersed in 10% TCA.. Due to the elastic nature of the thoracic aorta, it was necessary to stretch it to it's original one centimeter length in order to obtain segments. Segments were washed twice in 200μl of 10% TCA and methyl esterified proteins were quantified as described above.

To determine the distribution of radiolabel in rat tissues, 0.2 grams of the following tissues, brain, lung, liver and muscle, were homogenized in liquid N_2 . Each sample was then resuspended in 0.2 ml phosphate buffered saline, pH 7.4, containing 1% SDS (w/v). The samples were decolorized with 0.2 ml of 5.25% sodium hypochlorite and acidified with 0.2 ml glacial acetic acid. Aliquots of 0.2 ml were added to 4.0 ml of scintillation fluid to detect any radiolabel present in the samples. Radiolabel present in blood plasma was determined by adding 0.1 ml of plasma to scintillation fluid as above. Additionally, as a control, the left

saphenous leg vein was analyzed for radiolabel content as described for the injured blood vessel segments.

2.4. Results

In the course of evaluating the therapeutic potential of intravenous administration of PIMT and AdoMet, isolated segments of rat inferior vena cava were tested for their content of PIMT substrates. Unexpectedly, substrates in the vascular wall became radioactively methylated even in the absence of exogenously added methyltransferase. Furthermore, this radioactivity could subsequently be released as a volatile compound following treatment with alkaline solutions (Figure 2.1), suggesting that the linkages were methyl esters to blood vessel proteins. Several experiments were performed to examine the nature of this methylation. Methylation of blood vessel substrates was found to be optimal at physiological body temperatures (Figure 2.2.) The lack of a reaction a low temperature and heat inactivation of the reaction suggests strongly that an enzymatic process is involved rather than a non-catalyzed reaction (Kimzey and McFadden 1994).

To confirm that a methyltransferase is involved, radiometric assays were performed in the presence of either S-adenosyl L-homocysteine or sinefungin, potent competitive inhibitors of all known methyltransferases. These inhibitors caused in a sharp reduction of radioactively labeled substrates, as did addition of unlabeled AdoMet (Table 2.1).

Figure 2.1. Perfusion of blood vessels with ^{3}H-AdoMet results in methyl esterification of substrates both in the presence and absence of exogenously added PIMT . Immediately after isolation and washing, the lumen of one centimeter segments of inferior vena cava were perfused with RPMI 1640 cell culture medium, pH 7.4, containing radiolabeled AdoMet +/- purified rabbit PIMT. Following the conclusion of the radiometric assay, base volatile radioactivity was quantitated as described under Experimental Procedures. Solid bar represents veins perfused with radiometric assay mixture plus exogenous PIMT. Open bar represents volatile radioactivity detected in the absence of added PIMT. Error bars represent +/- s.e.m. of triplicate measures.

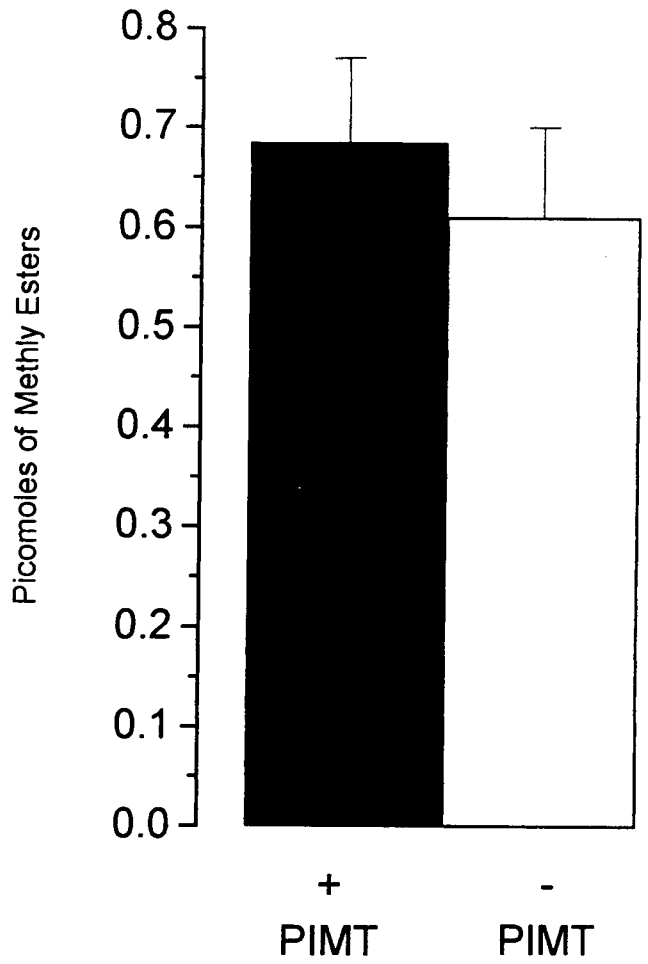


Figure 2.1

Figure 2.2. Blood vessel methylation is enzymatic. One centimeter segments of inferior vena cava were incubated with the radiometric assay mixture described under experimental procedures at 4° C and 37 ° C for thirty minutes. The 95 ° C bar represents a vein that was pre-incubated at 95 ° C for five minutes and then at 37° C in the presence of the radiometric assay mixture for thirty minutes. The assays were terminated by addition of 10% TCA and volatile radioactivity quantitated as described under Experimental Procedures. The solid bar represent veins incubated at 4° C, the striped bar represents veins incubated at 37° C and the open bar represent veins subjected to 95 ° C, followed by a 37° C incubation. Error bars represent +/- s.e.m. of duplicate measures.

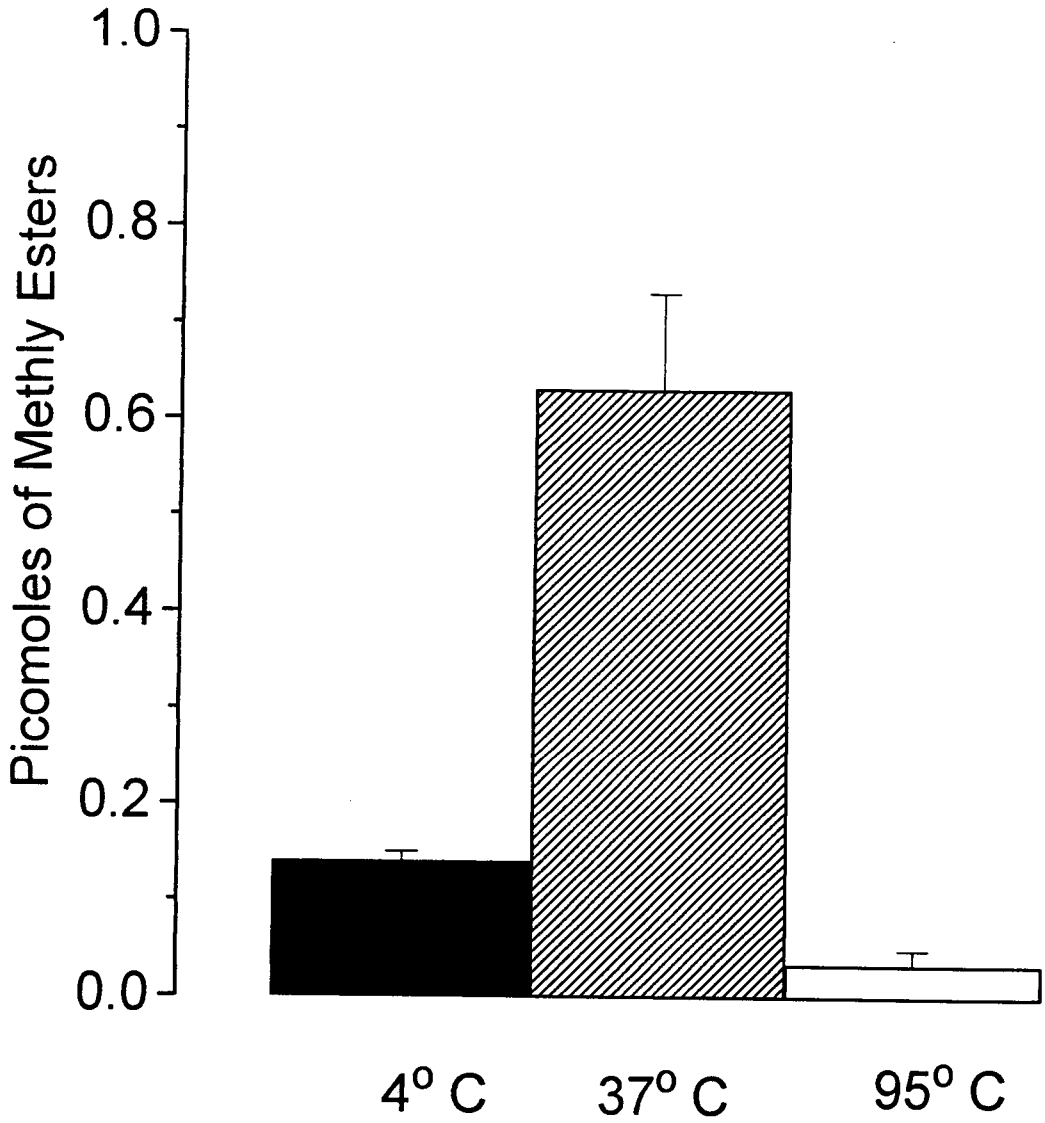


Figure 2.2

Table 2.1. Effects of various compounds upon enzyme activity. For thirty minutes prior to the radiometric assay, one centimeter vein segments were immersed in RPMI 1640, pH 7.4 containing the compounds listed. The radiometric assay and quantification of radioactivity was carried out as described under Experimental procedures. The percent decrease was determined by subtracting the mean of duplicate measures for each compound from the mean of duplicate measures for an untreated vein

Compound	% Decrease
No Treatment	0
5.6μM AdoMet (unlabeled)	53
40μM S-adenosyl homocysteine	91
10μM Sinefungin	93

The class of carboxyl methyltransferase involved in the methyl esterification of blood vessels was next investigated by characterizing the pH lability of the methyl moiety attached to the substrate (Paik and Kim 1990). A defining attribute of protein D-aspartyl/L-isoaspartyl carboxyl methyltransferases (PIMT), is the lability of their substrate's methyl ester moiety under mild alkaline conditions (Clarke 1985). The experiment was set up by incubating vein segments first with [^{3}H-methyl]-AdoMet in order to allow the endogenous enzyme activity to radiolabel substrates. Next, individual radiolabeled veins were exposed to a wide range of pH's to determine the pH sensitivity of the methyl ester moiety. At slightly alkaline pH, substrates radiolabeled by the endogenous blood vessel enzyme released their radiolabeled methyl moiety as a volatile product (figure 2.3). The tendency of the endogenous methyltransferase's substrates to lose their methyl moieties at relatively mild alkaline pH, is indicative of the involvement of an isoaspartyl methyltransferase in ester formation. Methylation of ovalbumin, a model isoaspartyl containing protein, by purified PIMT, followed by treatment with the same range of pH buffers, gave a similar methyl group lability profile (Figure 2.3), showing that the nature of the methyl ester linkage catalyzed by the blood vessel enzyme is the same.

In other tissues, PIMT has been shown to be localized intracellularly (Boivin et al, 1995; Gingras et al, 1991). The ability of endogenous blood vessel PIMT to utilize AdoMet delivered from the lumen to methylate protein

Figure 2.3. pH lability of methylated vein substrates. One centimeter segments of vein which had been radiometrically methylated as described under Experimental Procedures were treated with buffers of various pH. The release of volatile radioactivity was then measured after an incubation period of twenty minutes at room temperature. Closed symbols show the pH profile of methylated blood vessel substrates. Open symbols show pH profile of ovalbumin methylated by rabbit PIMT. Squares show the percent of the total volatile radioactivity that was lost as a result of each pH treatment. Circles show percent of volatile radioactivity remaining after each pH treatment. Error bars represent +/- s.e.m. of duplicate measures.

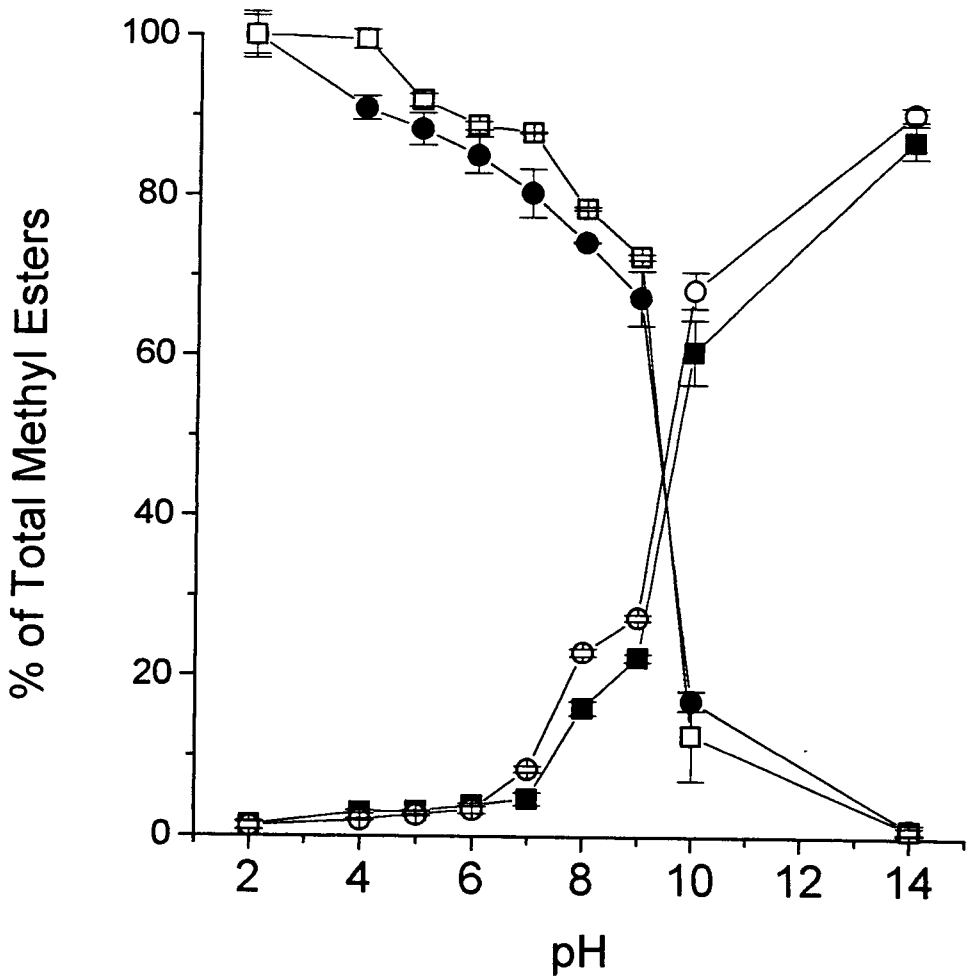


Figure 2.3

substrates is revealing. It suggests that in the course of blood vessel injury, PIMT leaks from the cells of this tissue, but remains trapped or cannot otherwise diffuse away from the blood vessel. This hypothesis implies that this trapped form of PIMT arises from cells located deep within the blood vessel wall, since PIMT leaking from cells near the lumen would likely be able to diffuse away. A series of experiments were designed to test this theory. First, since PIMT from other tissues has been shown to be effectively solubilized with non-ionic detergents (Lowenson and Clarke 1992; Pema et al. 1993), the lumens of veins were extracted with Triton X-100 and subsequently perfused with radiolabeled AdoMet to determine if PIMT activity was diminished. This treatment resulted in an approximate six fold increase in detectable methylation over untreated veins (Figure 2.4a). This is consistent with the hypothesis that PIMT becomes trapped within the blood vessel wall. Extraction with non-ionic detergent allows increased permeability of radiolabeled AdoMet into the blood vessel wall and also releases intracellular PIMT exposing it to new protein substrates, resulting in increased methylation.

Second, proteins circulating through the lumen of a blood vessel are likely to be inaccessible to methylation by deeply trapped PIMT. To determine if this is the case, TX-100 stripped veins were perfused with radiolabeled AdoMet and ovalbumin. No apparent methylation of luminal substrates was detectable (Figure 2.4b) yet, incubating purified rabbit PIMT along with radiolabeled AdoMet and ovalbumin in the vein lumen showed that there are no

Figure 2.4. Effects of TX-100 extraction upon methylation by endogenous PIMT of vessel wall substrates and luminal substrates. A) Prior to perfusion with radiolabeled AdoMet, the lumen of one centimeter segments of inferior vena cava were extracted with 1.0% TX-100 in RPMI 1640, pH 7.4 for twenty minutes on ice. The vein segments were then washed three times with PBS to remove excess detergent. Each segment was then perfused with RPMI 1640, pH 7.4 containing radiolabeled AdoMet for twenty minutes at 37° C. The solid bar represents untreated veins, the open bar, TX-100 extracted vein. B) Veins prepared as just described were perfused with RPMI 1640, pH 7.4 containing radiolabeled AdoMet one milligram of ovalbumin for twenty minutes at 37° C. At the end of the radiometric assay, the ovalbumin in the perfusate was recovered and precipitated with 10% TCA. Volatile radioactivity associated with the ovalbumin was quantified as described under experimental procedures. Solid bars represent ovalbumin perfusate from untreated veins, open bars, ovalbumin perfusate from TX-100 extracted veins. C) As a positive control, veins prepared as described in panel A, were incubated with ovalbumin as described in panel B, that also contained 0.6 units of purified rabbit PIMT, to determine if a methylation inhibitor was present. Solid bars represent ovalbumin perfusate from untreated veins, open bars, ovalbumin perfusate from TX-100 extracted veins. Error bars denote +/- s.e.m. of duplicate measures for all panels.

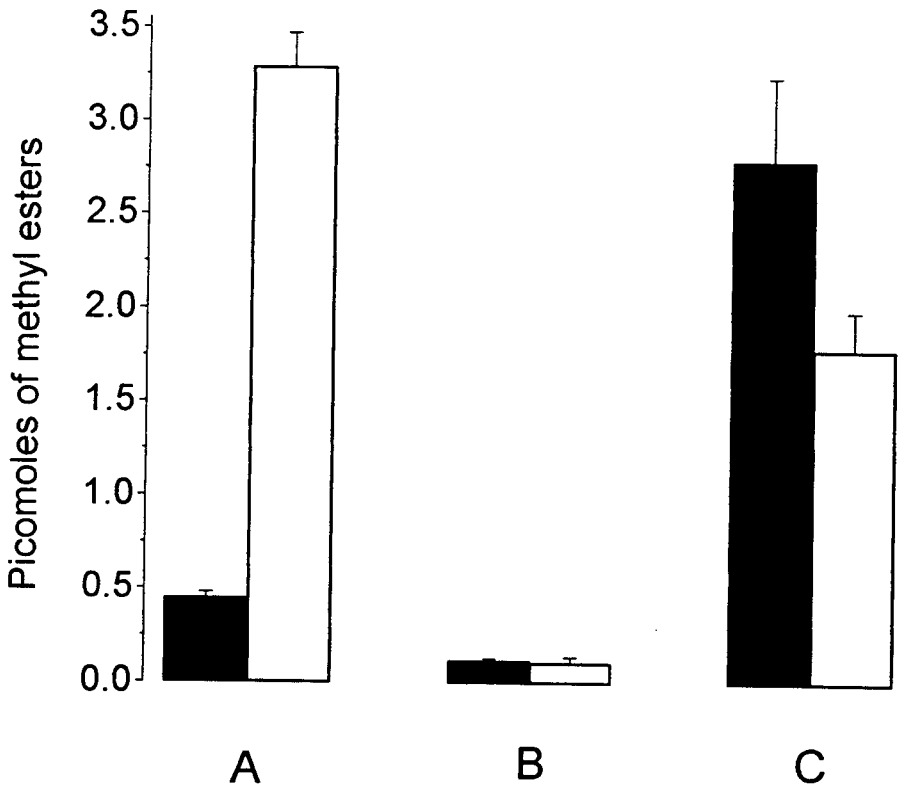


Figure 2.4

inhibitors of protein methylation present in blood vessel lumens (Figure 2.4c). The results of these experiments imply that the endogenous PIMT is unable to methylate substrates present in the lumen of blood vessels because the methyltransferase is trapped deeper within the blood vessel wall.

Third, to show that PIMT is normally localized intracellularly, blood vessel walls were enzymatically disassociated to obtain fractions enriched in the two major cell types of blood vessels; endothelial cells and smooth muscle cells. Lysates prepared from smooth muscle cells and endothelial cells of inferior vena cava revealed that smooth muscle cells contain greater than 65% of the PIMT activity, with the remainder found in the endothelial cell fraction (Table 2.2). Slightly different results were obtained with thoracic aortas. In this case, enzymatic activity was distributed almost equally among the two cell fractions. The localization of significant levels of PIMT within smooth muscle cells lends additional support to the hypothesis that this enzyme becomes trapped within the blood vessel wall, since these cells are located deep within this structure.

To more fully characterize blood vessel PIMT, several experiments were done. First, physically disrupting blood vessels by homogenization in liquid N_2 with mortar and pestle, was found to be capable of releasing greater than 95% of the PIMT activity in this tissue in a soluble form. This released PIMT activity was capable of methylating ovalbumin with a substrate K_{Mapp} of about 7μM, a value within the 0.5μM - 10μM range typically reported for this enzyme (Figure 2.5)

(McFadden et al. 1983; Aswad 1995). Second, immunoblotting coupled with activity assays confirm that PIMT can be detected in a form that is extracted by non-ionic detergent as well as a form resistant to detergent extraction (Figure 2.6). These results correlate well with those presented in Table 2.2, suggesting that the most of the detergent soluble PIMT activity may arise from endothelial cells and the detergent resistant activity from smooth muscle cells, which are found deeper within the blood vessel wall. The finding that PIMT is localized within cells of the blood vessel wall, strongly suggested that our initial observation of detectable methylation of blood vessel proteins following luminal perfusion with radiolabeled AdoMet, is a result of blood vessel injury. To quantify the extent of injury occurring during the isolation procedure as well as to determine if other enzymes can become trapped, an assay employing the well known intracellular enzyme, lactate dehydrogenase (LDH) was used. It was found that even under gentle isolation procedures, around 10% of the cells in blood vessels are compromised (Table 2.3). As expected, progressively harsher treatments results in greater LDH release, similar to what is seen for PIMT activity. Thus, "trapping" of enzymes from compromised cells within the blood vessel wall is likely to be a general phenomenon.

Table 2.2. Localization of endogenous enzyme. Shown in the table is the distribution of endogenous enzyme activity between endothelial cells and smooth muscle cells for both veins and arteries. +/- s.e.m. are shown in parentheses.

Blood Vessel	% Endothelial	% Smooth Muscle
Inferior Vena Cava	33.8 (0.08)	66.2 (0.13)
Thoracic Aorta	48.4 (0.17)	51.6 (0.11)

Figure 2.5. Kinetics of substrate methylation by crude extracts of veins and arteries. Extracts of veins and arteries, prepared as described under Experimental Procedures, were added to a radiometric assay mixture containing 0.2M citrate, pH 6.0, various concentrations of ovalbumin and radiolabeled AdoMet. The assays were allowed to proceed for twenty minutes at 37° C, before being terminated with 10% TCA. Volatile radioactivity was quantitated as described under Experimental Procedures. Solid circles represent enzyme extracts from arteries and open circles represent enzyme extracts from veins. Error bars denote +/- s.e.m. of duplicate measures.

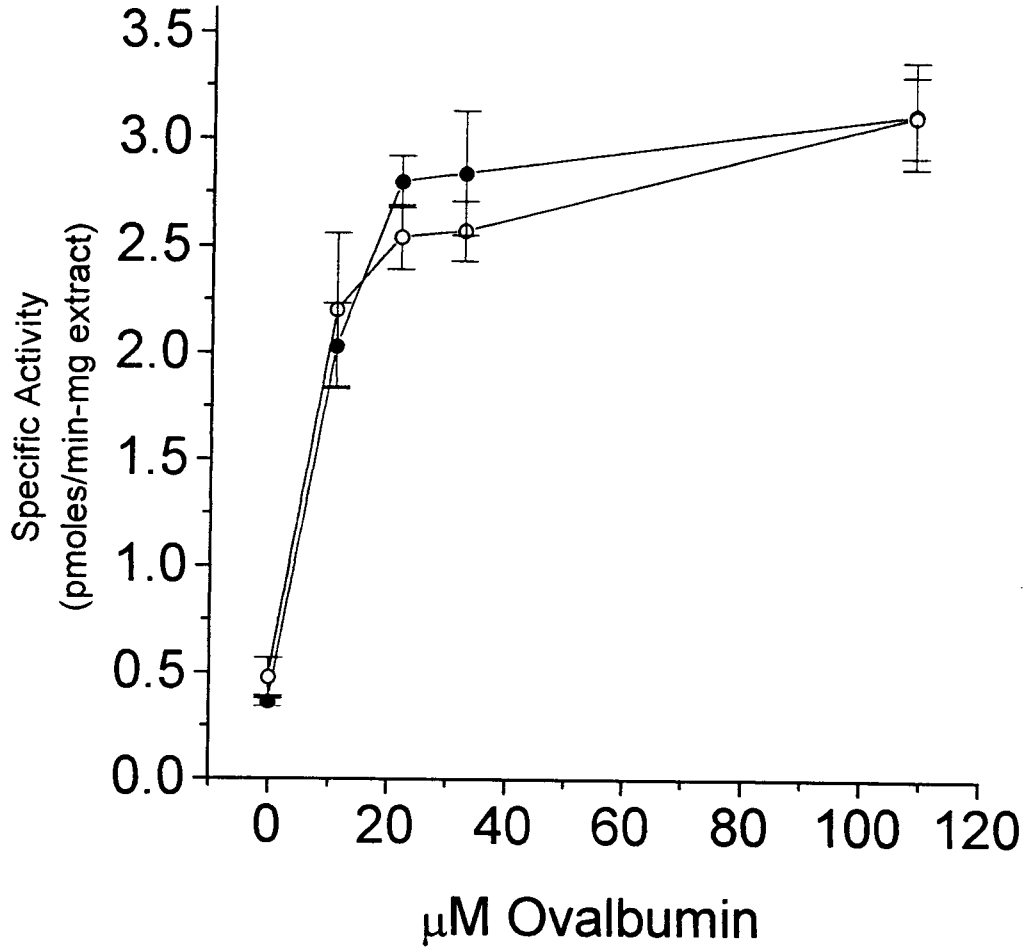

Specific Activity
(pmoles/min-mg extract)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
20
40
60
80
100
120
μM Ovalbumin

Figure 2.5

Figure 2.6. Immunological identification and activity of PIMT in vein and artery extracts. The upper portion of the figure represents the level of PIMT activity in various blood vessel subfractions relative to the total amount of enzyme activity present in one centimeter of vein or two centimeters of aorta. Error bars represent s.e.m. of duplicate measures. The lower portion of the figure shows immunodetection of PIMT against anti-PIMT sera after SDS-PAGE and transfer to nitrocellulose of the same preparations used to determine activity in the upper portion of the figure. Molecular weight is shown on the right.

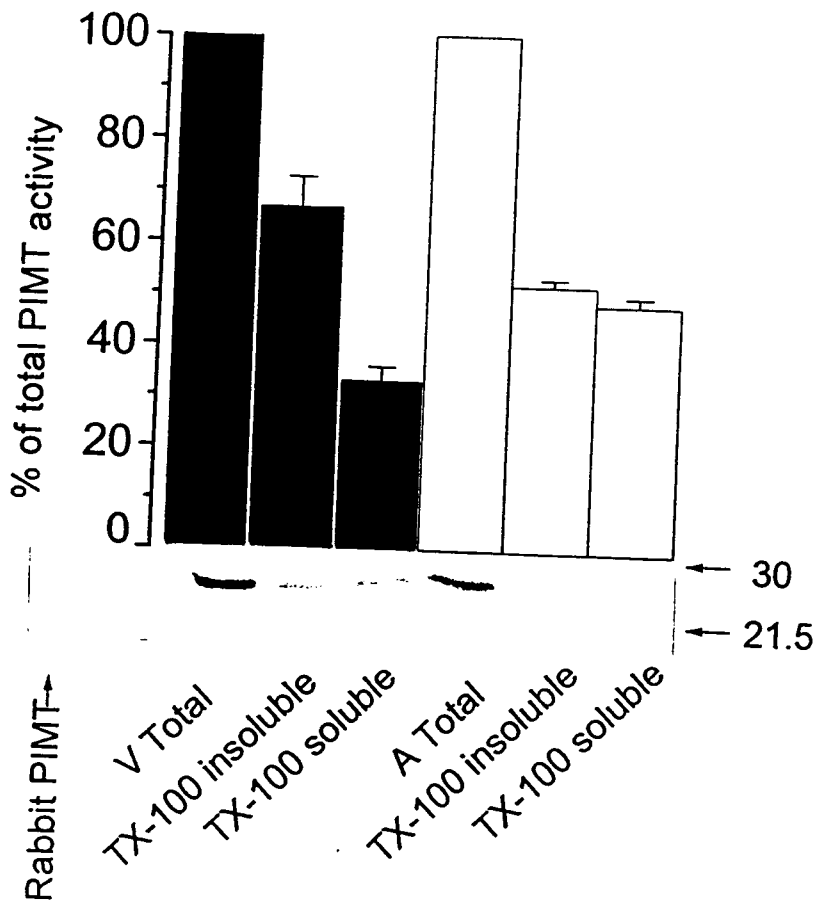
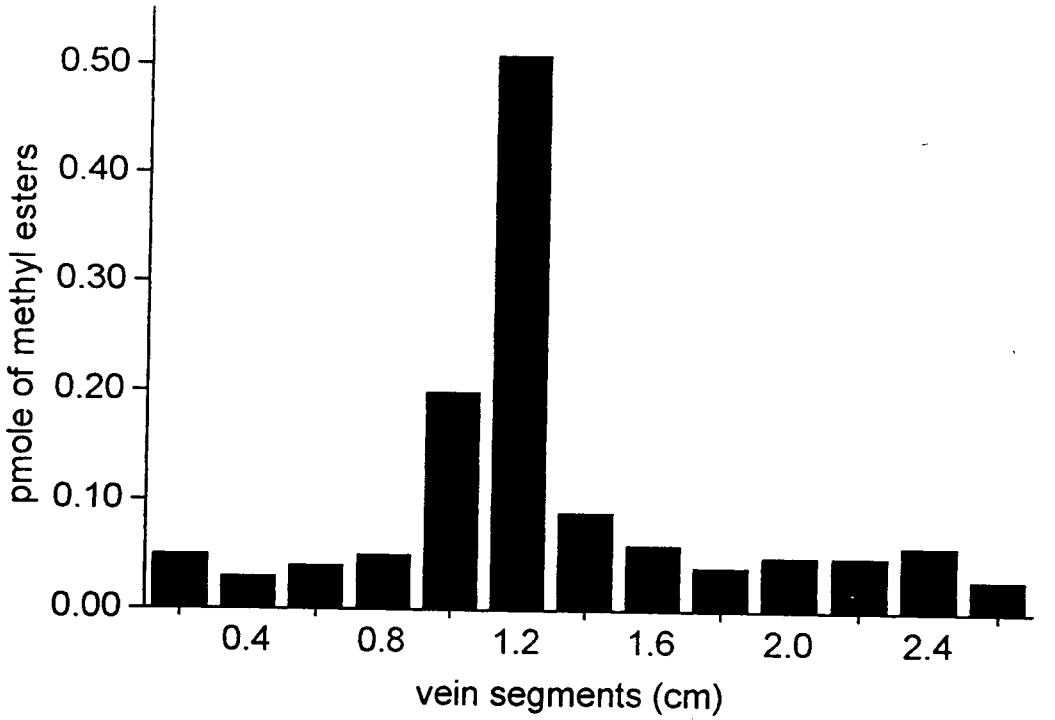

100
80
60
40
20
0
% of total PIMT activity
30
21.5
Rabbit PIMT
V Total
TX-100 insoluble
TX-100 soluble
A Total
TX-100 insoluble
TX-100 soluble

Figure 2.6

Table 2.3 Extent of cell lysis in various vein preparations. One centimeter segments of veins were treated as follows prior to assaying for released lactate dehydrogenase (LDH) activity; no treatment (Intact), extraction with 1% TX-100 (TX-100) or uniformly crushed with forceps for thirty seconds (Crush). Each vein segment was then luminally perfused with NADH and pyruvate, as described under Experimental Procedures. At the end of four minutes at room temperature assays were stopped and any decrease in absorbance at 340 nm was measured. Percent of lysis was calculated relative to total LDH activity present in aqueous extracts of homogenized veins. Numbers in parentheses denote s.e.m. of duplicate measures.

Preparation	% Cell Lysis
Intact	10.2 (1.2)
Crush	25.1 (2.4)
TX-100	41.2 (3.6)

Figure 2.7. Methylation by PIMT is confined to the site of *in vivo* blood vessel injury. A) Inferior vena cava: bars represent volatile methyl esters present in each 0.2 cm segment. Site of injury was approximately at one centimeter in the 2.6 cm of vein. B) Thoracic aorta: bars represent volatile methyl esters present in each 0.2 cm segment. Site of injury was approximately in the center of the one centimeter artery. Base volatile radioactivity was quantified as described under Experimental procedures.


0.50
0.40
0.30
0.20
0.10
0.00
pmole of methyl esters
0.4
0.8
1.2
1.6
2.0
2.4
vein segments (cm)

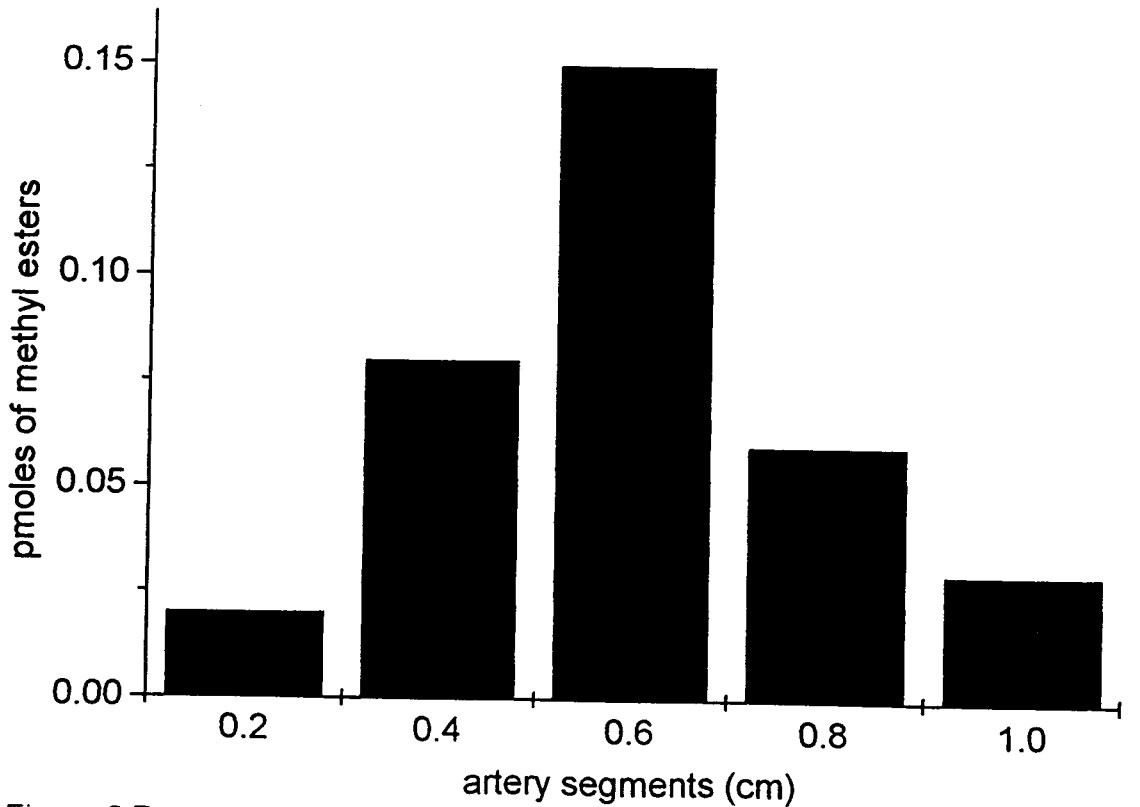

0.15
0.10
0.05
0.00
pmoles of methyl esters
0.2
0.4
0.6
0.8
1.0
artery segments (cm)

Figure 2.7

Finally, the *in vitro* phenomenon of PIMT methylation within injured blood vessels, was examined *in vivo*. Small regions of thoracic aorta and inferior vena cava of anaesthetized rats were injured prior to administration of radiolabeled AdoMet into the left ventricle of the heart. Subsequent isolation of the affected artery and vein, clearly show that only the area of injury correspond to significant amounts of base volatile methylation (Figure 2.7 A&B). Similar results were obtained when radiolabeled AdoMet was administered by tail vein injection (data not shown). At least 62% of the radiolabeled AdoMet administered can be accounted for in the tissues assayed (Table IV) and the remainder is assumed to associated with tissues that were not assayed.

2.5. Discussion

This investigation is the first to demonstrate that blood vessel injury can lead to the trapping of cytosolic enzymes within the extracellular matrix of the vascular wall. Further, it also represents the first example that circulating levels of AdoMet can be utilized by PIMT released from injured blood vessels, to methylated substrates within the vessel wall, *in vivo*.

Detecting intracellular PIMT in the vascular wall was expected, but the inability to completely extract the enzyme activity after treating the lumen with vigorous extraction procedures is unprecedented, as far as we know. The necessity of using mechanical homogenization to release PIMT from vessels supports a model in which PIMT is trapped within the mesh-like extracellular matrix, which has been shown by others to exclude molecules on the basis of

size (Meyer et al. 1977; Pearce and Laurent 1977; Bert et al. 1980; Monos et al. 1995). Furthermore, the entrapment of PIMT explains how blood vessel injury could allow a normally intracellular enzyme to catalyze covalent modifications of matrix proteins with circulating AdoMet as methyl donor. This meshwork probably acts as a selective permeability barrier allowing small molecules, such as AdoMet or NADH, to diffuse into the vessel wall, yet prevents larger macromolecules, such as protein substrates present in the lumen, from interacting with enzymes trapped within or behind the extracellular matrix.

Table 2.4. Distribution of radiolabeled adomet in rat tissues after blood vessel injury. For each of the tissues listed in the table, with the exception of blood plasma and saphenous vein, 0.2 grams of tissue was analyzed. Each sample was prepared as described under experimental procedures. For the blood plasma, 0.1ml was precipitated with 10% TCA immediately after spinning out of blood cells. In the case of the saphenous vein, a one centimeter segment was obtained from the left leg and washed free of blood and subsequently immersed in 10% TCA. It is a control to demonstrate that no radiolabel is associated with uninjured blood vessels.

Tissue	% Total AdoMet
Plasma	37.5
Brain	1.2
Heart	6.2
Lung	1.7
Muscle	8.9
Saph. Vein	>0.01

Our in vitro studies showing that luminally administered radiolabeled AdoMet can penetrate the vascular wall and be utilized by endogenous PIMT can be understood in light of the finding that even gentle isolation procedures compromise up to 10% of the cells in blood vessels. Even briefly clamping a blood vessel with hemostatic forceps, can lead injury and PIMT release as our *in vivo* experiment revealed. This implies that surgical procedures directly involving the manipulation of blood vessels, such as grafting, angioplasty and atherectomy (Ahn et al. 1990) are likely to injury blood vessels and release PIMT into the extracellular spaces. Clearly, these extracellular spaces contain altered aspartyl residues, as were detected in the course of this investigation (see chapter 3). Thus, therapeutic administration of AdoMet (Cutrin et al. 1992; Angelico et al. 1994; Barak et al. 1994; Bell et al. 1994; Dunne et al. 1994; Loguercio et al. 1994) following these and other events could conceivably allow extracellular PIMT to methylate and subsequently restore any altered aspartyl residues in proteins to their native L-configuration.

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Chapter 3

The Extracellular Matrix is the Major Locus of Altered Aspartyl Residues in Blood Vessels.

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3.1. Abstract

Blood vessel injury has been previously shown to result in the release of intracellular stores of protein D-aspartyl/L-isoaspartyl carboxyl methyltransferase (PIMT), as well as other cytoplasmic enzymes into the extracellular matrix . Additionally, it is known that both *in vitro* and *in vivo*, PIMT is capable of utilizing luminally supplied AdoMet to methylate substrates at the site of injury (Weber 1996). However, until now, nothing was known about the altered aspartyl containing substrates methylated by PIMT as a result of blood vessel injury. In this report blood vessels were enzymatically and chemically dissected in order to characterize the distribution and quantity of these proteins in both inferior vena cava and thoracic aorta of rats. Analysis of inferior vena cava, found that 90% of the altered aspartyl residues in blood vessels are inaccessible to methylation by intracellular PIMT under physiological conditions. Subfractionation of inferior vena cava on the basis of solubility found that at least 40% of the altered aspartyl containing proteins in blood vessels are insoluble in non-ionic detergent containing buffers and are highly resistant to extraction by protein denaturants. Analysis of peptides revealed that the majority of the altered aspartyl groups in blood vessels are located extracellularly. Amino acid analysis of the highly methylated blood vessel subfraction, revealed the presence of hydroxyproline, indicating that collagen may be a major substrate for PIMT in the ECM. It is estimated that one centimeter of vein contains on the order of 5×10^{14} altered aspartyl residues involving 1% to 5% of the total extracellular protein.

3.2. Introduction

Blood vessels represent a complex tissue of several cell types situated in a three dimensional protein scaffold of extracellular matrix (ECM) (Figure 3.1). In the preceding manuscript, we described how blood vessel injury can lead to entrapment of intracellular enzymes, such as, protein D-aspartyl/L-isoaspartyl carboxyl methyltransferase (PIMT) and lactate dehydrogenase (LDH), within the extracellular meshwork. In this report we extend our prior observations by examining the distribution of proteins containing altered aspartyl groups in blood vessels and examine the ability of PIMT to covalently modify these substrates in the injured blood vessel.

3.3. Experimental Procedures

In all the experiments described, three month old, female Sprague-Dawley rats, weighing 200 grams were used as sources for veins and arteries. Details of how blood vessels were excised, perfused with radiometric assay mixture, enzymatically digested, and how volatile radioactivity was quantified are described in the preceding report (Weber 1996).

3.3.1 Blood Vessel Fractionation

One centimeter vein or artery segments were homogenized in liquid N_2 using a mortar and pestle. The homogenized material was resuspended by vortexing in 50 μl of phosphate buffered saline (PBS), pH 7.4. The soluble

Figure 3.1. Schematic of Idealized Blood Vessel. The figure depicts the common structural features found in blood vessels. The blood vessel lumen is lined by a single cell layer of endothelial cells. Immediately beneath is thin layer of extracellular proteins called the basal lamina. Endothelial cells and the basal lamina make up the blood vessel intima. The media consists primarily of smooth muscle cells and extracellular matrix proteins. The outermost blood vessel layer is known as the adventitia and consists of loose connective tissue and is sparsely populated with fibroblasts and sympathetic nerves. Arteries and veins share all of these common features, but differ somewhat in their proportions. For example, thoracic aortas, contain less smooth muscle cells in the medial layer and more elastic fibers than inferior vena cava which contain more smooth muscle cells and collagen .

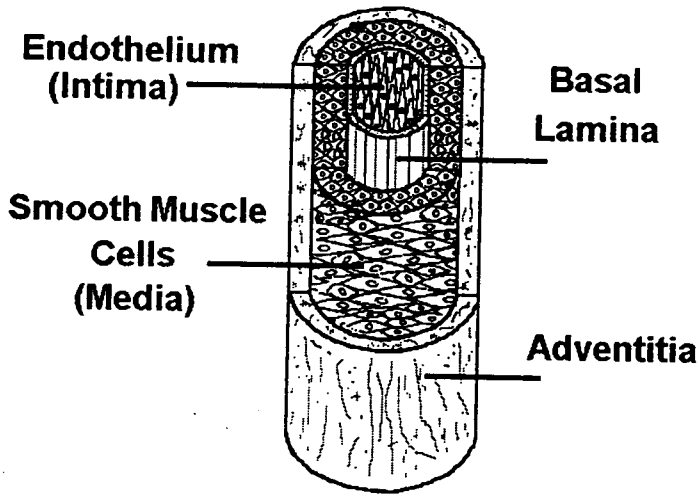
Endothelium
(Intima)
Basal
Lamina
Smooth Muscle
Cells
(Media)
Adventitia

Figure 3.1

material was then collected following centrifugation at 10,000 x g for five minutes at 4° C. This material was designated "water soluble" (WS). The insoluble material was then resuspended in 50µl of the above buffer containing 1% TX-100 (v/v) and vigorously mixed by vortexing. "Detergent soluble" (DS) material was collected by centrifugation as just described. Detergent insoluble material was further extracted with 2.5 M NaCl in 1% TX-100 to remove glycoaminoglycans [[Maurel, 1990 #68;Starcher, 1976 #433]. The remaining insoluble material (DI) was washed twice with ten volumes of water to remove excess salt and detergent and finally resuspended in 50 µl of PBS.

3.3.2 Proteolytic Digestion of Blood Vessel Protein

In some experiments, blood vessel subfractions were cleaved into peptides with cyanogen bromide (Adelstein and Kuehl 1970; Ozols 1991). Samples were suspended in 100 µl 70% formic acid containing 0.1M cyanogen bromide (Sigma) and reacted 18 hours under a nitrogen atmosphere. Solubilized material was collected following centrifugation at 13,000 x g for one minute and lyophilized as described below. Protein that was not solubilized by cyanogen bromide treatment was wash twice in 10 volumes of water and further digested with 20 µg of pepsin (Worthington Biochemical, 2x crystallized) in 100 µl of 0.5 M acetic acid for twelve hours at 37° C. The pepsin digested material and CNBr solubilized material was diluted to 1.0 ml H_20 and lyophilized two times using a centrifugal concentrator (Savant Speed-vac).

3.3.3 Amino Acid Analysis

Various artery and vein subfractions were hydrolyzed in 6M HCL at 110 ^{0}C for 24 hours under mild vacuum. Amino acid analysis using phenyl isothiocyanate (PITC) derivitization and isocratic reverse phase HPLC (Alltech C18 Econosphere, 5m, 4.6 X 250 mm) was used to quantify total amino acids in each protein fraction against a standard curve with detection at 254 nm (Green and Reagan 1992). The standard curve consisted of equal amounts of PITC derivitized glycine, alanine, proline and hydroxyproline. A single peak containing all of the derivitized amino acids was obtained with 60% acetonitrile (Burdick and Jackson, HPLC grade) at 1ml/min for 5.0 minutes. Specific amino acids (glycine, alanine, proline and hydroxyproline) were identified using the same column setup but gradient elution with a different solvent system. Solvent A: 10% methanol/90% H_2O containing 2.5 ml/L glacial acetic acid and 0.05ml/L acetone, pH 4.4. Solvent B: 90% methanol/10% H_2O containing 0.25 ml/L glacial acetic acid. Amino acids were eluted using a gradient of 5% B to 50% B over thirty minutes at a flow rate of 0.85 ml/min (Bhown et al. 1978; Harris et al. 1980). Specific peaks were identified using amino acid standards (Sigma).

3.3.4 Separation of Radioactive Peptides from [^{3}H-methyl]-AdoMet by Agarose Gel Electrophoresis

Since peptides are not quantitatively precipitated by TCA, an alternative means to separate excess [^{3}H-methyl]-AdoMet from methylated peptides was used. At the conclusion of radiometric assays with peptides, aliquots of reaction mixture were loaded onto 10% agarose gels (pH 2.4) and electrophoresed in a

horizontal mini-agarose gel apparatus (BRL, Gaithersburg, Md) for 30 minutes at 100 V. The excess [^{3}H-methyl]-AdoMet comigrates with the cathode dye, while the radiolabeled peptides are distributed between two dye fronts, with the majority of radiolabeled peptide migrating only one to two centimeters beyond the gel wells. Half centimeter gel slices of each lane were made and the radioactivity quantified as previously described (Weber and McFadden 1996). The total number of methyl esters present in each fraction was determined by taking the sum of the methyl esters present in each gel slice.

3.3.5 In vitro generation of altered aspartyl groups in blood vessel protein.

One centimeter vein segments were immersed in various buffers containing the following protease inhibitors: EDTA (1mM), aprotinin (2.5μg/ml), leupeptin (2.5μg/ml), pepstatin A (1μg/ml) and PMSF (100μg/ml). Sodium azide (0.02%) was added as a bacteriostatic agent. The solutions used were: 0.2M citrate; pH 6.0, PBS; pH 7.4, 0.2 M borate; pH 9.0, and NaOH, pH 12. Vein segments were incubated in these buffers for 24 hours at 37 °C. Vein segments were also incubated in PBS with protease inhibitors for 24 hours at the following temperatures: 4°, 25°, 37° and 50° C. Additionally, vein segments were incubated for 24, 72, and 168 hours at 37° C in PBS, pH 7.4 plus protease inhibitors. At the end of a given incubation, samples were frozen in liquid N_2 and stored at -20 °C. The samples were then homogenized in liquid N_2, using a mortar and pestle, and the detergent insoluble fraction (DI) obtained as described above. Any newly generated altered aspartyl groups in these samples

were detected by incubation with [^{3}H-methyl]-AdoMet and purified PIMT from rabbit erythrocytes.

3.4. Results

As previously reported, injured blood vessels release PIMT into the extracellular milieu. Subsequent administration of radiolabeled AdoMet, results in the methylation of blood vessel substrates by the released PIMT (Weber 1996). In order to characterize the quantity and distribution of PIMT substrates in the injured blood vessel a series of enzymatic and chemical dissections of the blood vessel wall were performed.

As a first approach, blood vessels were examined to determine if most of the altered aspartyl containing proteins are associated with endothelial cells (EC) or deeper sites with in the vessel wall. This was done by enzymatically stripping blood vessels of their endothelial lining. Examination of the released endothelial cells and the EC depleted blood vessels revealed that about 25% of the altered aspartyl proteins are associated with endothelial cells. The remaining altered aspartyl containing protein that is accessible by luminal perfusion, is located deeper within the vessel wall (Figure 3.2). Greater than 80% of the altered aspartyl containing protein that becomes methylated in EC depleted veins was found to be highly resistant to extraction by protein denaturants (Table 3.1).

Figure 3.2. Distribution of Proteins containing Altered Aspartyl Residues in Vein +/- Endothelium. Freshly isolated 1 cm segments of vein were perfused with bacterial collagenase IV for 20 minutes at 37° C in RPMI 1640, pH 7.4. This treatment has been shown to release at least 85% of endothelial cells, intact (Jaffe et al. 1973). The released cells were recovered by low speed centrifugation and washed twice with fresh cell culture medium. The cells were then incubated in cell culture medium containing purified rabbit PCM and ^{3}H AdoMet for 20 minutes at 37° C. The collagenase treated vein was washed twice to remove any remaining collagenase and then perfused with cell culture medium containing ^{3}H AdoMet for 20 minutes at 37° C. Error bars represent +/- s.e.m. of duplicate measures.

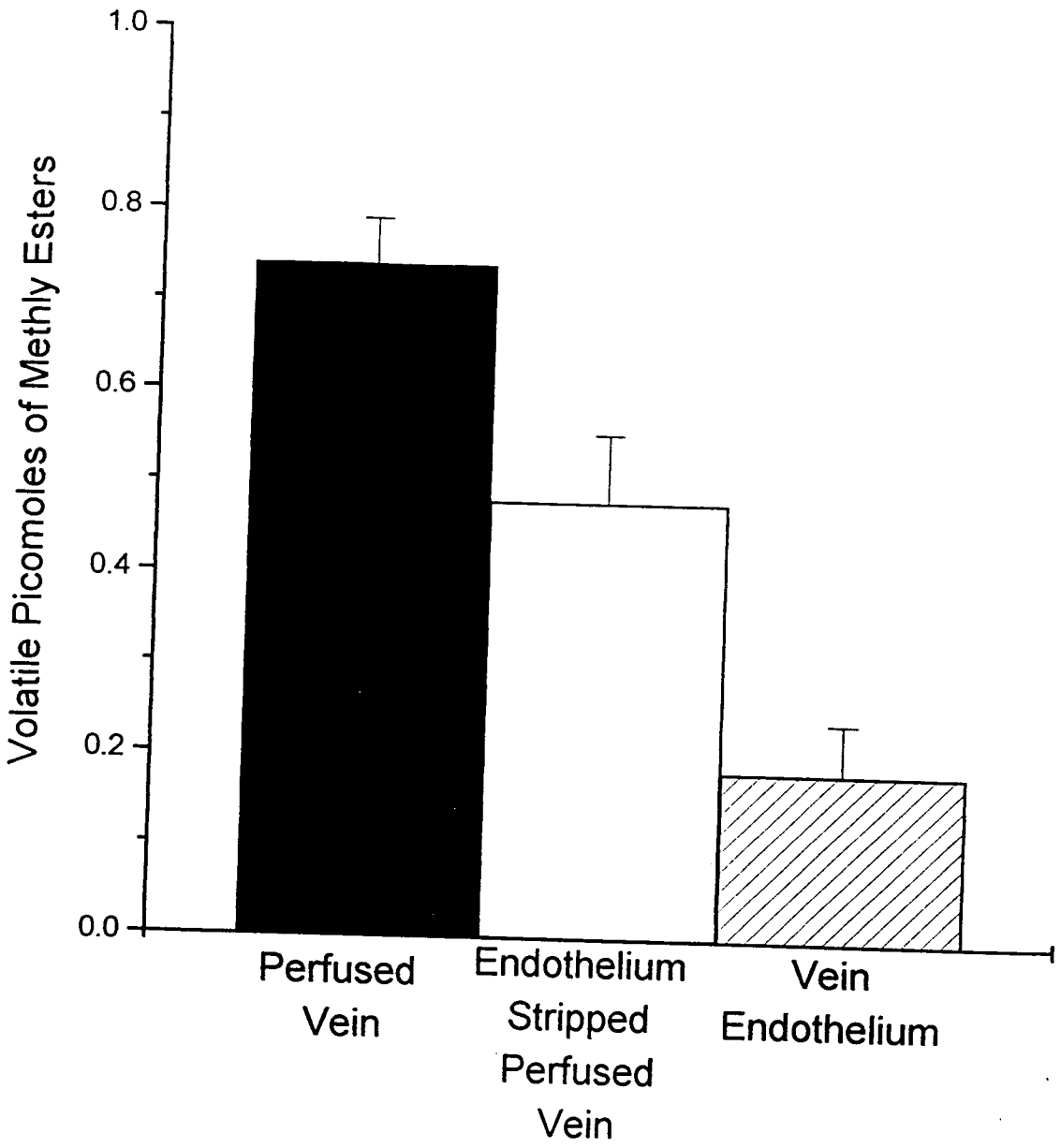
Volatile Picomoles of Methly Esters
1.0
0.8
0.6
0.4
0.2
0.0
Perfused Vein
Endothelium Stripped Perfused Vein
Vein Endothelium

Figure 3.2

Table 3.1. Ability of protein denaturants to extract methylated proteins from blood vessels. Following perfusion of vein segments as described under Experimental Procedures, unincorporated radiolabel was removed by washing the vessels with 10 volumes of 0.2M citrate, pH 6.0. The veins were then extracted in the listed solvents for 10 minutes at room temperature. The % of total extracted was determined by subtracting the mean of duplicate measures for each treatment from the mean of duplicate measures for and untreated vein.

Denaturant	% of Total Volatile Radioactivity
0.1% TX-100 / 1% SDS	15
0.1% TX-100 / 6M Urea	< 5
1% SDS / 3M Urea	< 12
80% Formic Acid	< 5
10% Methanol / 7% Acetic Acid	< 5
Chloroform:Methanol (2:1)	18

Since it is known that these highly insoluble proteins are arranged in a dense meshwork, it seemed likely that molecules of PIMT entrapped during blood vessel injury, would be diffusionally restricted and incapable of methylating all of the potential substrates within the extracellular milieu. This hypothesis was tested by chemically and physically disrupting the blood vessel architecture followed by addition of radiolabeled AdoMet to allow the released PIMT to methylate any substrates in the now disrupted meshwork. Non-ionic detergent extraction, or a cycle of freeze/thawing of veins led to a four fold increase in detectable methylation, while complete disruption of veins or arteries by homogenization in liquid N_2 resulted in a 10-12 fold increase in detectable protein methylation relative to untreated vein segments (Figure 3.3). Thus, the architecture of the blood vessel wall, plays a significant role in determining the extent of altered aspartyl containing proteins that are accessible to methylation by PIMT.

The above results indicated that the highly insoluble proteins of the blood vessel extracellular matrix, not only trap PIMT released during injury, but also may be the major substrate for the methyltransferase. Alternatively, during blood vessel injury, a wide variety of cellular proteins would be also expected to become entrapped within the extracellular meshwork of the blood vessel wall. These cellular proteins also could be major substrates for the methyltransferase yet appear insoluble and resistant to extraction since they are entrapped along with the methyltransferase. To measure if any particular category of protein

Figure 3.3. Physical disruptions of blood vessel architecture are necessary to expose the majority of isoaspartyl containing proteins. Immediately after isolation and washing, one centimeter vein segments were either extracted with 0.1% TX-100 in RPMI 1640 pH 7.4, for 20 minutes, frozen in liquid N_2 and thawed on ice, or frozen in liquid N_2 and ground to a fine powder with a mortar and pestle. Segments of thoracic aorta were also ground in a mortar and pestle as described for the vein. The detergent extracted vein was washed with cell culture medium twice and then perfused with [^{3}H-methyl]-adomet in RPMI 1640, pH 7.4 for 20 minutes at 37° C. The ground vein and artery were resuspended in RPMI 1640, pH 7.4, containing the same amount of ^{3}H-AdoMet and incubated as described for the detergent extracted vein. Error bars represent +/- s.e.m. of duplicate measures.

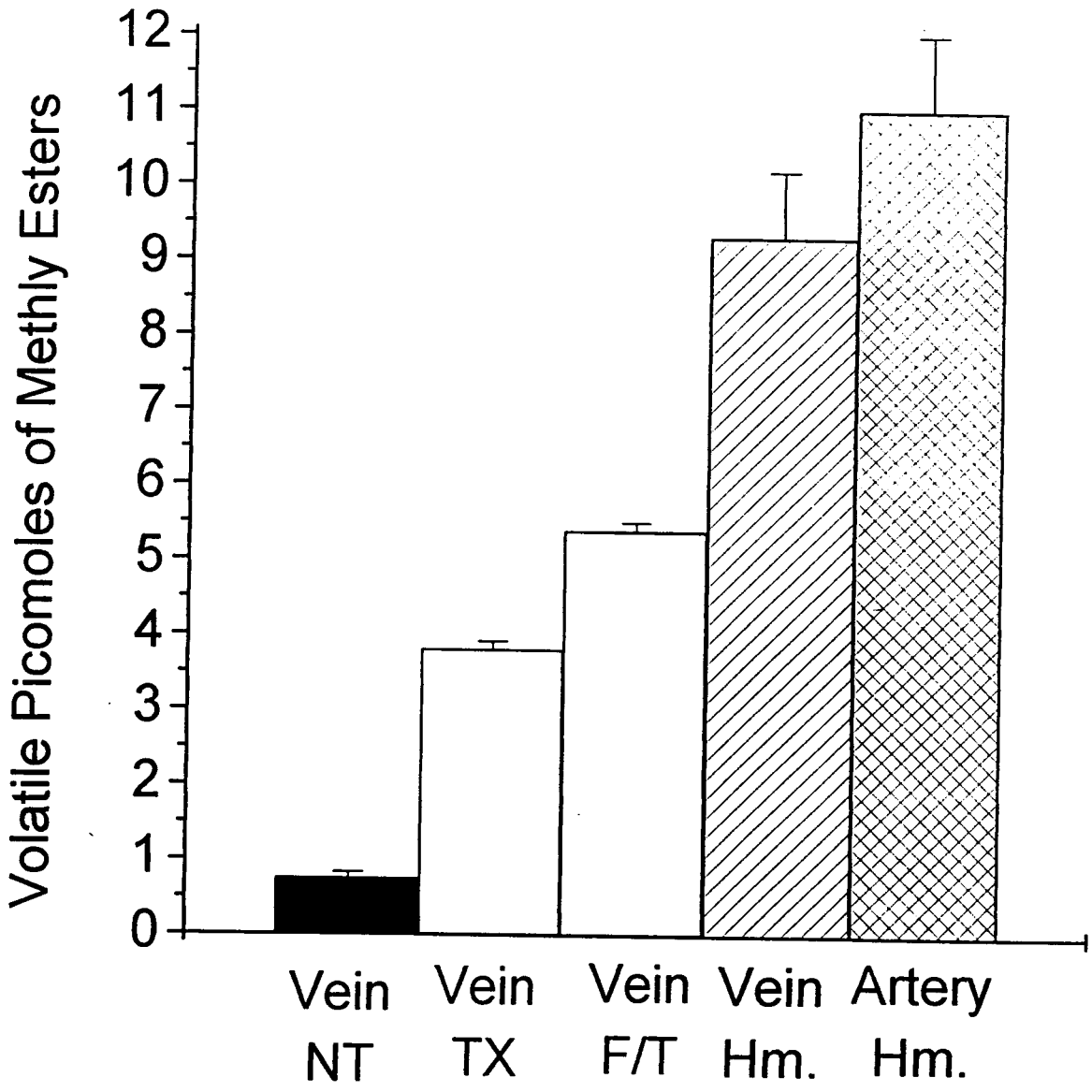
Volatile Picomoles of Methly Esters
12
11
10
9
8
7
6
5
4
3
2
1
0
Vein NT
Vein TX
Vein F/T
Vein Hm.
Artery Hm.

Figure 3.3

substrate present within an injured blood vessel is a major substrate for PIMT, radiolabeled AdoMet was added to vein homogenates and any proteins present were allowed to become methylated by the endogenous PIMT. Various protein fractions were recovered on the basis of solubility and examined for methyl ester content. Nearly equal distributions of methyl accepting proteins in the water soluble and detergent insoluble protein fractions was observed (Figure 3.4). Thus, substrates methylated by PIMT in an injured blood vessel are likely to be both cellular and extracellular in origin.

To quantify the amount of altered aspartyl containing proteins in blood vessel soluble and insoluble fractions, proteolytic cleavage methods were employed in order to completely solubilize proteins. This had several advantages, including elimination of any secondary or higher structures in the proteins, so that all altered aspartyl sites present are accessible to methylation by PIMT. Also, it ensured that only exogenously added PIMT would responsible for any observed methylation, since each blood vessel subfraction would be expected to contain variable amounts of the endogenous PIMT. On the basis of equimolar amounts of amino acids, the same concentration of peptides from each subfraction were assayed for altered aspartyl content using radiolabeled AdoMet and purified rabbit PIMT. Peptides derived from the detergent insoluble fraction of veins were found to be enriched four-fold in altered aspartyl residues, above the soluble vein proteins. For insoluble artery proteins, a greater than

Figure 3.4. Distribution of altered aspartyl containing proteins in vein subfractions. One centimeter segments of vein were subfractionated and subjected to the radiometric assay as described under Experimental Procedures. Bars show what percent of the total isoaspartyl containing protein in each fraction that is accessible to methylation by isoaspartyl methyltransferases.

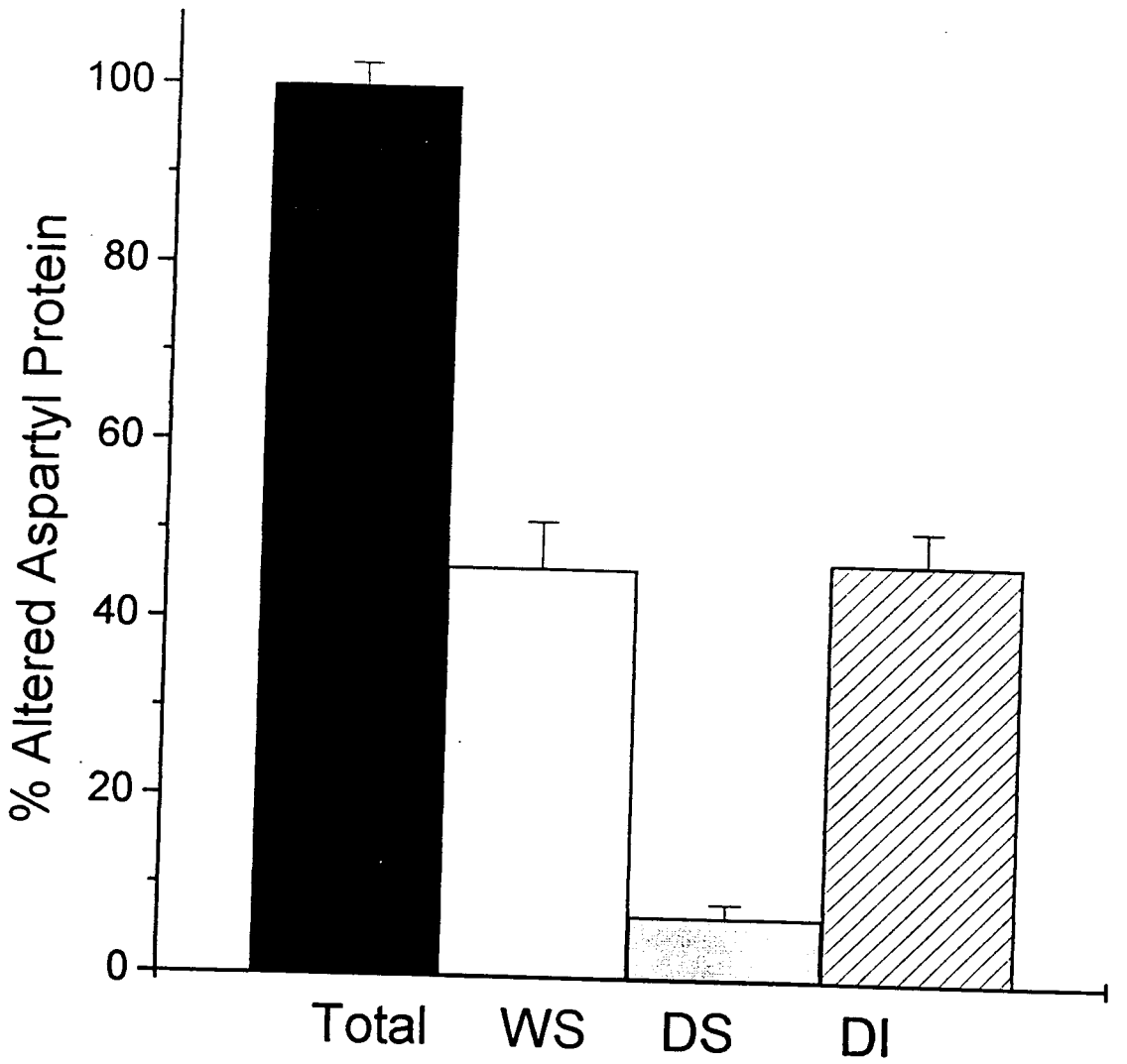

Figure 3.4

eight-fold enrichment in altered aspartyl containing proteins was found in this fraction as compared to artery soluble proteins (Figure 3.5).

By solubilizing the insoluble protein fraction of blood vessels as peptides, eliminating the endogenous PIMT activity present in the soluble protein fractions, and assaying equal amounts of peptides, it became clear that proteins in the extracellular matrix are the major altered aspartyl substrates in blood vessels.

Amino acid hydrolysis of this highly insoluble protein fraction, revealed that these proteins contain significant amounts of hydroxyproline, glycine and proline, suggesting that collagen may be one of the major methyl accepting proteins in this fraction (Figure 3.6).

To confirm that the results presented above did not arise during sample preparation, several control experiments were performed. First, since the formation of altered aspartyl residues within proteins is known to be affected by several well defined parameters, including time, temperature and alkaline pH (Brennan and Clarke 1995), veins segments were incubated for various times under each of these conditions. Only at extremes of time, temperature and alkaline pH could increases of methylatable substrates be detected (Figure 3.7). These conditions were much more extreme than those used during the experiments presented here. Thus, even though blood vessels injury can occur under mild handling procedures, blood vessel proteins are not similarly prone to altered aspartyl formation under the same conditions.

Figure 3.5. Methylation of equal amounts of peptides from subfractionated blood vessels. Peptides were generated by proteolytic digestion of vein subfractions as described under Experimental Procedures. Based upon amino acid analysis, one micro mole of amino acids in each peptide fraction were methylated for 60 minutes at 37° C in 0.2 M citrate pH 6.0 containing 15 mM ^{3}H-AdoMet (2.5Ci/mmol) and 1.2 units of rabbit erythrocyte PCM. The methylated peptides were then separated from free radiolabeled AdoMet by electrophoresis in 10% agarose gels. The bars represent the sum of the total number of volatile methyl esters present in each gel slice. Black bar equals the number of methyl esters contained in one micro mole of amino acids of the total protein fraction in a vein. Open bar represents methyl esters in one micro mole of amino acids from peptides in the water soluble subfraction. Striped bar shows the number of methyl esters in one micro mole of amino acids from peptides in the detergent insoluble fraction. Error bars depict +/- s.e.m of duplicate measures.

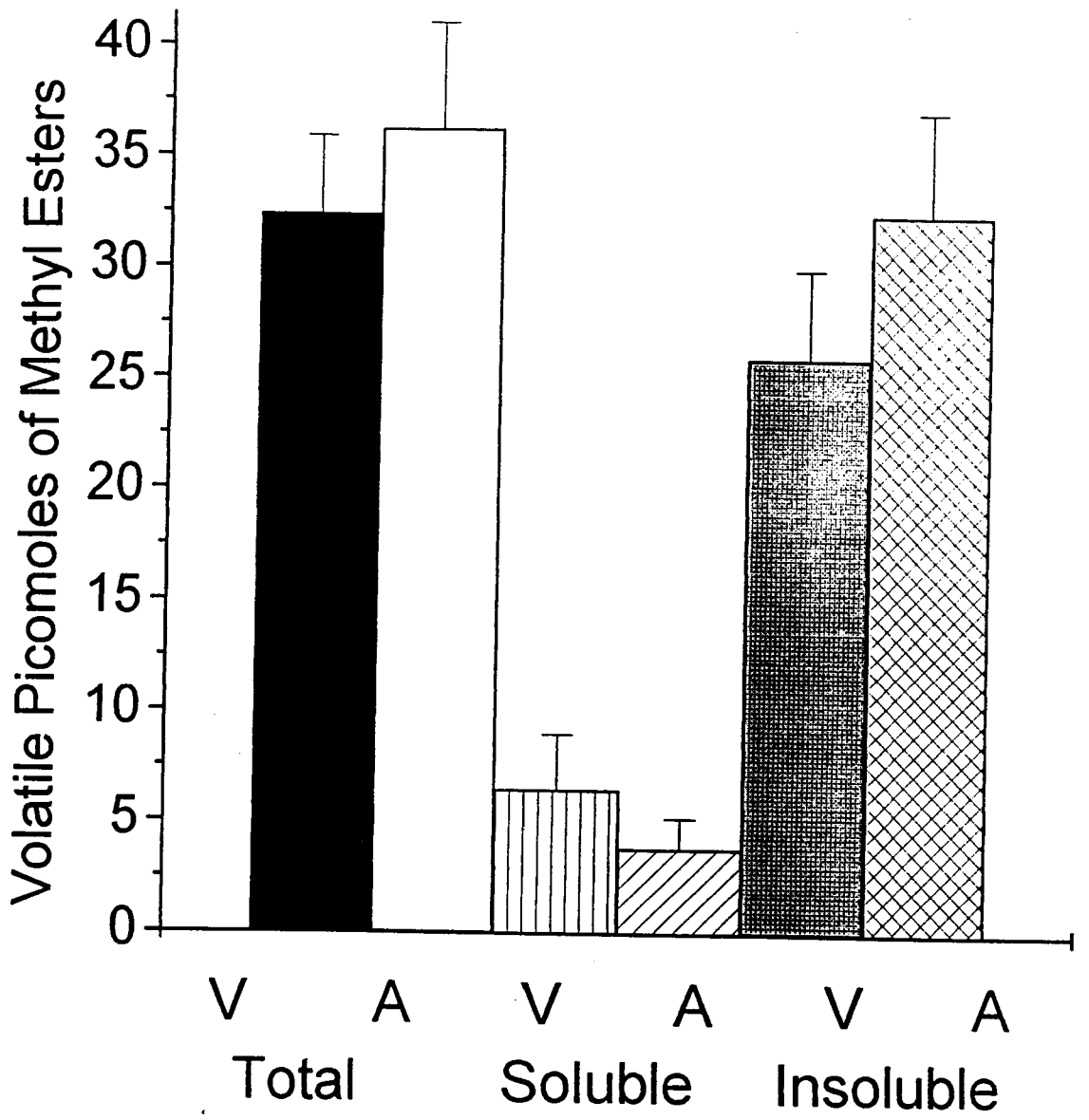
Volatile Picomoles of Methyl Esters
40
35
30
25
20
15
10
5
0
V
A
V
A
V
A
Total
Soluble
Insoluble

Figure 3.5

Figure 3.6. HPLC chromatogram of amino acids content of ECM from arteries and veins. Amino acid hydrolysis of the highly insoluble protein fraction of blood vessels (DI) and separation of PITC derivitized residues, reveals the presence of significant levels of hydroxyproline (Hypro), proline (pro) and glycine (gly), suggesting that collagen could be a major methyl accepting protein in this fraction.

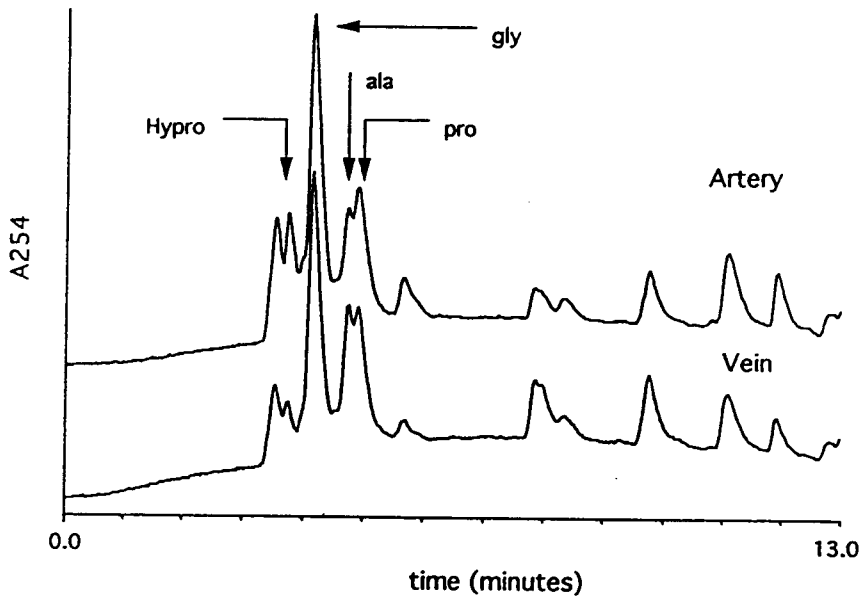

Figure 3.6

Figure 3.7. In vitro generation of isoaspartyl groups in blood vessel protein. One centimeter vein segments were incubated in buffers of various pH, or at several temperatures and times, in the presence of protease inhibitors, as described under Experimental Procedures. Open bars show picomoles of methyl esters in veins pre-incubated in buffers of various pH. Hatched bars show picomoles of methyl esters in veins pre-incubated at 37°C for various times. Solid bars show picomoles of methyl esters of veins pre-incubated in PBS pH 7.4, at various temperatures. The detergent insoluble subfraction was obtained as described and subjected to the radiometric assay using rabbit erythrocyte PCM and radiolabeled AdoMet in 0.2 M citrate, pH 6.0 at 37 °C for forty minutes. Error bars represent +/- s.e.m. of duplicate measures.

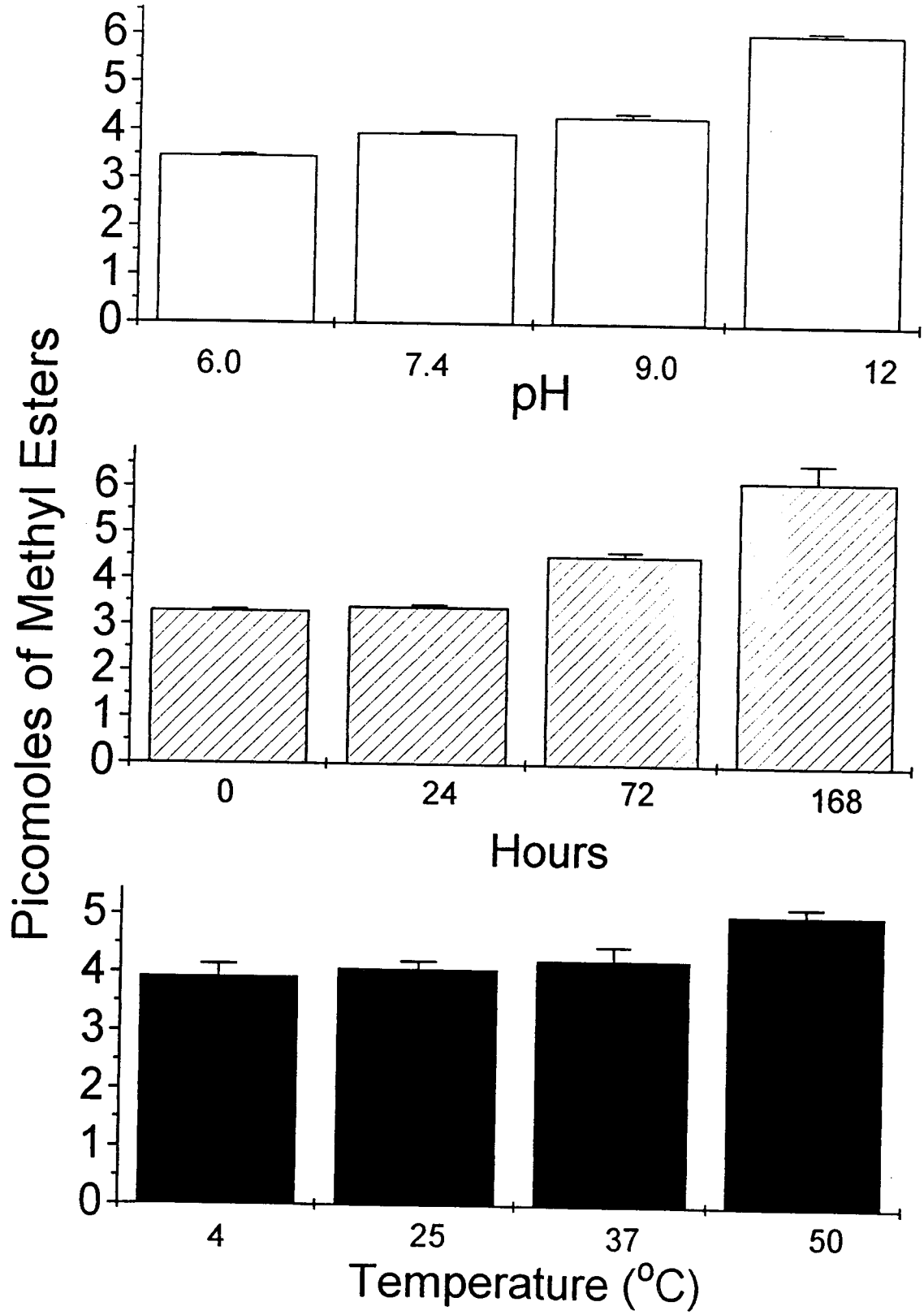
Picomoles of Methyl Esters
0
1
2
3
4
5
6
6.0
7.4
9.0
12
pH
0
24
72
168
Hours
0
1
2
3
4
5
4
25
37
50
Temperature (°C)

Figure 3.7

Second, the proteolytic conditions employed to generate peptides were examined to determine if they could cause the formation of altered aspartyl residues in the insoluble subfraction. To test this, bovine serum albumin (BSA) was exposed to the same proteolytic conditions as the blood vessel fractions. BSA, a naturally poor PIMT substrate, can become an excellent PIMT substrate under the appropriate conditions [McFadden, unpublished observation]. Increases in detectable groups were only seen after proteolytic digestion which would be expected due to the increased access of PIMT to sites that were inaccessible in the native protein (Figure 3.8). Thus, digestion of peptides under the conditions used, does not lead to the formation of altered aspartyl residues in peptides, but does allow altered residues that are already present but, inaccessible in the native proteins to become accessible to PIMT and subsequently detected following methylation.

3.5. Discussion

This study is the first description of the distribution of altered aspartyl residues in blood vessels. In an uninjured artery or vein, up to 90% of the altered aspartyl residues, are masked or otherwise inaccessible to endogenous PIMT under physiological conditions. This is based upon our previous work which demonstrated that PIMT in blood vessels is located intracellularly and the current work which found the majority of proteins containing altered aspartyl residues are located extracellularly.

Figure 3.8. Does proteolytic digestion cause formation of altered aspartyl residues. One milligram aliquots of bovine serum albumin were incubated with reagents (100μl) used to proteolytically cleave blood vessel proteins. **A**: negative control; 0.2 M Na^+ citrate buffer pH 6.0, overnight at 4° C. **B**: 0.5 M acetic acid, overnight at 4° C. **C**: 0.5 M acetic acid containing 20 μg pepsin, overnight at 4° C. **D**: 0.5 M acetic acid containing 20 μg pepsin and 5 μg of pepstatin A, overnight at 4° C. **E**: 70% formic acid, overnight at 37° C. **F**: 70% formic acid containing 0.1M CNBr, overnight at 37° . Following the incubations, each sample was brought up 1.0 ml with water and lyophilized. Subsequently, the samples were resuspended and subjected to a radiometric assay with purified PIMT and radiolabeled AdoMet. Methylated residues were quantified following agarose electrophoresis as described. Error bars represent s.e.m. of duplicate measures.

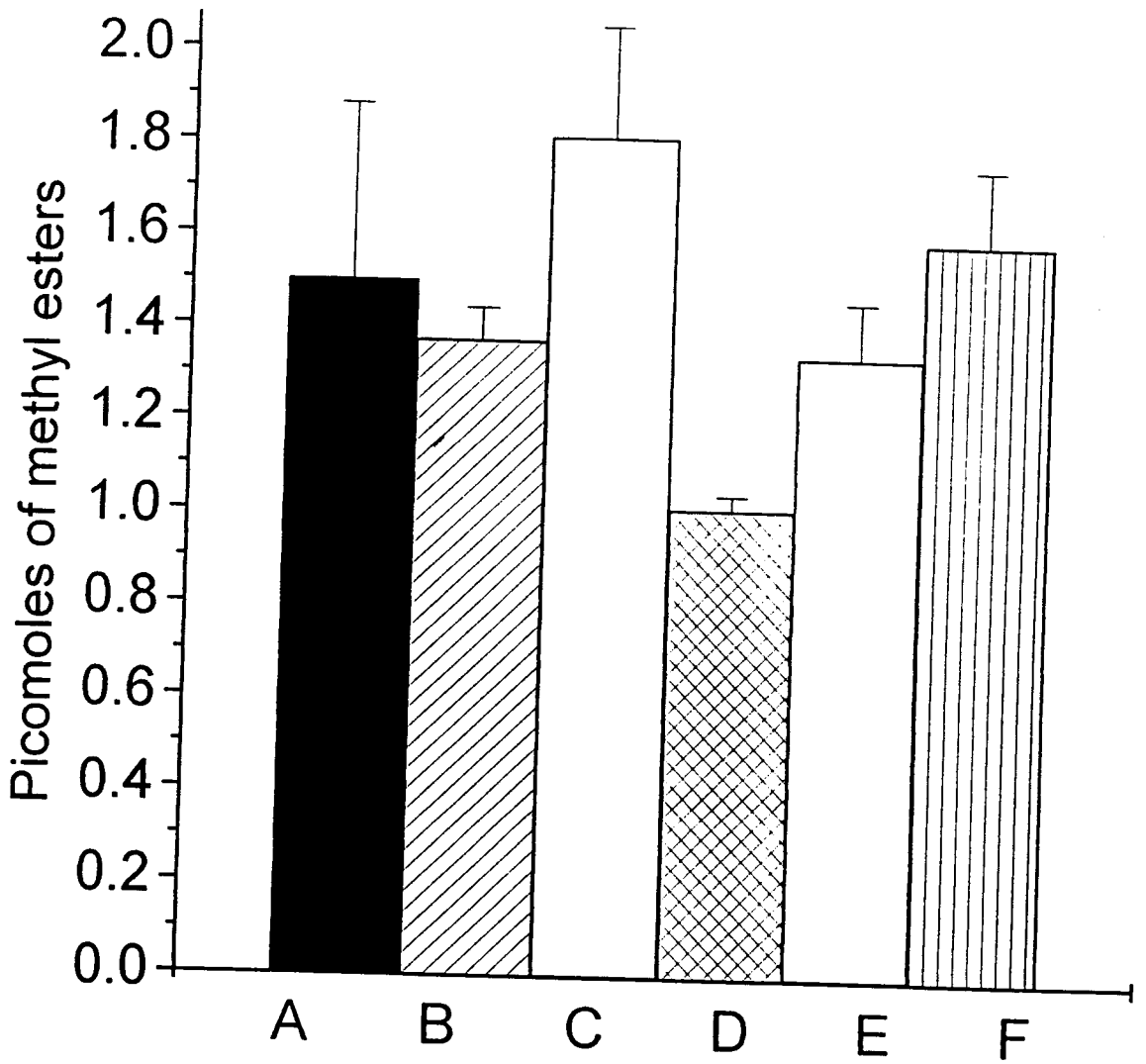
Picomoles of methyl esters
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
A
B
C
D
E
F

Figure 3.8

Progressively, increasing the extent of blood vessel injury, led to increasingly greater amounts of detectable altered aspartyl containing proteins, reflecting greater access by PIMT to altered aspartyl containing substrates. However, even in completely homogenized arteries or veins, only about 30% of the total amount of altered aspartyl residues present in blood vessels is accessible to methylation by endogenous PIMT. The remainder only becomes accessible and therefore detectable, following proteolytic cleavage. Using proteolytic cleavage and examining equal amounts of peptides from various blood vessel subfractions, it is clear that the highly insoluble proteins of the extracellular matrix are major repositories of potential PIMT substrates in this tissue. The significant amount of hydroxyproline present in this fraction suggests that collagen, an abundant and highly insoluble protein, is likely to be at least one of the PIMT substrates in the ECM.

Very little is known about the extent of altered aspartyl formation in complex tissues. Total amino acid analysis of the highly insoluble blood vessel protein fraction, coupled with our data from the methylation of peptides from this same fraction enabled us to calculate that 1.2% to 5.4% of the proteins in this fraction contain one or more altered aspartyl residues, assuming an average molecular weight of 50,000 - 200,000 Daltons, respectively for the proteins in this fraction. Our calculations correlate well with studies which have shown that in a wide variety soluble proteins, the formation of altered aspartyl residues is

sub-stoichiometric, typically ranging from 0.1% to less than 5.0% in a given protein population (Johnson and Aswad 1990; Chazin and Kossiakoff 1995).

The consequences of altered aspartyl accumulation in the largely structural proteins of the extracellular matrix is currently unclear. However, there are several well documented instances with enzymes, where these alterations have led to diminished protein function (Venkatesh and Vithayathil 1984; DiAugustine et al. 1987; Johnson et al. 1987; George-Nascimento et al. 1988; Friedman et al. 1991; Sun et al. 1992). In tissues such as blood vessels, proteins of the extracellular matrix, including collagen, play important structural roles and contain peptide sequences important for cell binding. Aspartyl residues are a key feature of these cell binding sequences (Yamada 1991; Humphries 1993; Yurchenco and O'Rear 1993; Service 1995). It is likely that an accumulation of altered aspartyl residues in these cell binding sequences, would impact this protein-protein organization. By our calculations, one centimeter of blood vessel contains on the order of $5x\ 10^{14}$ altered aspartyl residues in the highly insoluble protein fraction.

Our previous work found that even under gentle isolation procedures, up to 10% of the cells in blood vessels are compromised, as evidenced by detection of LDH leakage. Additionally, it was shown that a significant amount of endogenous PIMT from compromised cells can become trapped within the ECM. Thus, PIMT that is released by blood vessel injury and trapped within the

Thus, PIMT that is released by blood vessel injury and trapped within the extracellular matrix, is poised to utilize circulating AdoMet to methylate altered aspartyl containing extracellular proteins.

Acknowledgments

We thank Bill Amberg of OSU lab animal resources for help with obtaining the rats used in this study.

3.6. References

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Chapter 4

Injury-induced Enzymatic Methylation of Aging Collagen in the Extracellular Matrix of Blood Vessels

Darin J. Weber and Philip N. McFadden

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4.1. Abstract

As a result of blood vessel injury, intracellular enzymes such as protein D-aspartyl/L-isoaspartyl carboxyl methyltransferase (PIMT) become trapped within the meshwork of the vascular extracellular matrix. Previous work in this lab established that the extracellular matrix (ECM), is a major repository of altered aspartyl residues in both arteries and veins (Weber 1996). Digestion of these extracellular matrix proteins with cyanogen bromide (CNBr), followed by methylation with (PIMT), found that about 60% of the altered aspartyl residues in the ECM are solubilized by this treatment. The presence of hydroxyproline in amino acid hydrosolates of this fraction and acidic pH gel electrophoresis of methylated peptides, allowed the identification of collagen as the major PIMT substrate in the CNBr-soluble material. CNBr peptides derived from both type I and type III collagen were found to methylated. Chemical injury to blood vessel lumens with the non-ionic detergent Triton X-100, followed by luminal administration of radiolabeled S-adenosyl-methionine (AdoMet), further confirmed that collagen is a major substrate for the endogenous methyltransferase.

4.2. Introduction

In previous reports from this lab, it has been shown that blood vessel injury results in the release of intracellular enzymes such as PIMT and LDH into the intracellular spaces where they become entrapped. In vitro it was demonstrated that small molecules such as AdoMet and NADH are able to penetrate the extracellular meshwork from the lumen and subsequently be

utilized by enzymes trapped there. This finding may have physiologically relevant consequences, since it is well known that blood vessel injury damages the integrity of the plasma membranes of both endothelial and smooth muscle cells (Hawkins et al. 1988; Fingerle et al. 1989; Majesky et al. 1990; Lindner and Reidy 1991; Douek et al. 1992; Hassenstein et al. 1992; Lindner et al. 1992; Aliev et al. 1993; Hanke et al. 1994). PIMT appears to represent a novel example in that it's substrate specificity extends to the proteins of the extracellular matrix itself. Thus, in vivo administration of radiolabeled AdoMet into injured blood vessels enabled us to show that methylation of substrates occurs only at the site of injury. Partial characterization of these substrates found that they are extremely resistant to extraction by strong protein denaturants and likely to consist of proteins localized in the extracellular spaces. This report focuses on identifying the major PIMT substrates of the extracellular matrix, as they also represent the largest population of altered aspartyl proteins in blood vessels.

4.3. Materials and Methods

Cyanogen bromide soluble and insoluble fractions were obtained from rat blood vessels and the insoluble fraction subsequently solubilized by pepsin digestion as previously described in the preceding manuscript (Weber 1996).

4.3.1 Amino Acid Analysis

Phenylisothiocyanate (PITC) pre-column derivitization was used in all amino acid analyses (Furst et al. 1990; Green and Reagan 1992) . Total amino acids were quantified as described in the preceding manuscript. Content of alanine, hydroxyproline, glycine and proline in each sample was determined using standard curves following gradient reversed phase HPLC (Alltech C_{18} Econosphere, 5μ, 4.6 mm X 250 mm). Solvent A: 10% methanol/90% H_2O containing 2.5 ml/L glacial acetic acid and 0.05ml/L acetone, pH 4.4. Solvent B: 90% methanol/10% H_2O containing 0.25 ml/L glacial acetic acid. Amino acids were eluted using a gradient of 5% B to 50% B over thirty minutes at a flow rate of 0.85 ml/min with detection at 254 nm (Bhown et al. 1978; Harris et al. 1980). Since aspartic acid and glycine co-elute in this solvent system, a different solvent system, using the same column setup was employed in order to determine the content of aspartic acid and asparagine in each sample (Yang and Sepulveda 1985). Solvent A: 0.14 M sodium acetate/ 0.05% triethylamine/ 6% acetonitrile, pH 6.35. Solvent B: 60% acetonitrile. Gradient elution was employed using 0% B to 10% B over ten minutes at a flow rate of 1.0 ml/min. Standard curves of aspartic acid and asparagine were used for quantification.

4.3.2 Methylation of Peptides by PIMT and [^{3}H-methyl]-AdoMet

Equal quantities (based upon amino acid content) of CNBr-soluble and insoluble peptides from arteries and veins were assayed for their content of altered aspartyl residues by methylation using 2.0 units of protein D-aspartyl/L-isoaspartyl carboxyl methyltransferase (PIMT) (EC 2.1.1.77) purified

from rabbit erythrocytes (Gilbert et al. 1988) and 15 μM of S-adenosyl-L-[^{3}H-*methyl*]-methionine (AdoMet) (New England Nuclear) at a specific radioactivity of 2.5 Ci/mmol prepared in 0.2 M Na^+ citrate pH 6.0. Each altered aspartyl assay (50 μl) was carried out at 37 °C for 40 minutes. Reactions were terminated by addition of an equal volume of 0.13% TFA in water. Samples were stored on ice until HPLC injection.

4.3.3 HPLC of Peptides Radiolabeled by PIMT and [^{3}H-methyl]-AdoMet

Radiolabled peptides were separated by multi-step gradient reversed phase HPLC (Vydac protein C_4 , 4.6 mm X 250 mm) at a flow rate of 1.0 ml/minute and detection at 214 nm. Solvent A: 0.13% triflouroacetic acid (TFA) /H_2O. Solvent B: 0.1% TFA/90% acetonitrile. Resolution of peptides was achieved using the following solvent program: (time(minutes), %B); 0,1; 1, 20; 5, 25; 7.5, 31.6; 12, 34.3; 15, 35; 16, 80. Twenty 1.0 ml fractions were collected from each run. ^{3}H-methyl esterified peptides were detected as a volatile product (^{3}H-methanol) following base hydrolysis in 0.1 M sodium hydroxide.

4.3.4 Acidic pH Electrophoresis of Radiolabeled Peptides

Equal quantities (based on total amino acid content) of CNBr-soluble and insoluble peptides were assayed for altered aspartyl groups as described above. Reactions were terminated by mixing with an equal volume of 2X pH 1.4 gel loading buffer and stored at -20 °C until needed. Peptides were separated by polyacrylamide gel electrophoresis on the basis of size using a 4% (pH 1.4)

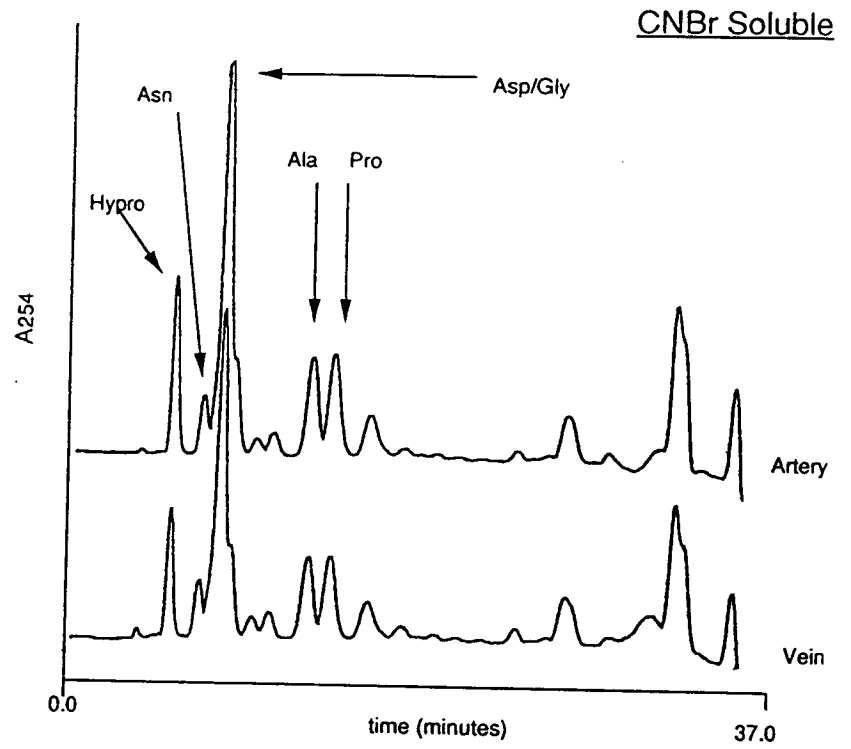
stacking gel and a 12% (pH 2.4) resolving gel, in a water- cooled electrophoresis apparatus (Owl Scientific). Gels were prepared and radiolabeled peptides quantified as described (Weber and McFadden 1996). In some cases, methylated peptides were separated from excess radiolabeled AdoMet in pH 2.4 10% agarose gels as previously described (Weber and McFadden 1996).

4.4 Results

As we previously reported, significant amounts of the amino acid hydroxyproline, were detected in the highly methylated and highly insoluble protein fraction of blood vessels (Weber 1996). This modified amino acid is reportedly found only in collagen. The experiments presented here were designed to determine if collagen is indeed a major PIMT substrate in the extracellular matrix.

Proteolytic cleavage methods followed by amino acid analysis indicated that the majority of hydroxyproline containing protein can be selectively solubilized with cyanogen bromide (CNBr) (Figure 4.1). Additionally, this fraction also contains detectable levels of aspartyl and asparaginyl residues. The CNBr-insoluble protein fraction was found to be relatively devoid of hydroxyproline and contains diminished levels of aspartyl and asparaginyl amino acids (Table 4.1). This result strongly suggested that the CNBr-soluble fraction contained collagen peptides and that this fraction would also likely contain most of the substrates for PIMT since the majority of aspartyl and asparaginyl

Figure 4.1. Distribution of hydroxyproline in CNBr-soluble and insoluble ECM proteins. Following subfraction of blood vessels, the highly insoluble present were digested with CNBr as described under Experimental Procedures. PITC derivitized amino acid hydrosolates were then resolved by HPLC. Specific amino acids were identified based upon the elution times of amino acid standards.


CNBr Soluble
Asp/Gly
Asn
Ala
Pro
Hypro
A254
Artery
Vein
0.0
time (minutes)
37.0

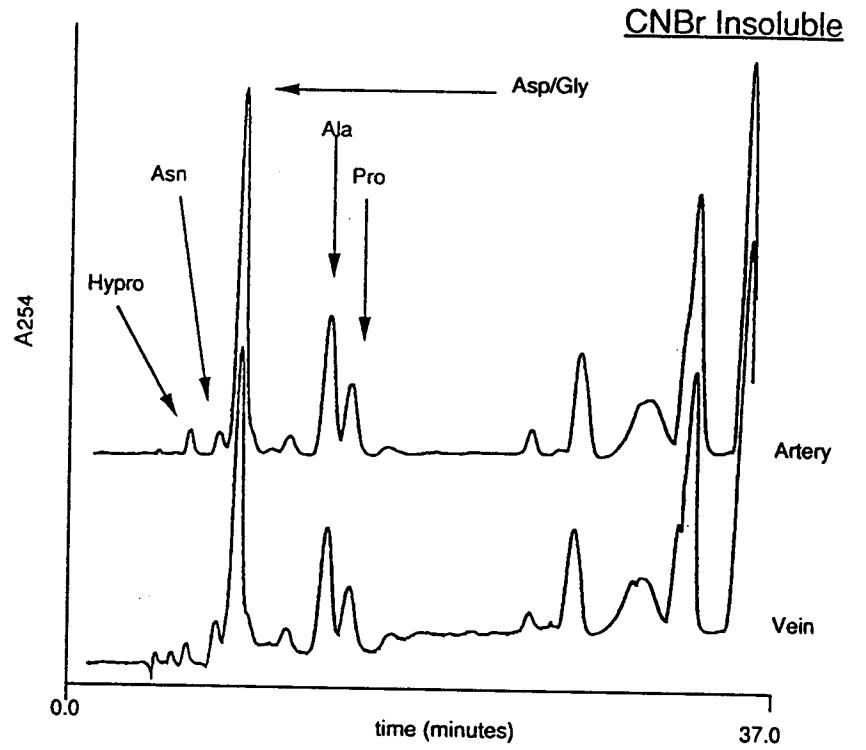

CNBr Insoluble
Asp/Gly
Ala
Asn
Pro
Hypro
A254
Artery
Vein
0.0
time (minutes)
37.0

Figure 4.1

Table 4.1. Mole % of Selected Amino Acids in CNBr-soluble and Insoluble Fractions. Amino acid hydrosolates of each fraction were separated by HPLC and specific amino acids identified based upon the elution times of amino acid standards and standard curves of each amino acid of interest. Peak areas were used to calculate the moles of each amino acid in each fraction, relative to the total number of moles of all amino acids present in the fraction. Error bars represent s.e.m. of duplicate separations.

	Artery Mole %		Vein Mole %	
Amino Acid	Soluble	Insoluble	Soluble	Insoluble
HyPro	8.8 (1.8)	2.1 (.47)	8.2 (.91)	1.7 (.53)
Asp	4.5 (.87)	1.9 (.32)	4.8 (1.6)	2.3 (.37)
Asn	4.3 (1.4)	2.2 (.58)	4.2 (.62)	2.1 (.44)

residues are located there. Further evidence for this hypothesis was provided by methylating equal amounts of peptides in each fraction with purified rabbit PIMT and radiolabeled AdoMet. The methylated CNBr-soluble and CNBr-insoluble were then resolved on acidic pH polyacrylamide gels. Surprisingly, no peptide bands were observed after staining with Coomasie in the CNBr-insoluble fraction (data not shown). This suggests that only very small peptides are present in this fraction after pepsin digestion. In contrast, very distinct peptide bands were observed in the CNBr-soluble fraction (Figure 4.2). Cyanogen bromide digests of commercially obtained collagen standards were used to positively identify the methylated peptides as fragments of collagen (Bornstein and Sage 1980). To be certain that the observed methylation associated with the collagen CNBr peptides was not due to co-migration of unknown peptides of similar molecular weight, a different separation technique was employed. Methylated CNBr-soluble and CNBr-insoluble fragments were separated by reverse phase HPLC and column fractions assayed for methyl esterified peptides (Figure 4.3). This method also enabled the total amount of altered aspartyl containing peptides in each fraction to be determined. In arteries, nearly 70% of PIMT substrates in the extracellular matrix are CNBr-soluble with the remaining 30% being CNBr-insoluble. For veins the relative proportions are 66% soluble and 34% insoluble (Table 4.2). This finding confirmed that peptides in the CNBr-soluble fraction are major PIMT substrates. It also revealed that the peptide content of the CNBr-insoluble fraction is markedly different from the

Figure 4.2. Electrophoretic identification of methylated CNBr peptides. Following methylation of CNBr-soluble peptides as described above, aliquots were resolved on acidic pH polyacrylamide gels. Coomasie stained bands were excised and analyzed for content of methyl esters as described. Histograms reflect the amount of picomoles of methyl esters found in each band. Bands corresponding to various collagen types were identified following cyanogen bromide digestion of commercially prepared collagen standards (Sigma). Greek letters refer to monomer chains of collagen. Subscript indicates which monomer chain. Roman numerals in parenthesis identify the type of collagen species. CB refers to cyanogen bromide fragment. The numbers identifying the cyanogen bromide fragments are used for historical reasons.

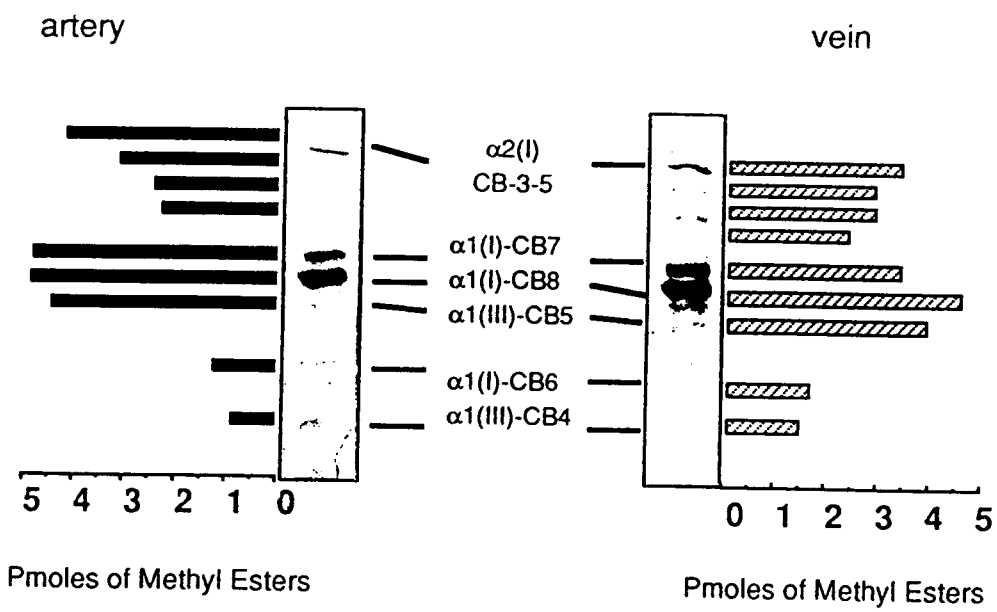
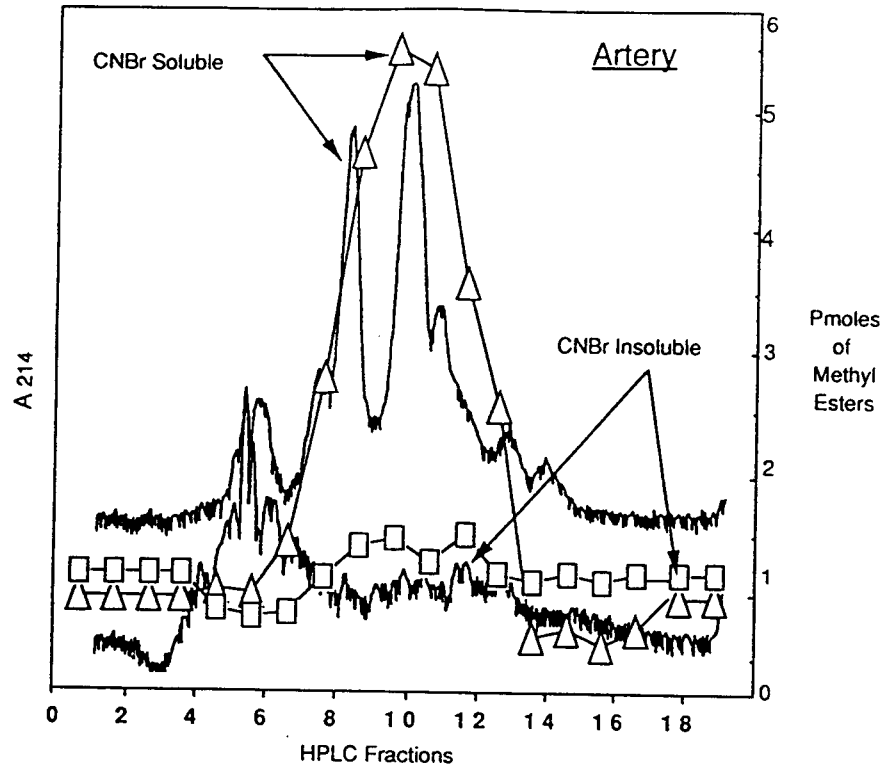

artery
vein
α2(I)
CB-3-5
α1(I)-CB7
α1(I)-CB8
α1(III)-CB5
α1(I)-CB6
α1(III)-CB4
5 4 3 2 1 0
0 1 2 3 4 5
Pmoles of Methyl Esters
Pmoles of Methyl Esters

Figure 4.2

Figure 4.3. Analysis of Methylated CNBr-soluble and insoluble peptides by HPLC. Equal amounts of peptides from the CNBr-soluble and CNBr-insoluble fraction were methylated by purified PIMT and radiolabeled AdoMet as described under Experimental Procedures. At the conclusion of the incubation period, the reaction mixtures were injected onto the HPLC. Nineteen 1.0 ml fractions were collected and assayed for the presence of methyl esterified peptides. Upper panel: HPLC chromatograms of artery CNBr-soluble and CNBr-insoluble peptides. Open triangles show the picomoles of methyl esters in each fraction of CNBr-soluble peptides and the open squares show the same for CNBr-insoluble peptides. Lower Panel: HPLC chromatograms of vein CNBr-soluble and insoluble peptides. Open squares show the picomoles of methyl esters in each fraction of CNBr-soluble peptides and open circles the same for CNBr-insoluble peptides.


CNBr Soluble
Artery
CNBr Insoluble
A 214
Pmoles
of
Methyl
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0
1
2
3
4
5
6
0 2 4 6 8 10 12 14 16 18
HPLC Fractions

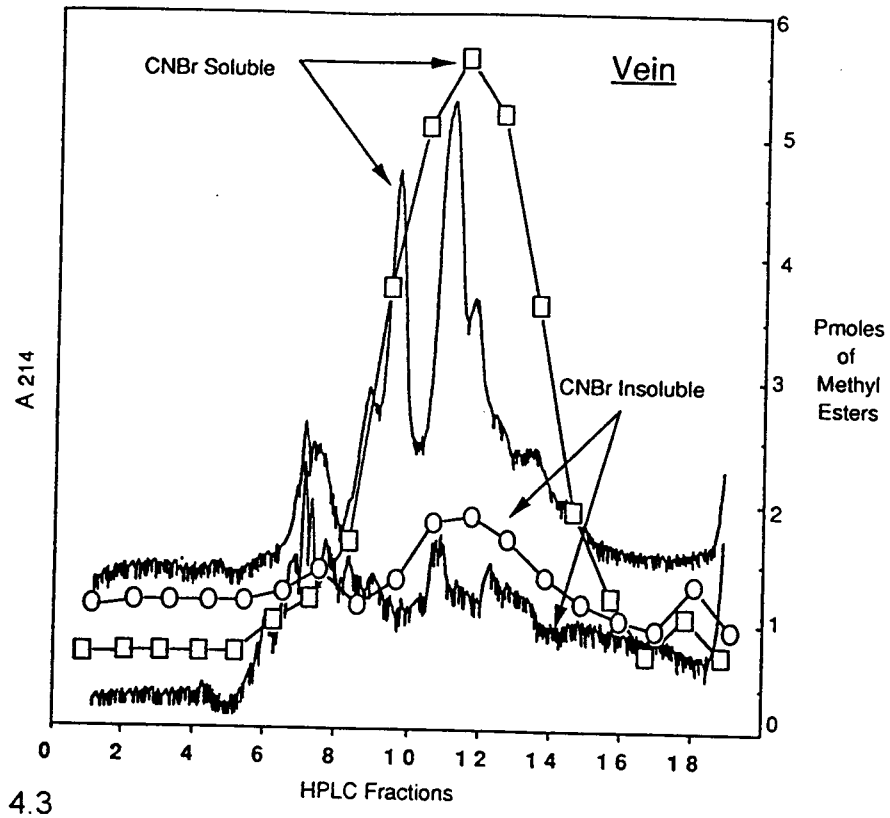
CNBr Soluble
Vein
CNBr Insoluble
A 214
Pmoles
of
Methyl
Esters
0
1
2
3
4
5
6
0 2 4 6 8 10 12 14 16 18
HPLC Fractions

Figure 4.3

Table 4.2. Picomoles of Methyl Esters in CNBr-soluble and Insoluble Peptides. Equal amounts of peptides from the CNBr-soluble and CNBr-insoluble fractions were methylated with PIMT and radiolabeled AdoMet as described above. Methylated peptides were separated from excess free [^{3}H-methyl]-AdoMet by electrophoresis in 10% agarose gels. Gels slices were excised and content of methyl esters present in each fraction determined as described under Experimental Procedures. Numbers in parenthesis represent s.e.m. of duplicate methylations.

Fraction	Artery	Vein
Total	33.37 (2.16)	35.19 (2.63)
CNBr-soluble	23.02 (1.37)	23.22 (1.72)
CNBr-insoluble	10.35 (.62)	11.95 (.47)

CNBr-soluble fraction. Separating methylated CNBr peptides by HPLC first followed by acidic pH gel electrophoresis, strongly supports the hypothesis that collagen is the dominant PIMT substrate in the CNBr-soluble fraction (Figure 4.4). The pattern of methylation of peptides in the gel (Figure 4.2), indicates that altered aspartyl residues are distributed throughout the polypeptide sequence of collagen, rather than being localized to any specific fragment (Table 4.3).

It is clear that upon blood vessel subfractionation and proteolytic cleavage of the insoluble fraction that collagen acts as a major substrate for PIMT. However, an important question to be addressed is whether as a result of blood vessel injury, does PIMT that is trapped in the ECM, methylate collagen. To test this hypothesis, blood vessels were chemically injured by washing the lumens of vein segments with non-ionic detergent, followed by perfusion with radiolabeled AdoMet. The veins were then homogenized and all the protein present subjected to digestion by cyanogen bromide. The solubilized proteins were then separated by acidic pH gel electrophoresis (Figure 4.5). As can be seen, the endogenous PIMT, released as a result of chemical injury, is able to methylate proteins that when fragmented by CNBr, exhibit a mobility identical to collagen peptides. This evidence strongly suggests that upon blood vessel injury, that collagen is a substrate for the endogenous PIMT along with other yet to be identified fragments, which migrate differently than the major collagen fragments.

Figure 4.4 Two-dimensional separation of methylated CNBr-soluble peptides. CNBr-soluble peptides from veins were methylated exactly as described for figure 4.3. Methylated peptides were then separated by RP-HPLC and 1.0 ml fractions collected (1st dimension = hydrophobicity). Fractions nine to fourteen, which encompassed the major peak of radioactivity were lyophilized. Subsequently, each fraction was resuspended in pH 1.4 gel loading buffer and separated by acidic pH gel electrophoresis as described for figure 4.2 (2nd dimension = size). HPLC fractions corresponding to each gel lane are shown at top of figure and the gel bands excised shown to right of figure. Coomassie stainable bands, previously identified as CNBr peptides of collagen were excised and content of methyl esters determined. Gel bands are identified as follows: (letter, collagen CNBr fragment), a, α_2(I)-CB-5; b, α_1(I)-CB-7; c, α_1(I)-CB-8; d, α_1(III)-CB-5; e, α_1(I)-CB-6; f, α_1(III)-CB-4. For quantification of methyl esters in each fragment, see Table 4.3.

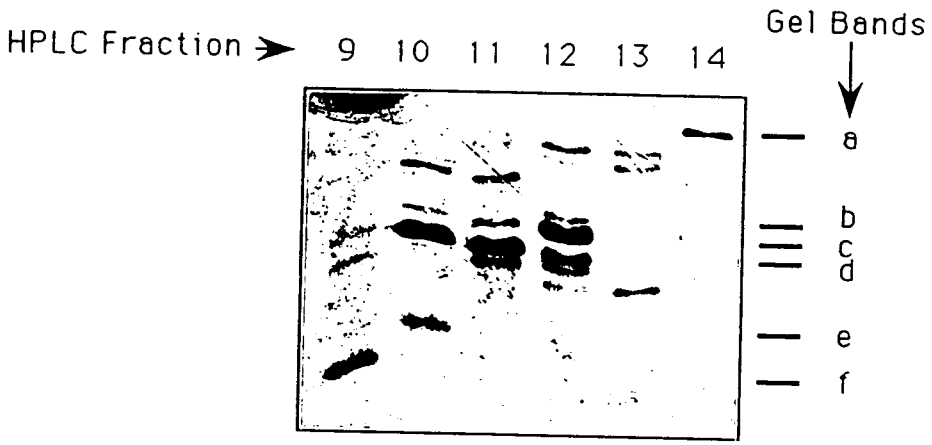

Figure 4.4

Table 4.3 Picomoles of methyl esters in CNBr fragments of collagen. Following a two dimensional separation of methylated CNBr-soluble peptides as described in figure 4.4, coomassie staining bands, previously identified as CNBr fragments of collagen were excised and methyl ester content determined. The picomoles of methyl esters for specific collagen CNBr fragments was obtained by summing the picomoles each identifiable CNBr fragment excised from each HPLC fraction. Percent of total picomoles refers to the total number of picomoles of methylated peptide in all HPLC fractions of the CNBr-soluble resolved material.

Band	Collagen CNBr Frag.	pmoles	% of Total
a	α_2(I)-CB 3-5	2.2	10
b	α_1(I)-CB-7	3.6	16
c	α_1(I)-CB-8	4.4	19
d	α_1(III)-CB-5	2.7	12
e	α_1(I)CB-6	1.4	6.0
f	α_1(III)-CB-4	1.3	5.7
Sum =		15.6	68 %

Figure 4.5. Following blood vessel injury, collagen becomes methylated by endogenous PIMT. The lumens of one centimeter vein segments were treated with 1% TX-100 and then perfused with radiolabeled AdoMet. Segments were the homogenized and all the protein present subjected to digestion with cyanogen bromide. Solubilized protein was then separated by acidic pH gel electrophoresis and stained with coomassie blue. One half centimeter gel slices were then made of the entire sample lane and assayed for methyl ester content as described above. For comparison, a vein segment was homogenized and then subjected to methylation by addition of radiolabeled AdoMet. Upper graph shows methyl esters in each gel slices for chemically injured blood vessels. Lower graph show methyl ester profile in gel slices of veins homogenized and then methylated. Note difference in scales between the two graphs. Error bars show the s.e.m. of duplicate samples run on the same gel. For simplicity, only a single sample lane for each treatment is shown.

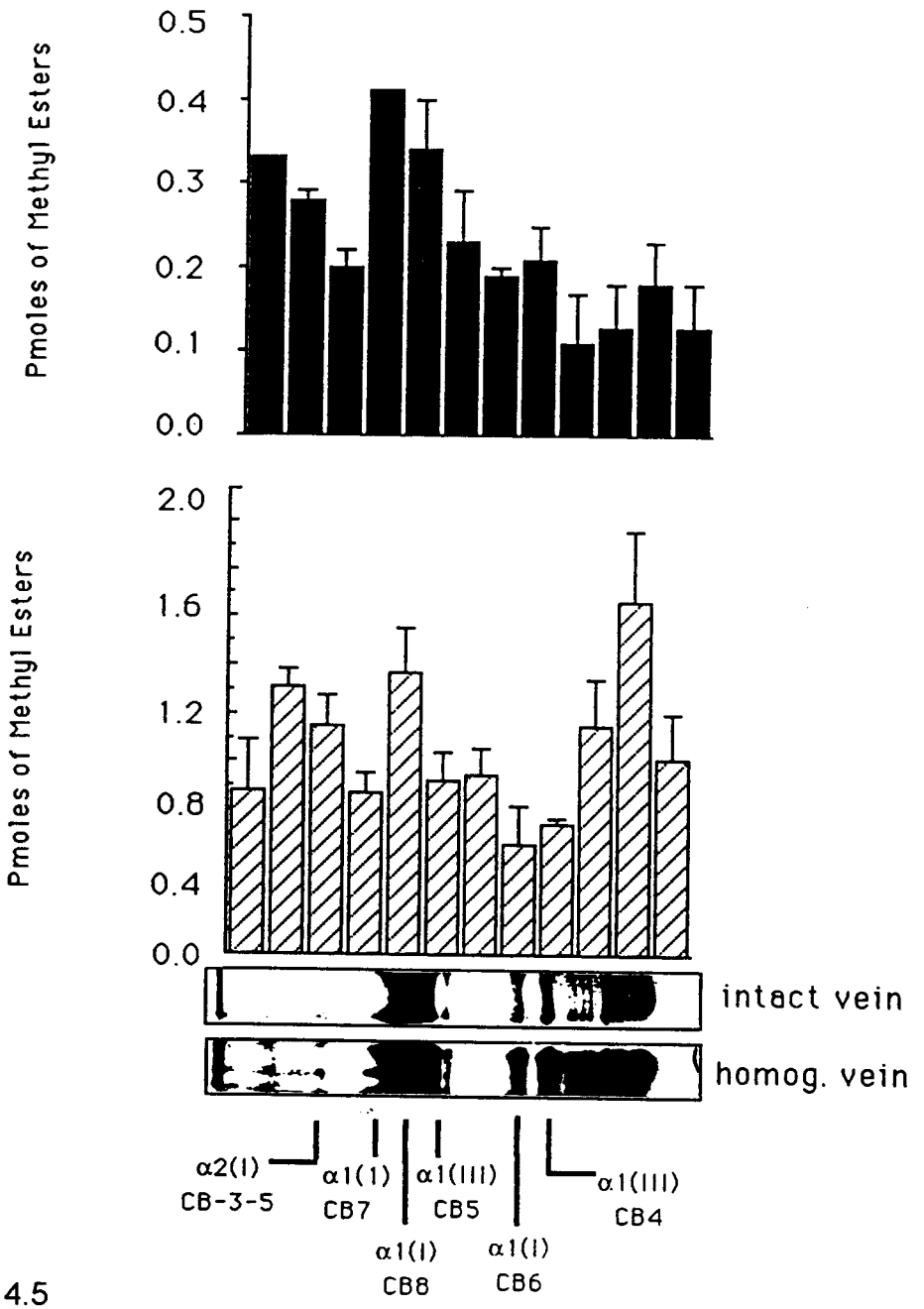
Pmoles of Methyl Esters
0.5
0.4
0.3
0.2
0.1
0.0
Pmoles of Methyl Esters
2.0
1.6
1.2
0.8
0.4
0.0
intact vein
homog. vein
α2(I)
CB-3-5
α1(I)
CB7
α1(I)
CB8
α1(III)
CB5
α1(I)
CB6
α1(III)
CB4

Figure 4.5

4.5 Discussion

The results presented in this study demonstrate that as a consequence of blood vessel injury, collagen, located in the extracellular matrix, becomes a major PIMT substrate.

To date, there are at least nineteen known proteins identified as collagens (Prockop 1995). In blood vessels, several fibril forming collagens (Types I, III & V) are major constituents of the medial layer with small amounts of network forming collagens (Type IV) found in the basal lamina. Type I collagen is the major form found in blood vessels (~60% of total) with Type III accounting for about 15% and Type V less than 5% (Morton and Barnes 1982; Murata et al. 1986; Maurel et al. 1990; Murata and Motoyama 1990).

Relying on the well known fact that each of the fibril forming collagens has a unique CNBr digestion pattern (Bornstein and Sage 1980; Sage et al. 1980; Maurel, Azema et al. 1990; Murata and Motoyama 1990; Takasago et al. 1992), enabled us to demonstrate that the two major types of collagen present in blood vessels (Type I & III), become methylated by PIMT following blood vessel injury. By separating these methylated peptides on the basis of both hydrophobicity (HPLC) and size (PAGE), and recovering nearly 70% of the total amount of methyl esters in the entire fraction in these peptides, minimizes the possibility that non-collagen peptides co-migrating with the identified fragments is the source of altered aspartyl residues. Other types of collagen species, if present, could not be identified due to their low abundance.

The finding that collagen contains altered aspartyl residues is not unexpected, since it known that in this tissue it is relatively metabolically stable (Johnson et al. 1993). Additionally, the extracellular matrix is probably normally devoid of PIMT, so there is no opportunity to eliminate these altered aspartyl residues by methylation-dependent mechanisms (reviewed in (Aswad 1995).

Though about 70% of the altered aspartyl residues in the extracellular matrix are located in the collagen-containing, CNBr-soluble fraction, about one-third of the altered aspartyl residues remain insoluble after digestion with cyanogen bromide. A likely candidate for the majority of protein in the CNBr-insoluble fraction is elastin, an abundant protein of the extracellular matrix that is unusual in that it lacks methionine and is nearly 95% non-polar amino acids in composition (Tanno et al. 1993). Though a positive identification of elastin could not be made with the techniques employed, circumstantial evidence comes from the amino acid analysis of the CNBr-insoluble fraction which shows that this fraction contains significant amounts of late eluting, and thus likely non-polar, amino acids.

At this time, the consequences on the vascular system of altered aspartyl residues in proteins of the extracellular matrix are not known. Above some threshold level it is likely that protein-protein interactions would be disrupted, since aspartyl residues are known to be critical amino acids in some cell binding sequences (Chen et al. 1991; Massia and Hubbell 1992; Cardarelli et al. 1994; Collen et al. 1994; Lin et al. 1994; Yamamoto and Yamamoto 1994).

Since it is known that PIMT released from injured blood vessels is capable of utilizing luminally supplied AdoMet to methylate substrates in the extracellular matrix (Weber 1996), it raises several interesting questions: Does endogenous methylation of collagen ever occur? If so, does this effect collagen metabolism in any way? The concentration of AdoMet in rat plasma is reportedly about 0.13 μM (Giulidori and Stramentinoli 1984), which is a magnitude lower than AdoMet's K_M for PIMT. So, while blood vessel injury no doubt places PIMT in contact with collagen, an important question that remains to be addressed is whether there is sufficient circulating AdoMet to promote physiological enzymatic modification at the site of injury.

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Chapter 5

General Discussion

5.1 Discussion

5.1.1. Extracellular matrix trapping of intracellular enzymes.

The existence of formerly intracellular enzymes trapped in the extracellular spaces, reacting with substrates in the blood stream was not previously known, but may offer insights into tissue responses as a result of injury. For example, coronary artery balloon catheterization (angioplasty) and vein graft bypass surgery, are both known to damage endothelial and smooth muscle cells of the vascular wall. Several groups have proposed that mitogens, such as basic fibroblast growth factor (bFGF), are released from injured cells which can then stimulate smooth muscle cells to proliferate and migrate into the intimal layer. This process, known as restenosis, quickly leads to partial obstruction of blood flow, causing the need for additional surgery (Ip et al. 1991; Hassenstein et al. 1992; Lindner and Reidy 1993; Hanke et al. 1994; Indolfi et al. 1995). The finding that intracellular proteins become trapped in the ECM, suggests that enzymes of intracellular origin could also be involved.

5.1.2. New biochemical methods instrumental to the thesis

Prior to the work presented here, no investigations had examined the occurrence of altered aspartyl residues in vascular tissues. The primary hindrance has been lack of suitable techniques. The lability of methyl esterified

proteins at neutral and above pH, prevents the use of common techniques, such as polyacrylamide gel electrophoresis, which typically utilize buffering systems at alkaline pH (Laemmli 1970). Though several acidic pH gel systems have been developed, the resolving power of complex mixtures of proteins is poor.

In the course of the work described in this thesis it was necessary to develop two techniques, to make possible the described work of mapping altered aspartyl sites in blood vessel proteins.

5.1.2.1 Acidic pH, discontinuous polyacrtylamide electrophoresis

The first technique is an acidic pH, discontinuous polyacrylamide gel electrophoresis system (see Appendix 1). The discontinuous gel system made it possible for the first time to obtain excellent resolution of complex mixtures of proteins and peptides, while at the same time, minimizing the loss of methyl esters from methylated proteins.

5.1.2.2 Acidic pH, 10% agarose gel system

The second method is an acidic pH 10% agarose gel system (see Appendix 2). This system made it practical to rapidly separate, methylated peptides from the bulk of excess radiolabeled AdoMet present in methylation reactions. This was necessary, since traditional methods of removing excess radiolabel from reaction mixtures, such as precipitation of protein with trichloroacetic acid, are not quantitative for peptides.

Without these two systems, it would not have been possible to show peptides derived from vascular collagen, are major PIMT substrate in the

extracellular matrix. Both of these techniques can be applied in tissues other than blood vessels and should be useful for research in the area of age-related protein modifications.

5.1.3. Possible consequences of altered aspartyl formation in collagen

At this time, the physiological significance of the accumulation of altered aspartyl residues, on the structural functions of collagen, are unknown. But in addition to structural roles, some collagens also contain cell binding motifs in which aspartyl residues are critical elements (Humphries 1993). The presence of an altered aspartyl residue in this motif would likely abolish any cell binding activity.

The collagen family of proteins are the most abundant proteins in vertebrates, and are relatively metabolically stable (Martin et al. 1985). Thus, in some tissues, collagen molecules may continue to accumulate progressively greater amounts of altered aspartyl residues until they are turned over. Yet, several groups have reported that the β-peptide linkage connecting L-isoaspartyl residues and its neighbor on the carboxyl side are resistant to hydrolysis by peptidases (Murray and Clarke 1984; Johnson and Aswad 1990), implying that complete degradation of the polypeptide may not be possible. This could impede some processes such as bone remodeling (Martin and Burr 1989).

Recently, an isoaspartyl dipeptidase was discovered in Escherichia coli, suggesting vertebrates may have an as yet undiscovered means of hydrolyzing these peptide bonds (Gary and Clarke 1995).

Though the physiological role that altered aspartyl residues play in the extracellular proteins of the vascular system is poorly defined, this thesis has demonstrated that they are the major repository of altered aspartyl residues in blood vessels. Since PIMT, an intracellular protein, is the only known enzyme capable of restoring these altered sites, blood vessel injury may inadvertantly mitigate the accumulation of altered aspartyl residues at sites accessible to the injury-released methyltransferase.

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Appendices

Appendix 1

Electrophoretic Identification of Altered Aspartyl Containing Protein Substrates of Protein (D-Aspartyl/ L-Isoaspartyl) Carboxyl Methyltransferase

Darin J. Weber and Philip N. McFadden

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A1.1 Introduction

The several classes of S-adenosyl-L-methionine-dependent protein methyltransferases are distinguishable by the type of amino acid that they modify in a substrate protein. The protein carboxyl methyltransferases constitute the subclass of enzymes which incorporate a methyl group into a methyl ester linkage with the carboxyl groups of proteins. Of these, protein (D-aspartyl/L-isoaspartyl) carboxyl methyltransferase, EC 2.1.1.77 (PIMT) specifically methyl esterifies aspartyl residues that through age-dependent alterations are in either the D-aspartyl or the L-isoaspartyl configuration (Aswad and Deight 1983; Lou and Clarke 1987). There are two major reasons for wishing to know the identity of protein substrates for PIMT. First, the proteins which are methylated by PIMT in the living cell, most of which have not yet been identified, are facets in the age-dependent metabolism of cells. Second, the fact that PIMT can methylate many proteins in vitro, including products of overexpression systems, can be taken as evidence of spontaneous damage that has in these proteins occurred since the time of their translation.

The biggest hurdle in identification of substrates for PIMT arises from the extreme base-lability of the incorporated methyl esters, which typically hydrolyze in a few hours or less at neutral pH. Thus, many standard biochemical

Figure A.1.1 Schematic of acidic discontinuous gel system. The system employs a pH 1.4 stacking gel on top of a pH 2.4 resolving gel with chloride as the leading ion and acetate as teh trailing ion to stack the proteins tightly. The presence of the anionic detergent SDS allows separation of proteins on the basis of molecular weight. The low pH preserves labile protein methyl esters, and so allows the identification of age-altered substrates of PIMT

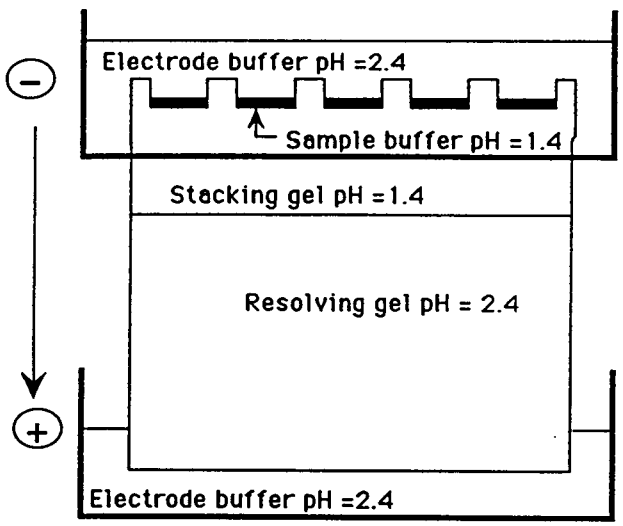
Electrode buffer pH =2.4
Sample buffer pH =1.4
Stacking gel pH =1.4
Resolving gel pH = 2.4
Electrode buffer pH =2.4

Figure A1.1

techniques for separating and characterizing proteins are not usefully applied to the identification of these methylated proteins. In particular, the electrophoresis of proteins by the most commonly employed techniques of SDS polyacrylamide gel electrophoresis result in a complete loss of methyl esters incorporated by PIMT, due to the alkaline pH of the buffers employed. Consequently, a series of systems employing polyacrylamide gel electrophoresis at acidic pH have been utilized in efforts to identify the substrates of PIMT. A pH 2.4 sodium dodecyl sulfate (SDS) system (Fairbanks and Avruch 1973) using a continuous sodium phosphate buffering system has received the most attention (Bower and Bates 1955; Aswad and Deight 1983; McFadden et al. 1983; Barber and Clarke 1984; O'Conner and Clarke 1984; Johnson and Aswad 1985; O'Conner and Clarke 1985; Lou and Clarke 1987; Neuhoff et al. 1988; Gingras et al. 1991; Sellinger and Wolfson 1991; Johnson et al. 1993). The main drawback of this system is that it produces broad electrophoretic bands . Acidic discontinuous gel systems using cationic detergents (MacFarlane 1984), have proven useful in certain situations (Freitag and Clarke 1981; O'Conner and Clarke 1983; O'Conner et al. 1984; O'Conner and Clarke 1984; Ohta et al. 1987; Gingras et al. 1994; Aswad and Guzzetta 1995) and can be recommended if the cationic detergent is compatible with other procedures that might be utilized by the investigator (e.g., immunoblotting, protein sequencing). Recently, we have developed an electrophoresis system which employs SDS and an acidic discontinuous buffering system (Figure A1.1) . This procedure results in sharp

electrophoretic bands and would be a good choice for investigators wishing to adhere to SDS as the anionic detergent. This system is described below and examples of its ability to resolve proteins are provided.

A1.2 Materials

A1.2.1 Equipment

1. Slab gel Electrophoresis Unit. We have used the mini- gel electrophoresis units from Idea Scientific (10 cm x 10 cm x 0.1cm) with great success, as well as the Sturdier large format gel system from Hoeffer Scientific Instruments (16 cm x 18 cm x 0.15 cm).
2. Electrophoresis power supply, constant current.
3. X-ray film and photo dark room.
4. Scintillation counter.

A1.2.2 Reagents

1. 40% (w/v) acrylamide stock solution containing 37:1 ratio of acrylamide to N,N'-methylene-bis-acrylamide (Biorad).
2. Resolving gel buffer: 0.1 M Na H_2PO_4 (Sigma), 2.0 % SDS (United States Biochemical (USB), ultrapure), 6 M urea (USB, ultrapure) pH 2.4 with HCl.
3. Modified Clark and Lubs Buffer(C & L buffer): 25.0 ml of 0.2 M NaCl to 26 ml of 0.2M HCl bring to a final volume of 0.l L. The buffer pH should be ~1.4 (see Note 1).

4.Stacking gel buffer (2X): 2.0 % (w/v) SDS, 6.0 M urea and C & L buffer such that the C & L buffer makes up 66% (v/v) of the total volume with the remaining 34% consisting of water and other buffer components. Buffer will be 0.033 M in NaCl. Re-adjust pH to 1.4 with HCl.

5. Sample solubilization buffer (2X): 2.0 % (w/v) SDS, 6.0 M urea, 10% glycerol ((USB), ultrapure), 0.01 % pyronin Y dye (Sigma) and C & L 33 % (v/v) of the total volume with the remaining 67% consisting of water and other buffer components. Buffer will be 0.0165 M in NaCl. Re-adjust pH to 1.4 with HCl.

6. Electrode Buffer (1X): 0.03 M Na H_2PO_4, 0.1 % SDS, 0.2M acetate, pH 2.4 with HCl.

7. Gel Polymerization catalysts: 0.06 % $FeSO_4$, 1.0 % H_2O_2, 1.0% ascorbic acid, prepared fresh in separate containers.

8. Colloidal Coomassie G-250 protein stain stock solution: 125 g ammonium sulfate, 25 mls 86% phosphoric acid, 1.25 g Coomassie brilliant blue G-250 (Sigma), de-ionized water to 1.0 L. The dye will precipitate, so shake well immediately before use. Stable indefinitely at room temperature (Neuhoff, Stamm et al. 1988).

9. De-stain solution: 10% (v/v) Acetic acid.

10.Flourography solution: 1.0 M sodium salicylate brought to pH 6.0with acetic acid (Chamberlain 1979).

11.X-ray film. Kodak X-Omat AR or equivalent.

A1.2.3 Gel Recipes

A1.2.3.1 12 % Acrylamide Resolving Gel pH 2.4

The following volumes are sufficient to prepare one 7.5 cm x 10.5 cm x 0.15 cm slab gel:

2X Resolving Gel Buffer	3.75 ml
40 % acrylamide (37:1)	2.25 ml
0.06 % $FeSO_4$	0.06 ml
1.0 % ascorbic acid	0.06 ml
0.3 % H_2O_2	0.06 ml.

A1.2.3.2 4.0 % Stacking Gel pH 1.4

The following volumes are sufficient to prepare one 3.0 cm x 10.5 cm x 0.15 cm stacking gel:

2X stacking gel buffer	3.75 ml
40 % acrylamide (37:1)	0.75 ml
0.06 % $FeSO_4$	0.06 ml
1.0 % Ascorbic acid	0.06 ml
0.3% H_2O_2	0.06 ml

A1.3. Methods

A1.3.1 Sample solubilization

1. An equal volume of 2X solubilization buffer is added and the samples are heated in a 95° C heating block for no more than 30 seconds (see Note 2).

2. To separate samples under reducing conditions, it is critical to add the reducing agent just prior to the addition of the solubilization buffer. Up to 10 μg per gel protein band can be resolved with this system; total sample loaded in a single well should not exceed about 100 μg.

A1.3.2 Gel Preparation

A1.3.2.1 Resolving gel

1. Add all the components listed under 2.3.1, except the 0.3 % H_2O_2, together in a small erlenmeyer side-arm flask.

2. De-gas the solution for at least 5.0 minutes using in-house vacuum.

3. Assemble together gel plates and spacers that have been scrupulously cleaned and dried.

4. Using a pen, make a mark 3.0 cm from the top of the gel plates to denote the space left for the stacking gel.

5. To the de-gassed gel solution, add the H_2O_2 catalyst. Gently mix solution by pipeting the solution in and out several times. Avoid introducing air bubbles into the solution.

6. Quickly pipet the acrylamide solution between the glass gel plates to the mark denoting 3.0 cm from the top of the gel plates.

7. Carefully overlay the acrylamide solution with about a 2.0 mm layer of water saturated butanol using a pasteur pipet, so that the interface will be flat upon polymerization.

8. Allow the gel to polymerize at room temperature until a distinct gel-butanol interface is visible.

9. After polymerization is complete, pour off the overlay and gently rinse the top of the gel with de-ionized water. Invert the gel on paper towels to blot away any remaining water between the gel plates.

A1.3.2.2 Stacking gel:

1. Add all the components listed under 2.3.2, except the 0.3 % H_2O_2, together in a small erlenmeyer side-arm flask.

2. De-gas the solution for at least 5.0 minutes using in-house vacuum.

3. Soak the well forming combs in the the H_2O_2 catalyst solution while preparing the stacking gel.

4. To the de-gassed gel solution, add the H_2O_2 catalyst. Gently mix solution by pipeting the solution in and out several times. Avoid introducing air bubbles into the solution.

5. Quickly pipet the acrylamide solution between the glass gel plates to the very top of the gel plates.

6. Remove the combs from the H_2O_2 catalyst solution, shake off some of the excess solution and insert the comb in between the gel plates by angling the

comb with one hand and guiding the comb between the plates with the other hand.

7. Ensure the comb is level relative to the top of the resolving gel and that no bubbles are trapped under the comb.

8. After the stacking gel has completely polymerized, carefully remove the comb. Remove any unpolymerized acrylamide from each well by rinsing with de-ionized water (see Note 3).

A1.3.3 Electrophoresis

1. Assemble the gel in the electrophoresis unit.

2. Add sufficient electrode buffer to cover the electrodes in both the upper and lower reservoirs.

3. Remove any air bubbles trapped under the bottom edge of the plates with a bent 25 gauge needle and syringe containing electrode buffer.

4. Rinse each well with electrode buffer immediately before adding samples.

5. Load all wells with 40ul-60ul of protein samples; load 1X solubilization buffer into any empty wells.

6. Run the gels at 15 mA, constant current, at room temperature for 4-6 hours, or until proteins of interest have been adequately resolved (see Note 4).

7. Fix the gel in 12 % (v/v) TCA with gentle shaking for 30 minutes.

8. Pour off fixative and mix 1 part methanol with 4 parts with colloidal Coomassie G-250 stock solution. Slowly shake gel with staining solution ~12 hours (see Note 5).

9. Pour off staining solution. Any non-specific background staining of the gel can be removed by soaking the gel in 10% (v/v) acetic acid. Little destaining of protein bands occurs even after prolonged times in 10% acetic acid.

A1.3.4 Detection of Radioactively Methylated Proteins

Several protocols exist for radioactively methylating proteins with PIMT (Lou and Clarke 1987; Aswad and Guzzetta 1995). Following electrophoresis, gel bands containing radioactively labeled proteins can be detected by fluorography or scintillation counting of gel slices.

A1.3.4.1 Fluorography of gels

1. If gels have been stained they are destained under using 10% methanol/7% acetic acid to de-colorize the gel bands.
2. Expose the gel to flourography solution for 30 minutes at room temperature with gentle shaking.
3. The gels are then placed on a piece of filter paper and dried under vacuum without heat for 3 hours.
4. In a dark room, x-ray film (Kodak X-Omat AR) is pre-flashed twice at a distance of 15 cm with a camera flash unit fitted with white filter paper (3M) to act as a diffuser.
5. The gel is placed in direct contact with the film and taped into place. For future alignment, puncture holes in an asymmetric pattern in a non-crucial area of the gel-film sandwich. A 25 gauge needle is useful for this purpose.

6. After sealing in a film cassette, the cassette is wrapped with aluminum foil and exposure takes place at -70° C for several weeks.

7. After exposure, remove the cassette from -70° C and allow to warm to room temperature. Develop film in dark room. An example of this technique is shown in Figure A1.2.

Figure A1.2. Comparison of discontinuous acid gel with continuous acid gel. The following experiment tested for the presence of age-altered proteins in a commercial preparation of collagenase. The collagenase preparation (Sigma, type IV) was methylated with purified rabbit erythrocyte PIMT and ^{3}H-AdoMet. Aliquots from the same methylation reaction were then resolved on (top) 12% discontinuous acid gel, described in text, or (bottom) 12% continuous acid gel system according to the method of Fairbanks and Auruch. Lane: Rainbow molecular weight markers (Amersham); Lane 2: 18 μg of methylated collagenase were loaded on each gel. Following electrophoresis and staining, 0.5 cm gels slices were treated with base to detect radioactivity as described under section A.3. Both gel systems are capable of preventing the loss of methyl esters from protein samples, but the discontinuous system provides much higher resolution of individual polypeptide bands.

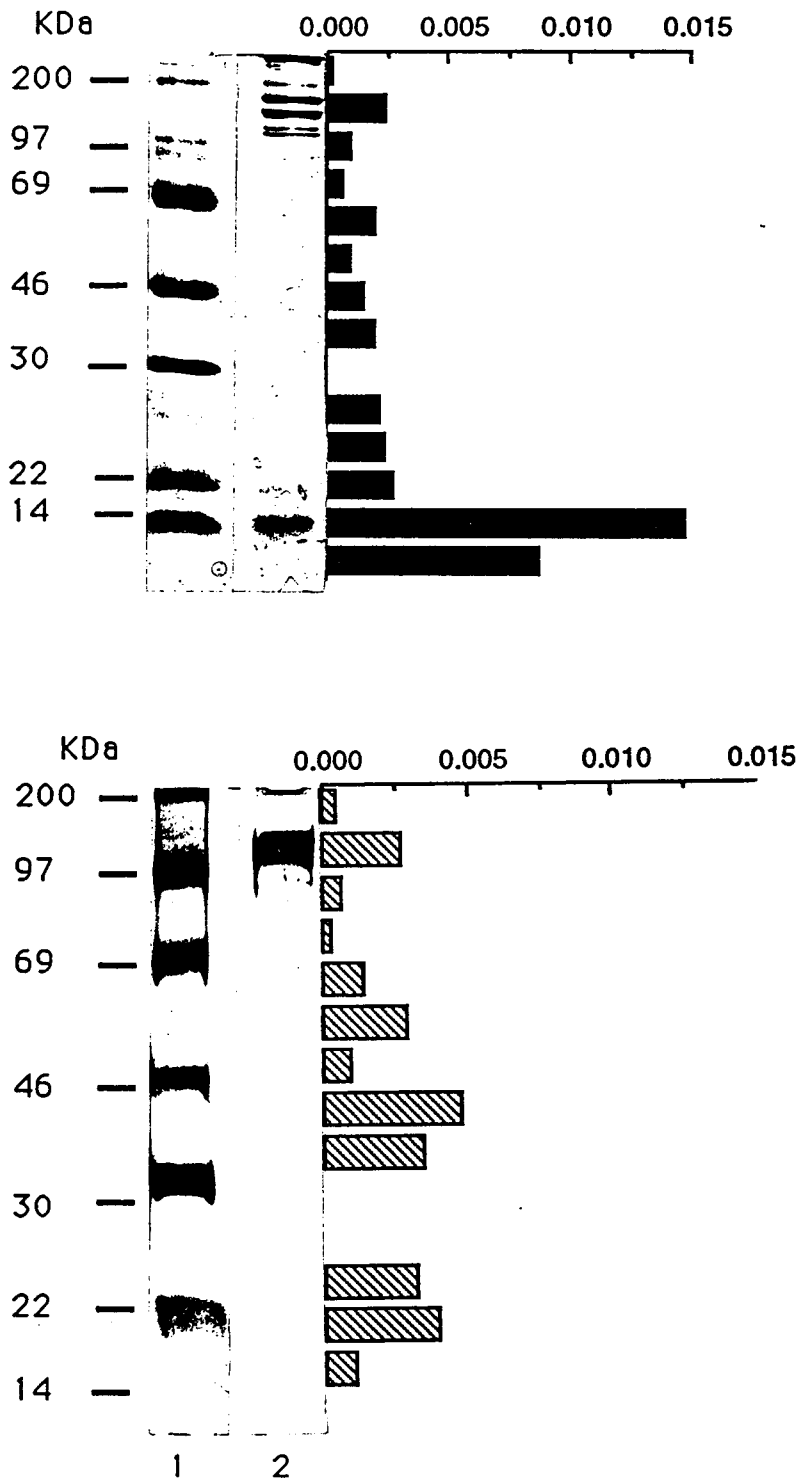
Methyl Esters (pmoles)
KDa
0.000
0.005
0.010
0.015
200
97
69
46
30
22
14
KDa
0.000
0.005
0.010
0.015
200
97
69
46
30
22
14
1
2

Figure A1.2

A1.3.4.2 Scintillation counting radioactive methanol evolved by base hydrolysis of protein methyl esters in gel slices

1. Stained gels are soaked in 10% acetic acid containing 3% (v/v) glycerol for 1.0 hour, placed upon a piece of filter paper and dried under vacuum without heat for 3.0 hours. The glycerol keeps the gel from cracking and pliable for the steps described below.

2. Using a ruler and fine tip marking pen, a grid is drawn directly upon the surface of the dried gel.

3. A sharp scalpel is then used to precisely cut out uniform slices from each gel lane. Alternatively, selected bands can be individually excised from the gel.

4. 4.0 ml of scintillation fluid is added to each 20 ml scintillation vial. A glass 1 dram vial is then placed inside the scintillation vial, carefully avoiding spilling scintillation fluid into the 1 dram vial.

5. Each dried gel slice is then placed in the inner dram vial of a scintillation vial.

6. After all the gel slices have been placed in a separate inner 1 dram vial, 0.3 ml of 0.2 M sodium hydroxide is added to each inner vial. The scintillation vial is then immediately tightly capped and allowed to sit undisturbed for at least 3 hours. Several hours are required for any volatile methanol that has formed by methyl ester hydrolysis to equilibrate and partion into the organic scintillation fluid where can then be detected by the scintillation counter.

8. Controls for measuring the efficiency of equilibration are performed by using a ^{14}C methanol standard which is added to an inner 1 dram vial containing a

non-radioactive gel slice and base hydrolysis solution. This is then placed inside a scintillation vial and allowed to equilibrate along with the other samples.

9. Figures A1.3 and A1.4 show examples gels that have been sliced and counted in a scintillation counter under the conditions just described.

A1.4. Notes

1. The buffering system employed in the stacking gel and sample solubilization buffer is based on a modification of the Clark and Lubs buffering system. NaCl is employed rather than KCl of the original system, because K^+ ions cause SDS to precipitate out of solution.

2 .On occasion, the solubilization buffer will contain precipitates, these can be brought back in to solution by briefly heating the buffer at 37° C . The solubilization buffer is stable for at least two weeks at room temperature; by storing the buffer in small aliqouts at -20° C, it is stable indefinately.

3. Gels can be stored for up to two weeks at 4°C by wrapping them in damp paper towels and sealing tightly with plastic wrap.

4. Since dye front is a poor indicator of protein migration, use of pre-stained molecular weight markers, such as the colored Rainbow markers from Amersham allow the progress of protein separation to be monitored by simply identifying the colored bands which are coded according to molecular weight.

5. It is essential that all fixing and staining steps occur at acidic pH. The colloidal Coomassie G-250 procedure described under Methods has several advantages: it is acidic, simple to perform and has higher sensitivity than other

dye based staining methods including those using Coomassie R-250. Additionally, non-specific background staining is very low so only minimal de-staining is necessary to visualize protein bands.

6. Avoid using higher glycerol concentrations or prolonged incubation of the gel in this solution. Otherwise the gel will be sticky after drying and contracts sharply away from the paper backing upon cutting.

Figure A1.3. Coomassie staining and autoradiography of complex protein mixtures by acidic discontinuous SDS gel electrophoresis. The following experiment was performed to measure the varieties of age-altered proteins in cell cytoplasm. (A) Coomasie G-250 stained acidic discontinuous gel. (B) Autoradiogram of same gel. Lane 1: Cytoplasmic proteins, following incubation of PC12 cells with ^{3}H-S-adenosyl-L-methionine (AdoMet). Lane 2: Cytoplasmic proteins following incubation of PC12 cytoplasm with 3H-AdoMet. Lane 3: Cytoplasmic proteins, following incubation of intact PC12 cells with 3H-AdoMet and purified rabbit PIMT. Lane 4: Cytoplasmic proteins following incubation of lysed PC12 cells with 3H-AdoMet and rabbit PIMT. Lane 5: Positive control; 20 μg of 3H-AdoMet and rabbit PIMT. Cells, lysates and subfractions were incubated with 1 unit of enzyme, 300 pmoles AdoMet in a final volume of 50 μl of 0.2 M citrate, pH 6.0 for twenty minutes at 37 °C.

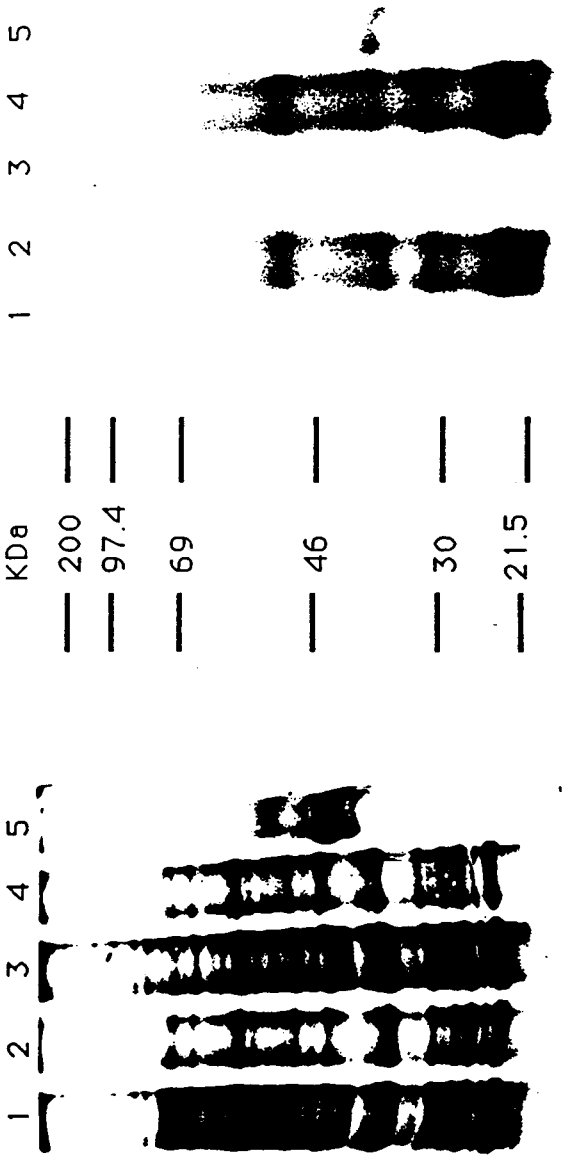

Figure A1.3

Figure A1.4. Coomassie staining and radioactive methyl ester determination in gel slices of electrophoresed proteins from diseased human brain tissue. Extracts prepared from homogenates of Alzheimer's dieseased brain (obtained from Department of Pathology, Oregen Health Sciences University) were methylated in vitro with purified rabbit PIMT and 3H-AdoMet. Methylated proteins were separated on 12% discontinuous, acid pH gels. Radioactivity in each gel slice was quantified with scintillation counting as desrcibed under section A.3. Top: Distribution of methyl accepting proteins in tissue that was insoluble in the non-ionic detergent, Triton X-100. Middle: Distribution of methyl acceptor proteins in tissue that was soluble in aqueous homogenization buffer. Bottom: Distribution of methyl acceptor proteins in crude homogenates of AD brain. Incubation conditions are similar to those described for figure A.3.

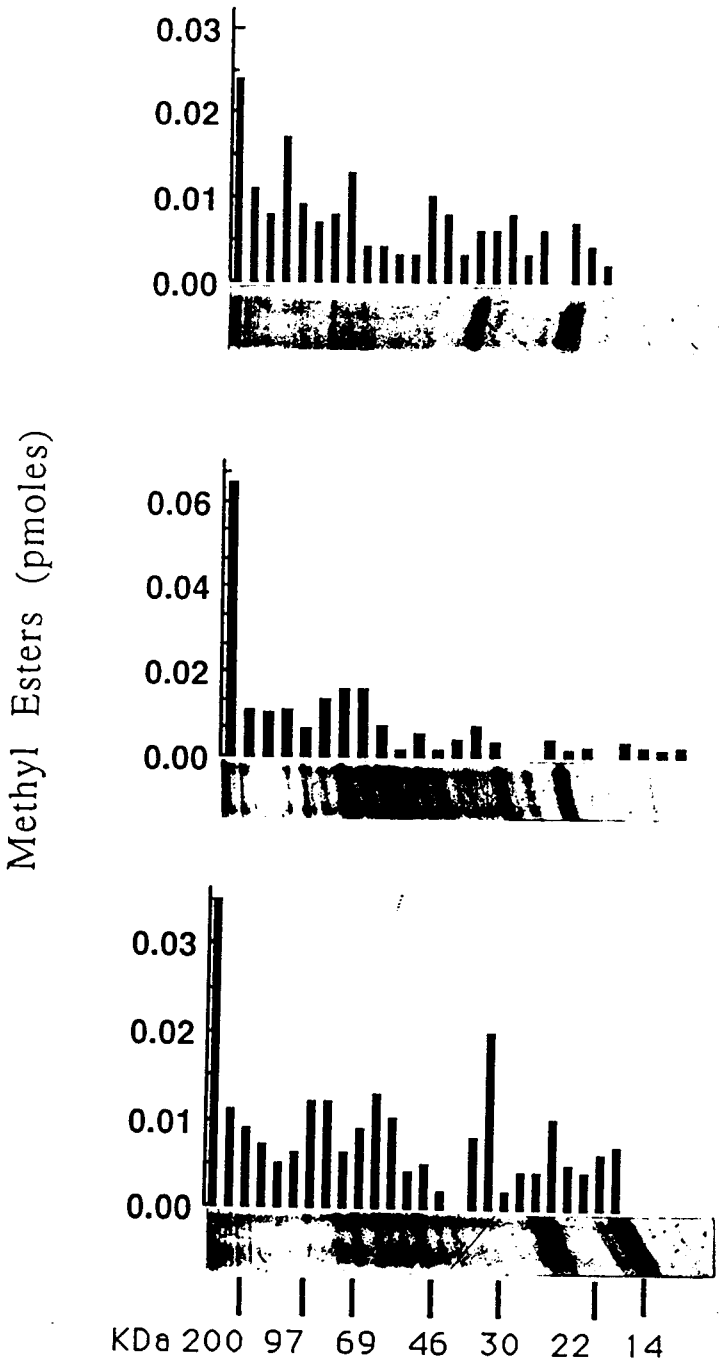
0.03
0.02
0.01
0.00
0.06
0.04
0.02
0.00
0.03
0.02
0.01
0.00
Methyl Esters (pmoles)
KDa 200 97 69 46 30 22 14

Figure A1.4

A1.5 References

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Appendix 2

Protein Methylation in the Nervous System

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A2.1 Introduction

The purpose of this chapter is to describe the types of protein methylation that might be confronted in investigations of the nervous system, and to summarize technical approaches which have proven to be useful in elucidating the chemistries and identities of methylated proteins. Several distinct protein methyltransferase activities have been described in nervous tissues, and there are undoubtedly more to be discovered. It has often been the case that identification of a methylation pathway in the nervous system has led to the discovery of a similar system in some other tissue. An excellent recent example is the characterization in the brain of isoprenylated protein methyltransferases (Ben et al. 1993; Paz et al. 1993; Klein et al. 1994) which have proven to have important activities in many other cells (Clarke 1992b).

Nervous system protein methyltransferases discovered thus far can be categorized on the basis of their amino acid group specificities (e.g. lysine vs. arginine vs. aspartic acid). However, issues of whether sequence specificities play a role in methylation patterns are much less clear. There are many other outstanding questions as well: How many proteins does a given enzyme methylate? How abundant and how subtly different are the isozymes of a given category of protein methyltransferase? What biological factors regulate the expression and activities of these protein methyltransferases? And most hotly debated: What are the functions of protein methyltransferases? Though all of these questions remain unanswered, both general and specific observations can

be made regarding changes in the proteins that become methylated. In general, both N-methylation and carboxyl methylation will cause small shifts in a protein's pKa as a result of changes in charge distribution within the protein due to the methyl group. The consequences of such shifts in pKa are presently unknown. Specifically, in several proteins, including histones, ribosomal proteins, myelin and calmodulin, N-methylation has found to modulate activity (Rattan et al. 1992) and in some cases, protease resistance (Lischwe 1990). Carboxyl methylation in prenylated, C-terminal cysteinyl residues on several GTP binding proteins (Ong et al. 1989; Ota and Clarke 1989; Fung et al. 1990; Lerner et al. 1992; Clarke 1992b; Giner and Rando 1994; Ghomashchi et al. 1995; Volker et al. 1995) has been postulated to have a role in signal transduction. Clearly, both N-linked and carboxyl methylation of proteins play important roles in the nervous system, however the precise function of these post-translational modifications remain to be elucidated.

There is a solid foundation of research methods from which to study nervous system protein methylation. Most fundamental are the methods used to describe protein methyltransferase reactions on the basis of it's amino acid group specificity. So far, every nervous system protein methyltransferase has been found to be either a carboxyl O-methyltransferase or an N-methyltransferase. The carboxyl O-methyltransferases include enzymes that methyl esterify the carboxyl groups of proteins, including the side chains of age-altered aspartyl residues, the C-terminal carboxyl groups of certain proteins,

and the side-chain of glutamic acid (exclusively in bacteria). The N-methyltransferases include enzymes that methylate nitrogen atoms of lysine, arginine, histidine, and the amino terminus.

The differing chemical stabilities of carboxyl O-methylated sites and N-methylated sites can be used as a diagnostic early in the characterization of a protein methylation reaction. Which category of reaction is identified will then dictate the repertoire of approaches that can most effectively be used in the further characterization of the methylation reaction.

A recurring theme of research with protein methyltransferases is that structure leads to function -- the identification and characterization of methylated proteins has often led to the discovery of novel forms of cellular function and regulation. A recent example is the series of investigations that yielded the identity of the major methylated protein in brain extracts as protein phosphatase 2A (Lee and Stock 1993; Xie and Clarke 1993; Favre et al. 1994; Xie and Clarke 1994a; Xie and Clarke 1994b). The finding that the C-terminal leucine of this protein undergoes carboxyl O-methylation is interesting from many perspectives, notably as an interface between methylation and phosphorylation-control of the nervous system. Perhaps future elucidations of methylated proteins will lead to other such unexpected discoveries.

Several excellent reviews of the field of protein methylation have been published. A particularly helpful reference for those embarking on the study of a protein methylation reaction is a book on the subject edited by Paik and Kim

(Paik and Kim 1990). Other very useful reviews on protein methylation and the substrates for protein methyltransferases can be found in the following references (Clarke 1992b; Clarke 1993; Aswad 1995).

A2.2 Methodological differences in studies of protein N-methylation and carboxyl-O-methylation.

The single greatest factor in determining the choice of methods for characterizing a protein methylation reaction is the distinction between nonhydrolyzable N-linkages and hydrolyzable carboxyl O-linkages. There are three likely approaches: a) If a protein is N-methylated, many of the standard techniques of biochemistry (e.g. SDS PAGE, electrophoretic immunoblotting, and Edman degradation) can generally be employed as tools in establishing the identity of the protein. b) If the protein is carboxyl O-methylated on its C-terminal carboxyl group, these same standard techniques can probably also be used, with a few precautions taken to avoid the hydrolysis of the relatively stable methyl ester linkage. c) If the protein is carboxyl O-methylated on an altered aspartyl residue, a less commonly used subset of acidic techniques will be required since the methyl ester linkage will spontaneously hydrolyze under the conditions used for most standard biochemical techniques.

Some of the most commonly found types of methylated proteins are those that fall into category "c" because even though many species of proteins are likely to be N-methylated (Najbauer et al. 1992) or carboxyl O-methylated at their C-termini (Clarke 1992b), essentially all cellular proteins have the potential for

being methylated on the carboxyl groups of age-altered aspartic acids (O'Conner and Clarke 1984).

The methyltransferase that catalyzes the methylation of altered aspartyl residues is protein (D-aspartyl/L-isoaspartyl) methyltransferase (E.C. 2.1.1.77). This enzyme is also known as protein isoaspartyl methyltransferase (PIMT) in recognition of the fact that the first synthetic substrate for this enzyme contained an L-isoaspartyl residue (Aswad and Guzzetta 1995). This enzyme is ubiquitous, and notorious for its ability to transfer a methyl group to the substoichiometric age-altered fraction of nearly any protein population. The highly labile methyl esterification catalyzed by PIMT has been shown to be capable of converting altered aspartyl residues in proteins back to their normal L configuration (Johnson et al. 1987). In vitro studies with calmodulin have shown that formation of isoaspartyl residues in calcium binding sites prevents binding and that this binding activity can be restored following methylation with PIMT and subsequent de-methylation (Johnson et al. 1987) . The finding that PIMT activity is highest in the brain, a tissue composed mostly of post-mitotic cells, is intriguing (Aswad 1995) and suggests PIMT may play a role in maintaining the functional state of proteins. Reports of the presence of significant levels of isoaspartyl residues in β-amyloid plaques in the brains of Alzheimer's Disease victims lend support to this hypothesis (Payan et al. 1992; Roher et al. 1993; Tomiyama et al. 1994; Iversen et al. 1995). PIMT activity is

therefore responsible for an incredibly complex pattern of methyl group incorporation into protein mixtures such as those found in neuronal cells.

A2.2.1 Hydrolytic lability of methyl groups incorporated by protein carboxyl-O-methyltransferases

Four amino acid sites have been shown to be carboxyl O-methylated by nervous system protein carboxyl O-methyltransferases: the alpha carboxyl group of L-isoaspartic acid; the beta carboxyl-group of D-aspartic acid; the C-terminal carboxyl group in isoprenylated L-cysteines; and the C-terminal carboxyl group in L-leucine. The generic distinguishing feature of protein carboxyl O-methylation as opposed to protein N-methylation is the lability of the methyl ester linkage to hydrolyzing chemical conditions. All of the known carboxyl O-methylation linkages are severed by strong acid (e.g. 6M HCl, 100C, 6 hours) and strong base (e.g. 1M NaOH, 37C, 6 h) (Terwilliger and Clarke 1981; Paik and Kim 1990). Furthermore, the methyl ester linkages to L-isoAsp and D-Asp are hydrolyzed by mild alkaline conditions as a result of a hydrolysis mechanism that involves intramolecular displacement of the methyl ester by the amide nitrogen of the neighboring amino acid (Murray and Clarke 1984; Johnson and Aswad 1985). The product of methyl ester hydrolysis is methanol. Therefore, if proteins containing radiochemical methyl groups (see below) yield radiolabeled methanol upon hydrolytic treatment, this is a strong positive indication of the involvement of a protein carboxyl O-methylation reaction. By varying the pH of the hydrolysis reaction it is possible to ascertain whether or not

the ester is of the extremely base-labile variety involving L-isoAsp and D-Asp carboxyl O-methylation (Clarke et al. 1988).

The simplest method for determining if the product is radioactive methanol is a vapor-phase assay in which volatile products are captured directly into scintillation cocktail for radiometric measurements (Murray and Clarke 1984; Chelsky et al. 1989; Aswad and Guzzetta 1995). (Under some conditions of hydrolysis, the breakdown of methylarginine can also yield a potentially volatile product, methylamine; but methylamine can be kept nonvolatile by performing the vapor-phase assay at low pH.)

Because methyl esterified amino acids are unstable to hydrolytic chemical conditions, it is not possible to recover and analyze the free carboxyl O-methylated amino acids by routine acid hydrolysis procedures used in amino acid analysis. Other methods are therefore necessary to positively identify the amino acid group which is methyl esterified. The most commonly employed method is to degrade the polypeptide as much as is practical with proteases, followed by an analysis of the proteolytic digestion products for free amino acids with the methyl group still present (McFadden and Clarke 1982; Xie and Clarke 1993) . In the case of prenylated cysteine residues, the C-terminal methylated product in rod outer segment membrane proteins was detected following proteolytic digestion together with performic acid oxidation to remove the prenyl moiety (Ong, Ota et al. 1989; Ota and Clarke 1989); and similar procedures led

to the positive identification of C-terminally methylated cysteines in brain G protein gamma subunits (Fung, Yamane et al. 1990).

A2.2.2 Hydrolytically stable protein methylation

Conversely to what is found for carboxyl O-methylation, hydrolytic stability of a methyl group in a protein is strong evidence for N-methylation. Extensive reviews are available which cite the many known examples of proteins that are N-methylated (Park and Paik 1990; Clarke 1992a). While many of these studies have utilized tissues and cells outside of the nervous system, it is clear that numerous proteins which have been characterized as being N-methylated are present in many tissues and are therefore likely candidates for methylated proteins in the nervous system. A means of increasing the sensitivity of detection of N-methylated protein substrates in PC12 pheochromocytoma cells involves pretreating the cells for a lengthy period with a pharmacological inhibitor of methylation, and then incubating the now hypomethylated cell extracts with radiolabeled S-adenosylmethionine (Najbauer et al. 1991; Najbauer, Johnson et al. 1992).

The amino acids that are N-methylated include lysine, arginine, and histidine. Each of these modified amino acids can be recovered after acid hydrolysis of the protein (Park and Paik 1990). For lysine, the single nitrogen atom of the side chain can be mono-, di- or tri-methylated. Calmodulin (Siegel et al. 1990), actin, myosin, and histones are well-known proteins that are methylated on lysines and, of course, these proteins are abundant in the

nervous system. For arginine modification, examples are known of methyl group incorporation into a single nitrogen atom in the guanidinium group of the side chain (monomethylarginine); methyl groups incorporated into both nitrogen atoms of the side chain (N^G,N'^G-dimethylarginine); and two methyl groups incorporated into one of the nitrogen atoms of the side-chain (N^G,N^G-dimethylarginine). Arginine methylation has been observed in histones, myelin basic protein and other proteins (Ghosh et al. 1990). Histidine modification occurs on actin, myosin, and histones among other proteins (Park and Paik 1990).

A2.3 Radiochemical methyl groups, and their incorporation into proteins of the nervous system

The commercial availability of radiochemically labeled methyl groups as S-adenosyl-L-[methyl-^{3}H]-methionine and S-adenosyl-L-[methyl-^{14}C]-methionine, as well as, L-[methyl-^{3}H]- and L-[methyl-^{14}C]-methionine is fundamentally responsible for the spate of discovery of methylated proteins that still continues. It is important to consider the chemical and radiochemical characteristics of these commercially available reagents. It is also important to optimize the assays and incubation conditions through which proteins are radioactively methylated.

A2.3.1 Radiochemical characteristics of methyl group donor compounds

As is true of any comparison of molecules which are singly labeled by either tritium or carbon-14, the specific radioactivity of tritium can theoretically be almost 500 times as high as the specific radioactivity of carbon-14 (e.g. 29 Ci

$mmol^{-1}$ for carrier-free tritium versus 62 mCi $mmol^{-1}$ for carrier-free carbon-14). Even higher specific activities of S-adenosyl-L-[methyl-^{3}H]-methionine are available since up to 3 tritium atoms can be present per methyl group. Thus, for a given number of radiolabeled molecules it is technically possible to achieve the highest number of incorporated dpm by using tritium labeling. This is not always the overriding concern, however. Depending on the specific application it may be more important to take advantage of the relatively greater decay energies of beta particles emitted by carbon-14. For example, the greater decay energy of carbon-14 can be helpful if the radiolabeled protein is to be detected as an electrophoretic band by autofluorography (Skinner and Griswold 1983).

Another practical consideration that may weigh in favor of the use of carbon-14 as the radiolabel is the typically greater degree of radiation decomposition of tritiated compounds. Manufacturers of radiolabeled S-adenosylmethionine and methionine have endeavored to package their materials with stabilizers against radiation decomposition. However, the rate of radiation decomposition of tritiated S-adenosylmethionine is typically about 1% per month as compared to 1% per year for S-adenosylmethione labeled by carbon-14.

Suppliers of [^{14}C-methyl]- and [^{3}H-methyl]-S-adenosylmethionine include American Radiolabeled Chemicals, Inc., Amersham, Inc., ICN, and New England Nuclear. [3H-methyl]S-adenosylmethionine is sold by Sigma. Both L-[methyl-^{3}H]-methionine and L-[methyl-^{14}C]-methionine are sold by each of these

manufacturers. The availability of S-adenosylmethionine and methionine which is radiolabeled in a position other than the methyl group can be useful in certain control experiments. For example, a radiolabel transfer reaction that is catalyzed by hemoglobin is evidently a methyl transfer reaction since radiolabeled methyl groups are incorporated from S-adenosylmethionine, but not the radiolabeled carboxyl moiety (Kimzey and McFadden 1994).

A2.3.2 Radiolabeling procedures in the methylation of proteins

The starting point for essentially all studies of protein methylation is to perform the radioactive labeling of proteins. Broadly speaking there are two ways in which this can be done: *in vivo* cell metabolism and *in vitro* reconstitution assays.

A2.3.2.1 Protein methylation procedures in intact cells

The *in vivo* approach allows the machinery of living cells to accomplish this radiolabeling in the course of cellular methylation metabolism. The radioactive precursor can be methionine (either L-[methyl-^{3}H]-methionine or [L-[methyl-^{14}C]-methionine), since cells are thought to not take up S-adenosylmethionine, but are generally capable of taking up methionine and converting a portion of this methionine into S-adenosylmethionine. A popular approach is to use cultured neuronal cells in such radiolabeling, including clonal lines such as PC12 cells (Najbauer, Johnson et al. 1991; Johnson et al. 1993) and murine neuroblastoma cells (O'Dea et al. 1987). Brain tissue slices have

also been used in the study of protein methylation in intact nervous system cells (Wolf and Roth 1985). The medium in which the cells are incubated should be adjusted in its nonradioactive methionine content so that the specific radioactivity of the radiolabel is high enough to result in the incorporation of detectable levels of methyl group radioactivity in the proteins of interest. For relatively short-term labeling experiments of less than a few hours, methionine-free culture media can be used with additions of high specific radioactivity methionine (including carrier-free radiolabel). For longer term incubations the cells may require higher chemical concentrations of methionine for growth, and so the addition of unlabeled methionine will dilute the specific radioactivity and decrease the sensitivity with which radioactively methylated proteins can be detected.

Of course, the direct incorporation of radiolabeled methionine into protein will also occur in most cells through protein translation. In the case of protein carboxyl O-methylation, the incorporation of radioactive methyl esters can be detected above the much larger background of protein backbone radiolabeling by isolating the proteins, exposing them to an alkali treatment, and measuring volatile radioactive methanol (Kloog et al. 1983; Chelsky, Sobotka et al. 1989). Another way to work around the high background due to backbone radiolabeling is to make use of a protein synthesis inhibitor to block protein translation, with the assumption being made that at least some aspects of protein methylation will continue. A good example of the latter approach is the use of cycloheximide to

block protein translation in HeLa cells during their incubation with [^{3}H-*methyl*]methionine (Ladino and O'Connor 1992). A further interesting possibility is that some cells can in fact directly take up radiolabeled S-adenosylmethionine and use it in the methylation of their proteins (Barten and O'Dea 1989).

A2.3.2.2 In vitro protein methylation procedures

Since nearly every type of living cell contains many types of methyltransferases and their substrates, there is a near certainty that when cell extracts and subcellular fractions are incubated with radiolabeled S-adenosylmethionine there will be a resulting incorporation of methyl groups into proteins. Techniques of gel electrophoresis (see below) are often used in conjunction with such radiolabeling in order to resolve the various methylated species of proteins. Many discoveries of protein methyltransferases and their substrates have been made with this basic approach. For example, guanine nucleotide-dependent carboxyl methylation of proteins was investigated by incubating cell membranes with S-adenosyl-[^{3}H-*methyl*]-methionine and then measuring how the incorporation of radiolabel into particular electrophoretically separated proteins was affected by including GTP analogs in the incubation mixtures (Backlund and Aksamit 1988). As another example, the identification of protein phosphatase 2A as a C-terminally methylated substrate (Lee and Stock 1993; Xie and Clarke 1993; Favre, Zolnierowicz et al. 1994; Xie and Clarke 1994a; Xie and Clarke 1994b) ultimately stemmed from long-standing

observations of highly methylated proteins formed during incubations of cytoplasm with radiolabeled S-adenosylmethionine (O'Conner and Clarke 1984).

A variation of the *in vitro* approach is to use cell extracts as the source of the enzymatic activity, and to include either recombinantly expressed proteins or synthetic polypeptide substrates as the methyl acceptor substrate. For example, the methylation of recombinant protein A1 by calf brain methyltransferase led to the identification of a specific arginine residue that is methylated in this protein component of the heterogeneous nuclear ribonucleoprotein complex (Rajpurohit et al. 1994). As another example, pathways in the maturation of C-terminally modified proteins can be studied by *in vitro* methylation of synthetic polypeptides that resemble protein carboxyl termini (Stephenson and Clarke 1990; Stephenson and Clarke 1992). High pressure liquid chromatography (see below) has often been used to confirm that polypeptide species being methylated by a cellular extract are indeed the synthetic polypeptide substrates (Murray and Clarke 1986).

To demonstrate that polypeptide radiolabeling *in vitro* is due to methyltransferase activity and not some other spurious process, a valuable control experiment is to test whether the radiolabel incorporation is blocked by micromolar concentrations of the end-product inhibitor of most methyltransferases, S-adenosylhomocysteine.

Recent work has shown that RNA methylation can potentially exhibit some of the expected characteristics of protein methylation (including

radiolabeled electrophoretic bands in SDS PAGE) (Hrycyna et al. 1994), and so tests of the nuclease stability of methylated macromolecules might also be an important control experiment to perform.

A2.3.2.3 Radiolabel quantitation

The quantitation of radioactivity and the conversion of dpm values to mole quantities requires accurate knowledge of the specific radioactivity of the radiolabel in the protein methylation reaction mixture. This is not too difficult in *in vitro* reactions if no residual quantities of S-adenosylmethionine are introduced from the cell extract or enzyme preparation; in this case the specific radioactivity should be that of the added radiolabel. If on the other hand the protein methylation reaction is performed by the uptake of radiolabeled methionine into cells, the specific radioactivity of the ensuing radioactive S-adenosylmethionine will depend on the amount of nonradioactive S-adenosylmethionine that is also present from cellular pools. In this case it is necessary to independently measure the chemical concentrations of S-adenosylmethionine and its radioactivity to arrive at a specific radioactivity that can be used to convert dpm incorporated into protein into mole quantities. Fractionation procedures for purifying S-adenosylmethionine and determining its concentration can be based on chromatography (Glazer and Peale 1978; Barber and Clarke 1983); alternatively, the concentration of S-adenosylmethionine can be measured by standardized enzymatic assays such as the hydroxyindole O-methyltransferase assay procedure (Baldessarini and Kopin 1966).

A2.4 Methylation procedures.

Of the several types of protein methylation reactions known, the study of the methylation of altered aspartyl sites is most dependent on the use of specialized techniques. These radiolabeled methyl ester moieties are very labile at neutral pH. Thus, methods of characterizing proteins with altered aspartyl residues must employ acidic pH, which has been shown to stabilize the methyl ester moiety.

As described under section A2.3.2, there are two general ways of methylating altered aspartyl residues in cells and tissues. One method utilizes the endogenous PIMT to methylate any proteins containing altered aspartyl residues in the intact cell, and the other employs exogenously added PIMT to methylate altered aspartyl residues in proteins from a wide variety of sources such as cell lysates, proteins isolated from specific subcellular compartments, or even highly purified proteins.

A2.4.1 In vivo methylation of altered aspartyl sites by PIMT

To identify which proteins are substrates for endogenous PIMT under in vivo conditions, cells are incubated with radiolabeled methionine and a suitable protein synthesis inhibitor, if necessary to prevent backbone radiolabeling (Chelsky, Sobotka et al. 1989). PIMT subsequently uses the radiolabeled

S-adenosylmethione produced in the cell to methylate proteins containing altered aspartyl residues that are accessible to the enzyme under physiological conditions. Following the period of radiolabeling, cell lysates prepared under acidic conditions can be prepared and the individual proteins resolved and analyzed by techniques described below.

A2.4.2 In vitro methylation of altered aspartyl sites

Under physiological conditions, PIMT endogenous to cells is localized primarily in the cytoplasm. Thus, intracellular methylation studies do not provide any information about the occurrence and distribution of many proteins containing altered aspartic acid that are inaccessible to methylation (O'Conner and Clarke 1983). This may include proteins enclosed in organelles, certain cytoskeletal proteins, and integral membrane proteins with extracellular domains. For these inaccessible proteins, an in vitro methylation assay is required. Cell extracts and subcellular fractions can be prepared in several ways, and then subjected to methylation with radiolabeled S-adenosylmethione and purified PIMT (Kloog, Axelrod et al. 1983; O'Conner and Clarke 1983; Barten and O'Dea 1989; Ladino and O'Connor 1992; Johnson et al. 1993). A typical approach for examining the distribution of proteins containing altered aspartic acid in cells involves subfractionating cellular proteins using differential solubility in various aqueous buffers. Suitable subfractionation buffers should avoid extremes of pH and ionic strength to avoid spurious alterations of aspartyl sites. Strong denaturants such as sodium dodecyl sulfate (SDS) or urea should

be avoided as they are incompatible with altered aspartyl detection assays. A generalized protocol for detecting altered aspartyl residues in proteins is described below:

Altered Aspartyl Detection Assay

1-2 U purified PIMT

100 μM radiolabeled AdoMet

10-20 μg of substrate protein

The components of this assay are incubated in a final volume of 50 μl in 0.2 M sodium citrate, pH 6.0 at 37 C for 20-30 minutes. PIMT (1unit = 1pmol methyl esters/minute) can be purified as described (Gilbert et al. 1988; Aswad and Guzzetta 1995). Acidic pH is used to stabilize any methyl esters formed during the reaction. This assay is broadly applicable to analysis of altered aspartyl residues in any protein-containing solution, ranging from cell lysates to highly purified proteins. Optimal reaction conditions can be determined as described in (Johnson and Aswad 1991). If a biochemical workup of native proteins is anticipated as the next step in the analysis, 10-20 μM of S-adenosylhomocysteine (AdoHcy) can be added to quench the reaction . Another common means of quenching the reaction is to inactivate the PIMT and stabilize methyl esters is by adding a denaturing, acidic electrophoresis sample buffer (see below).

A2.5 Quantitation of sites methylated by PIMT

Methylated proteins obtained by either of the two methods described under section 4.1, can be subjected to a variety of analyses, depending on the goal of the studies. However, special conditions must be employed in order to minimize the loss of methyl esters, which are labile at neutral and alkaline pH. For protein fractionation, it is imperative that cold temperatures and acidic conditions be employed to minimize hydrolysis of methyl esters during the isolation procedure. Additionally, depending on the preparation, isolation buffers should possibly include a broader spectrum of protease inhibitors than are used at neutral pH since proteases with acidic pH optimum will be favored under acidic isolation schemes (Weber and McFadden 1995).

A2.5.1 Quantitation of total levels of altered aspartyl residues in proteins

To measure the total level of abnormal aspartyl residues present in a specific sample, at the end of the altered aspartyl detection assay, the reaction is quenched by adding an equal volume of a 20% (v/v) solution of trichloroacetic acid (TCA). The highly acidic pH inactivates the enzyme, stabilizes protein methyl esters, and precipitates the protein substrates. The precipitated proteins are then washed twice by resuspension in five volumes of 10% TCA and centrifuged for five minutes at 5,000 x g to pellet the protein. This is done to separate any unincorporated radioactive AdoMet, which is soluble in 10% TCA, from the methyl esterified proteins in the pellet. The level of altered aspartyl residues in each sample is quantitated by taking advantage of the fact that the

hydrolyzed by alkaline pH. Treatment with base results in the release of radiolabeled methanol, a volatile compound. Each acid precipitated pellet is resuspended in 200 μl of 0.2 M sodium hydroxide for twenty minutes at room temperature in a tightly capped polypropylene tube. During this time, all the methyl esters will hydrolyze to form methanol. At the end of the hydrolysis period, one tenth volume of 3M citric acid is quickly added, and the tube re-capped. The acid stops the hydrolysis reaction and prevents the volatilization of any incidentally formed methylamine. Aliquots of the radiolabeled methanol containing samples are then transferred to a vial that is placed inside a scintillation vial containing scintillation fluid (Murray and Clarke 1984; Aswad and Guzzetta 1995). The methanol in the inner vial is given several hours to volatilize and partition into the organic scintillation fluid. The radioactivity is then quantitated in a scintillation counter and the volatilization efficiency corrected using an internal standard of radioactive methanol.

A2.5.2Quantitation of the total level of altered aspartyl residues in peptides

Since peptides are not quantitatively precipitated by TCA, an alternative means to separate excess radioactive AdoMet from the methylated peptides has been developed (Figure A2.1). Peptides which have been radioactively methylated by PIMT are loaded onto agarose gels and electrophoresed in a horizontal mini-agarose gel apparatus (BRL, Gaithersburg, Md) for 30 minutes at 100 V.

Figure A2.1 Diagram of acidic pH 10% agarose gel system for separating methyl esterified proteins of peprtides from unicorporated radiolabeled AdoMet remaining at the end of an altered aspartyl detection assay. Aliquouts of PIMT reaction mixtures (5 μl) are loaded into the centrally located sample wells and electrophoresed for 30 minutes at 100 volts. The system takes advantage of the small size of the radiolabeled AdoMet whcih will carry two positive charges at the acidic pH used in the system. As a small, doubly charged molecule, it will rapidly migrate toward the cathode, well ahead of any methyl esterified polypeptides, which primarily due to size, migrate much more slowly. Theo low molecular weight anodic and cathodic dyses included in the loading buffer delineate the maximal distribution of any radiolabeled peptides. A histogram of the typical localization of radiolabeled peptides between the two dye fronts is shown. Radiolabeled AdoMet runs at the leading edge of the cathodic dye on the right of the diagram.

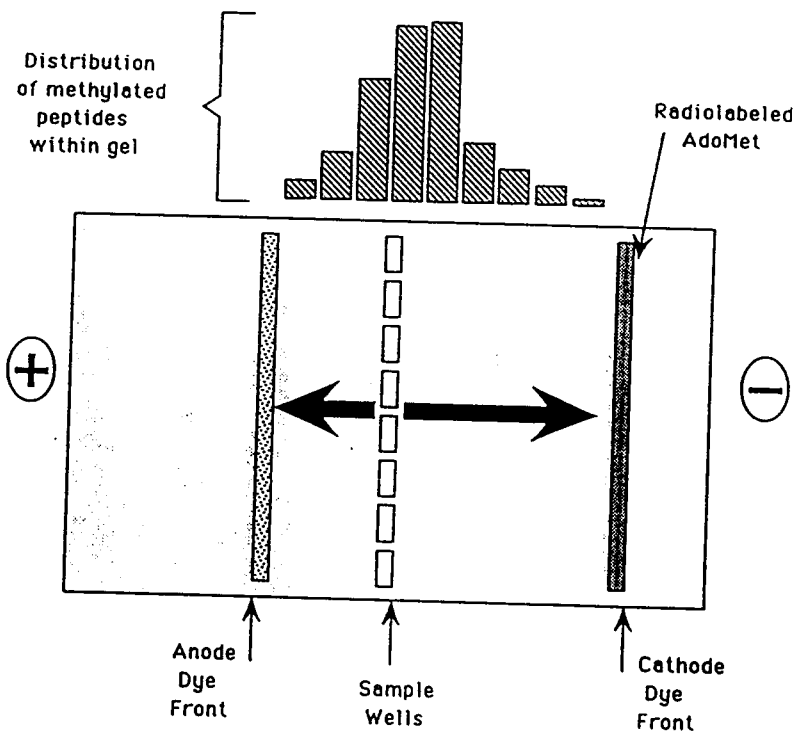
Distribution
of methylated
peptides
within gel
Radiolabeled
AdoMet
+
−
Anode
Dye
Front
Sample
Wells
Cathode
Dye
Front

Figure A2.1

The gels are made by preparing 10% (w/v) ultra pure agarose (BRL) in an Erlenmeyer flask containing a final volume of 100 mls of 50 mM NaH_2PO_4 pH 2.4. The flask is pre-weighed and the agarose melted by heating in a microwave. The flask is then reweighed and enough di$H_2$0 added to attain the original weight. While still hot, 20 mls of the viscous solution is cast into a mini-agarose gel caster and an 8-well comb is inserted into the center of the gel. After solidification, the comb is removed and the gel immersed in 150 mls of the same buffer used to prepare the gel. The 2X loading buffer consists of 100 mM NaH_2PO_4 pH 2.4, 5% ultra pure glycerol (USB), 0.5 mg bromophenol blue and 0.5 mg methyl green. The bromophenol blue migrates toward the anode while the methyl green migrates toward the cathode. Excess radiolabeled AdoMet comigrates with the cathode dye, while the radiolabeled peptides are distributed between the two dye fronts, with the majority migrating only one to two centimeters beyond the sample wells. Half centimeter gel slices of each lane are made and the radioactivity is quantified by volatilization assay as described above for TCA precipitated proteins. The total number of methyl esters present in each lane is determined from summing over all the gel slices. This method works equally well in separating radiolabeled S-adenosylmethione from high molecular weight proteins, with the proteins tending to migrate only short distances.

A2.5.3 Identification of proteins containing altered aspartyl residues

The initial stages in the characterization of the distribution of altered aspartyl containing substrates in cells and tissues are well suited to polyacrylamide gel electrophoresis. This method allows complex mixtures of proteins, such as those fractionated on the basis of differential solubility, to be resolved on the basis of molecular weight; and multiple samples can be resolved simultaneously. Unfortunately traditional methods of electrophoresis (Laemmli 1970), cannot be employed, since the system operates at an alkaline pH which causes hydrolysis of any PIMT-methyl esterified proteins. Consequently, a series of systems employing polyacrylamide gel electrophoresis at acidic pH have been utilized in efforts to identify altered aspartyl containing proteins (Fairbanks and Avruch 1973; MacFarlane 1984). The main drawbacks of these systems are that they either produce broad electrophoretic bands or use cationic detergents that may be incompatible with other protein analysis procedures. Recently a new electrophoresis system has been developed which employs SDS and an acidic discontinuous buffering system (Weber and McFadden 1996). This procedure results in sharp electrophoretic bands and would be a good choice for investigators wishing to adhere to SDS as the anionic detergent. An example of the ability of this system to resolved methylated altered aspartyl proteins is shown in figure 2.

A2.5.4 Localization of sites of altered aspartyl residues in protein sequences

To determine the total number of altered aspartyl residues in a given protein or a preparation of proteins, it is frequently necessary to fragment native

protein(s) into peptides using enzymatic or chemical cleavage techniques in order to disrupt secondary or higher order structures that prevent sites from being methylated by PIMT. Following a methylation reaction, any peptides that are methylated can be isolated and the amino acid composition determined.

Figure A2.2 Separation of methyl esterified, altered aspartyl containing proteins on 12% acidic pH polyacrlyamide gels. Various subcellular fractions of PC12 cells were assayed for the presence of altered aspartyl containing proteins. Protein fractions were obtained on the basis of solubility in various aqueous buffers. Approximately 1 x 10^6 cells under went a cycle of freeze/thawing in phosphate buffered saline (PBS), pH 7.4 containing protease inhibitors. Following centrifugation of the lysates at 10,000 x g for 10 minutes at 4 ^{o}C, any proteins recovered in the supernatant were designated "WS", for water soluble. The insoluble pellet was washed twice by resuspending in 200 μl of PBS and centrifugation as above. This pellet was resuspended in PBS containing 1% (v/v) Triton X-100. Following a 30 minute extraction period on ice, the supernatant was recovered by centrifugation as above and designated "DS", for detergent soluble. The pellet that was insoluble in TX-100 was washed twice as previously described and resuspended in 200 μl of PBS by brief sonication on ice. The fraction was designated "DI" for detergent insoluble. Based on a dye binding assay, equal amounts of protein in each fraction was methylated using the altered aspartyl detection assay described in section A.4. Equal aliquots of methylated proteins were then electrophoresed on a discontinuous, acid pH gel system as described (Weber and McFadden, 1996). Following staining of the gel with Coomassie blue, 0.5 cm gel slices of each sample lane were taken and volatile radioactivity determined as described. Top: Distribution of altered aspartyl containing proteins in the DS fraction. Middle: Distribution of altered aspartyl residues in the DI fraction. Bottom: Distribution of altered aspartyl residues in the WS fraction. The gel lanes follow the same order as the histograms representing the distribution of methyl esterified proteins. Molecular weights are shown at the bottom of the figure.

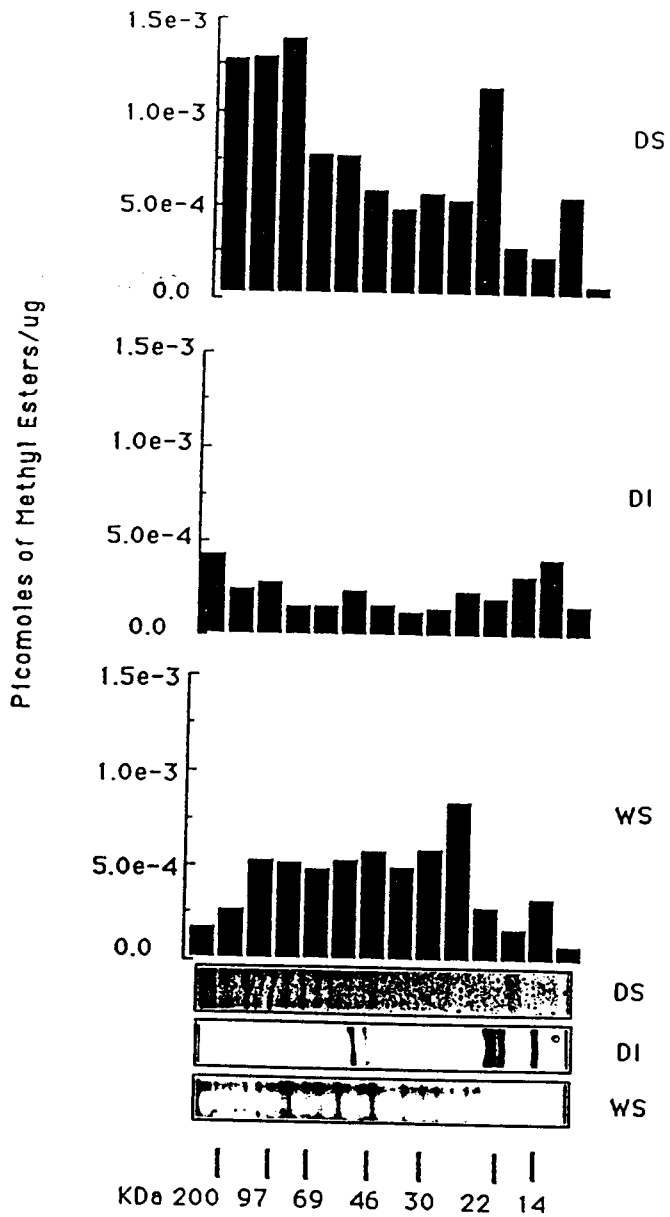
1.5e-3
1.0e-3
5.0e-4
0.0
DS
1.5e-3
1.0e-3
5.0e-4
0.0
DI
1.5e-3
1.0e-3
5.0e-4
0.0
WS
Picomoles of Methyl Esters/ug
DS
DI
WS
KDa 200 97 69 46 30 22 14

Figure A2.2

A2.5.4.1 Protein fragmentation methods

Cleavage methods have included trypsin digestion and cyanogen bromide cleavage (Aswad and Guzzetta 1995). The cleaved proteins can then be radioactively methylated using the altered aspartyl detection assay as described above, and the total amount of methylated residues present quantitated by electrophoresis on 10% agarose gels as described earlier. This method should also be useful for analyzing proteins, such as those of the neuronal cytoskeleton that contain altered aspartyl residues, but which are relatively insoluble under physiological conditions, and therefore incapable of being fully methylated in their intact form.

A2.5.4.2 Separation of individual methylated peptides

Reverse phase high performance chromatography (RP-HPLC) of fragmented proteins has proven to be the method of choice for characterizing the location of methylated residues within the amino acid sequence of single, purified proteins. All types of methylated amino acids can be localized in this manner. Even the alkali-sensitive methyl esters formed by PIMT can be localized by this approach since acidic, high-resolution solvent systems for HPLC are well-established. In one common procedure, a purified protein is first radioactively methylated by a purified methyltransferase. Following chemical or enzymatic cleavage, the methylated products are chromatographed on a

reversed phase column using a solvent system consisting of 0.1% triflouroacetic acid, water and acetonitrile (Ota and Clarke 1990; Aswad and Guzzetta 1995). Column fractions containing radioactively methylated peptides are then detected, and amino acid compositions are used to arrive at the identity of the methylated peptide. The amino acid sequence of a methylated peptide can also be determined using conventional sequence analysis or by mass spectrometry . If only substoichiometric amounts of a protein are methylated, as is generally true of PIMT reactions, the radioactively methylated peptide fragments may be only a small subpopulation of the peptide whose composition is actually measured. In many cases, the presence of the methyl group has led to only a very slight shift in the chromatographic behavior of a peptide, which allows the methylated peptide to be identified by the composition of its co-eluting, unmethylated neighbors (Ota and Clarke 1990; Lindquist et al. 1996)

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APPENDIX 3

A Heterogeneous Set Of Urea-Insoluble Proteins In Dividing PC12 Pheochromocytoma Cells Is Passed On To At Least The Generation Of Great-Granddaughter Cells.

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A3.1. Abstract

About 5 percent of the total cellular protein synthesized in exponentially dividing PC12 pheochromocytoma cells remains insoluble after extractions with aqueous buffer, nonionic detergent, and a strong denaturant, 6M urea. Single- and double-radiolabel pulse-chase labeling experiments with radioactive leucine indicate that for much of the 6M urea-insoluble protein there is either a lag between its synthesis and deposition in a urea-insoluble compartment and/or the urea-insoluble protein is comparatively stabilized from destruction. Given the doubling time of PC12 cells, much of the long-lived and urea-insoluble protein of PC12 cells is passed on for at least three generations. Electrophoretic analyses show there are many species of long-lived proteins in the 6M urea-insoluble fraction, displayed as a near-continuum of subunit molecular weights.

A3.2. Introduction

Age-related accumulations of insoluble protein in and around neurons is common to many neuropathologies, but the biochemical mechanisms underlying such accumulations are difficult to study. Because of their relative simplicity, model neuronal cell lines are promising systems in which to define intracellular factors that promote neuronal protein insolubilization and the intracellular deposition of inclusion material. The well-characterized PC12 pheochromocytoma cell (Greene, et al., 1987) displays many of the biochemical traits of neurons and so there is a possibility that protein insolubilization can be detected in these cells over conveniently short time intervals. Thus, while it is true that cultured cells continuously divide and live for relatively short periods, there may be an opportunity to explore the fundamental biochemistry of protein insolubilization in conveniently cultured neuronal cells.

For example, tyrosine hydroxylase is generally an easily solubilized neuronal enzyme (Shiman, et al., 1971) that catalyzes the first committed step in catecholamine biosynthesis, but a small portion of the immunodetectable tyrosine hydroxylase in cultured PC12 pheochromocytoma cells is resistant to solubilization with nonionic detergent and strong denaturants such as 6M urea (Weber and McFadden, see accompanying manuscript). Pulse-chase radiolabeling showed that there is a lag between synthesis of the enzyme and its deposition in insoluble form. This result led to the question of whether other cellular proteins may also enter a highly insoluble compartment.

A3.3. Experimental Procedures

A3.3.1 Culture media.

"Growth medium" consisted of RPMI 1640 culture medium supplemented with 10% heat-inactivated horse serum (Hyclone; Logan, UT), 5% fetal bovine serum (Hyclone), 100 units ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, and 20 mM sodium Hepes, pH 7.4. "Low-leucine pulse medium" was identical to growth medium except that it was prepared with a deficient RPMI 1640 culture medium containing 54 µM leucine (1/7 of the standard leucine concentration).

A3.3.2 Cell culture.

PC12 cells were obtained from the American Type Culture Collection and were cultured at 37°C under a 5% CO_2 atmosphere, using procedures based on those described (Greene, et al., 1987; Greene and Tischler, 1976). The cells were routinely subcultured into untreated T25 flasks (Falcon 3013) at a splitting ratio of 1:10 every seven days, giving an exponential doubling time of 2.1 days.

For radiolabeling experiments, cells in growth medium were plated onto 1.5 cm diameter polystyrene wells (24-well dishes, Falcon 3047) that had previously been coated for 1 hour with 0.88 µg of mouse-tumor collagen IV (Collaborative Research Products, Bedford, MA) in 0.5 ml 50 mM HCl and then rinsed with 1 ml of phosphate buffered saline (PBS). The plating density was 1-2 x 10^5 cells per well.

A3.3.3 Measurements of protein fate by single pulse-chase radiolabeling.

Growth medium was removed by aspiration, and the cells ($2x10^5$/well) were rinsed with 0.5 ml of low-leucine pulse medium. The cells were then pulse-labeled for 24 hours with 0.3 ml of low-leucine pulse medium containing 1 µCi of L-[U-^{14}C]leucine (315 mCi mmol^{-1}, New England Nuclear). After the ^{14}C-labeling period the cells were detached from their 1.5 cm dish and replated in 4 ml of growth medium onto 3.5 cm collagen-coated dishes. Cell culture was then continued for an additional variable chase period in the presence of growth medium, with the medium changed every one- to three-days to minimize reincorporation of radioactivity into newly synthesized protein, and to ensure that exponential division continued throughout the chase period.

A3.3.4 Measurements of protein fate by double pulse-chase radiolabeling.

Plating medium was removed from dividing cells ($1x10^5$/well), which were then rinsed with 0.5 ml of low-leucine pulse medium, and pulse-labeled for 24 hours with 10 µCi of L-[4,5-^{3}H]leucine (165 Ci mmol^{-1}, Amersham) in 0.3 ml of low-leucine pulse medium. After the ^{3}H-labeling period, the cells were rinsed twice with 0.5 ml low-leucine pulse medium, and the cells were pulse-labeled for 24 hours with 0.3 ml of low-leucine pulse medium containing 2 µCi of L-[U-^{14}C]leucine (315 mCi mmol^{-1}). After the ^{14}C-labeling period the cells were rinsed twice with 0.5 ml of growth medium, replated in 4 ml of growth medium onto 3.5 cm collagen-coated dishes, and then cultured for a chase period in the absence of added radiolabel.

A3.3.5 Extractions of cells.

After a given chase period, the cells were rinsed in their wells with ice-cold PBS, and then detached with repeated streams of phosphate-buffered saline from a Pasteur pipette. The cells were recovered by low-speed centrifugation, and were then lysed by freeze-thawing (-20°C) in 0.1 ml of lysis buffer, consisting of 25 mM sodium Hepes, pH 7.4; 1 mM disodium EDTA; 0.25M sucrose; 2 μg ml^{-1} aprotinin; 2 μg ml^{-1} leupeptin; 1 μg ml^{-1} pepstatin; and 0.5 mM phenylmethylsulfonyl fluoride. Centrifugation of the lysates (10,000 x g, 10 minutes) yielded the water-solubilized fraction (WS). The pellet from this extraction step was extracted by resuspension and centrifugation (10,000 x g, 10 minutes) from lysis buffer containing 0.1% (w/v) Triton X-100, yielding the detergent-solubilized fraction (DS). The pellet from this step was washed once with detergent-containing extraction buffer, and was then further extracted by resuspension and centrifugation (10,000 x g, 10 minutes) from lysis buffer containing 6M urea and 0.1% (w/v) Triton X-100, yielding the 6M urea-solubilized fraction (US). The pellet from this step was then washed once with detergent- and urea-containing extraction buffer, yielding a final pellet as the 6M urea-insoluble fraction (UI). Aliquots of non-electrophoresed proteins of the various solubility classes were mixed with sodium dodecyl sulfate (1%) to ensure complete suspension of all protein, mixed with 10 volumes of liquid scintillation counting cocktail (Scintiverse E, Fisher), and then assayed for radioactivity.

A3.3.6 Isolation of the histone fraction.

Following nonionic detergent extraction, each DI pellet was resuspended in 0.1mls of 0.25 N HCL containing 2mM dithiothreitol, as described (Bonner, et al., 1980). The pellets were completely dissociated by 5 seconds of sonication using a microtip sonicator. All operations were performed on ice. Following sonication, the pellets were centrifuged at 18,000 x g for 10 minutes at 4 degrees. The acid-soluble histones were then precipitated with 10 volumes of acetone overnight at -20°C, and the precipitated histones were collected by centrifugation as above for $^{3}H/^{14}C$ ratio measurements.

A3.3.7 Gel electrophoresis of US and UI proteins.

Aliquots of the US and UI fractions were mixed with SDS sample buffer for SDS polyacrylamide gel electrophoresis (Laemmli, 1970), using 12% (w/v) acrylamide/ bisacrylamide (37:1) cast into 10 cm long, 1.5 mm thick resolving slab-gels. Following electrophoresis the proteins were visualized by silver staining, and the gels were impregnated with 5% (v/v) glycerol and dried onto filter paper using a vacuum gel dryer. Gel lanes were sliced into evenly spaced segments (3mm) for scintillation counting measurements. For measurements of background radioactivity, slices of equivalent dimensions to the radioactive slices were excised from gel regions which contained no electrophoresed polypeptides. Gel slices were solubilized by heating them in capped scintillation to 60°C for 12h in 0.3 ml in a mixture of 4-volumes of 30% H_2O_2 and 1-volume of Clorox laundry bleach (use caution when mixing this reagent since

there is immediate gas evolution). Scintillation cocktail (5ml; Scintiverse E, Fisher) was then added, and the samples were counted as described below.

A3.3.8 Liquid scintillation counting

Radioactivity was measured in a Beckman LS 6000 scintillation counter. Quench correction was by the H-number method (Beckman Instruments) which employs an external ^{137}Cs source to quantify the shift in decay spectra due to quenching. For measurements of $^{3}H/^{14}C$ ratios, dpm values for both ^{3}H and ^{14}C were determined with at least 5% precision, and tests with standard radioactive samples showed that ratios were accurate as long as the ^{14}C dpm did not greatly exceed the ^{3}H dpm since high energy ^{14}C scintillations tend to mask the lower energy scintillations due to ^{3}H. In practice, accurate isotope discrimination was achieved by arranging the experimental conditions so that ^{3}H radioactivity was in relative excess over ^{14}C.

A3.4. Results

Successive extraction of [^{14}C]leucine-labeled PC12 cells with an aqueous buffer, a nonionic-detergent containing buffer, and a 6M urea-containing buffer yields on the order of 5% of the radiolabeled cellular protein as an insoluble residue (Table A3.1) Approximately the same fraction of cell protein remains insoluble when cells are lysed and extracted directly, rather than successively, in a buffer which contains both nonionic detergent and 6M urea (Table A3.1).

Table A3.1. Percentage of PC12 protein in various solubility classes.

Protein Fraction	Percentage of radiolabel recovered from cells labeled 24h with ^{14}C Leucine[a]	
	Trial 1[b]	Trial 2[c]
Water soluble	36 (5)	--
Detergent soluble	39 (3)	--
6M Urea soluble	19 (2)	--
6M Urea insoluble	6 (0.3)	4 (1)

[a]Values are averages of three determinations (std)

[b]Exponentially dividing cells extracted successively by the listed procedures

[c]Exponentially dividing cells extracted directly in detergent and 6 M urea containing buffer

Protein-dye assays also show that about 5% of the cellular protein is resistant to solubilization by denaturing buffers (data not shown).

The chase kinetics of [^{14}C]leucine from pulse-labeled cells was measured in order to estimate the average protein half-life in the different solubility classes. A 24-hour labeling period with [^{14}C]leucine was employed to ensure the extensive labeling of cellular protein, including that which is synthesized slowly. The loss of [^{14}C]radioactivity was fastest from the water-soluble class of protein, slowest from the 6M urea-insoluble protein, and intermediate in rate from the detergent-solubilized and 6M urea-solubilized protein (Figure A3.1).

Not surprisingly in consideration of the complex mixture of proteins in each of these fractions, the chase kinetics for each solubility class of protein were more complex than first-order. However, biphasic exponential decay curves lent reasonably good fits to the disappearance of radiolabel from proteins in the various fractions, allowing a grouping of proteins from each solubility class into two populations governed by exponential constants as determined by analytical curve-fitting (Table A3.2). By this method, the exponential half-life of almost half of the 6M urea-insoluble radioactivity is calculated to be about 8 days, a period greater than three cell-doubling times. The most rapid exponential half-life calculated from these measurements was for a component of the 6M urea-insoluble fraction, in which about half of the radioactivity exited with a half-life of 0.46 days (Table A3.2). Thus, in addition to

Figure A3.1. Loss of ^{14}C radioactivity from PC12 protein fractions as a function of chase time. Cells were labeled for 24 hours with 1 μCi per well of [^{14}C]leucine, and then chased for the indicated periods with nonradioactive medium containing excess nonradioactive leucine. Plotted here is the natural log of the portion of radioactivity remaining in the following protein fractions: l, water-solubilized; m, nonionic-detergent solubilized; n, 6M urea-solubilized; p, 6M urea-insoluble; +, acid-soluble histone-containing fraction. The smooth curves are plotted from double-exponential decay functions, $y = a_1e^{-k_1 t} + a_2e^{-k_2 t}$, that achieved the best-fit to the data as determined by nonlinear least squares curve fitting. The parameters a_1, a_2, k_1, and k_2 are given in Table A3.2.

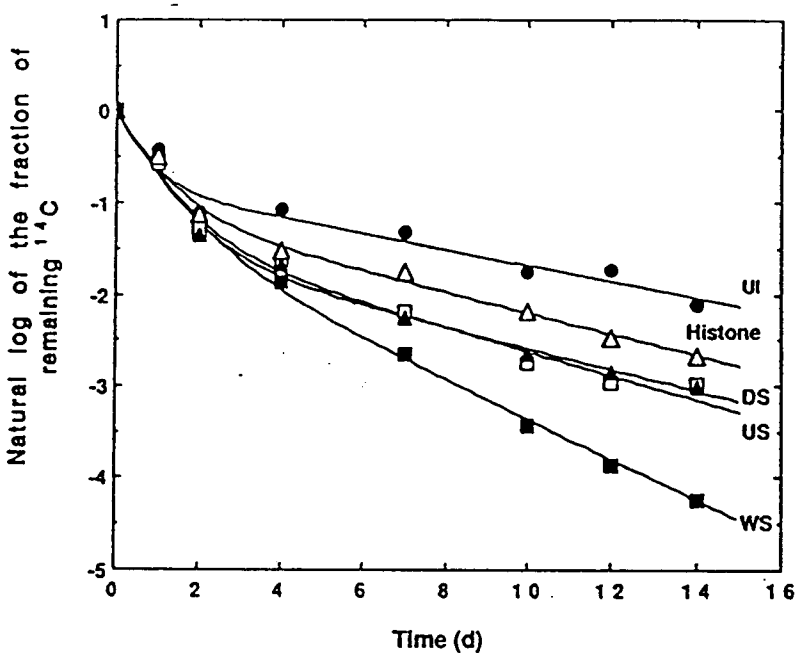

Natural log of the fraction of remaining ^{14}C
1
0
-1
-2
-3
-4
-5
0
2
4
6
8
10
12
14
16
Time (d)
UI
Histone
DS
US
WS

Figure A3.1

Table A3.2 Exponential half-lives of PC12 protein fractions

Protein Fraction	Fast component[a] (% of total)	Half-life of fast component[b] (d)	Slow component[a] (% of total)	Half-life of slow component[b] (d)
Water soluble	66.8%	0.660d	33.2%	3.05d
Detergent soluble	73.1%	0.706d	26.9%	5.25d
6M urea soluble	76.5%	0.711d	23.5%	6.03d
Insoluble residue	56.0%	0.459d	44.0%	7.95d
Acid-soluble histone-containing fraction	65.5%	0.653d	34.5%	6.04d

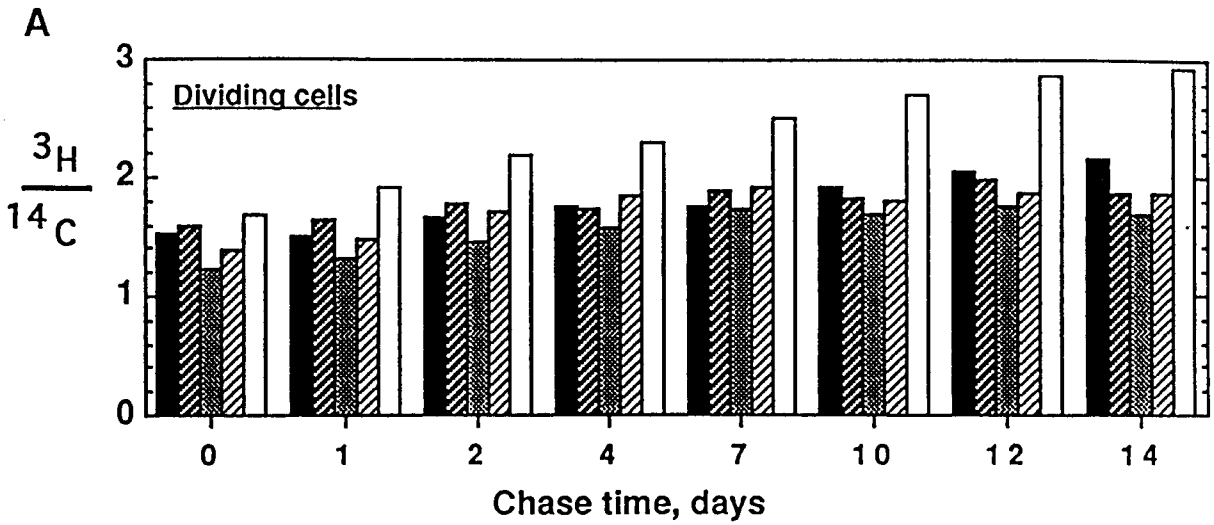
harboring the most stable protein in the cells, the 6M urea-insoluble fraction also rapidly loses a component of radiolabel.

As a check of our methods, the stability of the histone protein fraction was measured since it is generally known that these proteins are turned over in cells very slowly. The fact that our measurements indicated long half-lives for the acid-soluble fraction enriched in the PC12 histones therefore validates the measurements of similarly long half-lives for the urea-insoluble proteins (Figure A3.1, Table A3.2).

Double-isotope radiolabeling was then tested as an alternate means of following the fate of protein as a function of its solubility class. By this method, a 24-hour cohort of newly synthesized cellular protein was first radiolabeled by exposure of cells to [^{3}H]leucine, and then a second cohort was radiolabeled by a 24-hour exposure to [^{14}C]leucine. The two cohorts of radiolabeled protein were then followed over a chase period of 0 to 14 days (Figure A32). At 0 chase time, the 6M urea-insoluble protein exhibited a ^{3}H/^{14}C ratio that was similar to that in the other solubility classes of proteins. However, with increasing chase time the ^{3}H/^{14}C ratio in the 6M urea-insoluble protein steadily increased, presumably from destruction of the more rapidly turned over 6M urea-insoluble protein that would exhibit a low ^{3}H/^{14}C ratio, and/or from a lag-time that exists between synthesis of relatively soluble protein and its entry into the urea-insoluble compartment. The other solubility classes of proteins also showed increases in their 3H/14C ratios, though not as dramatically as for the 6M urea-insoluble fraction. The 6M urea-insoluble fraction in dividing cells was

Figure A3.2. $^{3}H/^{14}C$ ratios as a function of chase-time in proteins fractionated according to their solubility. Double isotope-labeling was performed as desribed in "Experimental Procedures". After each chase period, measurements were made of the $^{3}H/^{14}C$ ratio in the total cell protein and in the various solubility classes of cell protein. The shading of the bars decreases successively for the measurements of the ratio in the total cell protein, water-solubilized, detergent-solubilized, urea-solubilized, and urea-insoluble. Shown here are averages of triplicate measurements. For clarity, the error range is not shown, but typically ratio the standard error relative to the mean $^{3}H/^{14}C$ ratio was ± 15% or less.

Figure A3.2



thus indicated by double pulse-chase labeling to accumulate distinctly stable proteins, a trend similar to that obtained with single pulse-chase labeling.

The next question addressed was how heterogeneous are the stable proteins of the UI fraction. Cells were double-isotope radiolabeled, proteins were extracted according to their solubility class, and the proteins were electrophoresed in SDS polyacrylamide gels. While there were differences in the $^{3}H/^{14}C$ ratios among individual protein electrophoretic bands derived from the WS and DS fractions , these differences did not cluster according to solubility class (not shown). On the other hand, almost the entire lane of electrophoresed protein of the UI class exhibited a significantly higher $^{3}H/^{14}C$ ratio than other classes of cellular protein, including the WS, DS, and US proteins (Figure A3.3).

Though the $^{3}H/^{14}C$ ratio is seen to be nearly unvarying over long stretches of these gel lanes (Figure A3.3), this should not be interpreted as meaning that there was no protein in these gel slices; the typical gel slice in this experiment contained 1000- to 20,000 ^{3}H dpm and 100- to 5000 ^{14}C dpm, and therefore contained enough radioactive protein to yield very accurate ratio measurements. These measurements instead show that polypeptides of a near-continuum of molecular weights contribute to the relatively stability of the UI fraction.

In addition, several gel slices in the US and UI fraction exhibited $^{3}H/^{14}C$ ratios that were well-above average. The UI fraction exhibited a high $^{3}H/^{14}C$-ratio in the 43kD zone; and both the US and UI fractions exhibited high $^{3}H/^{14}C$-ratio in the zones below 21 kD (Figure A3.3).

Figure A3.3 SDS polyacrylamide gel electrophoresis and $^{3}H/^{14}C$ ratio measurements of cell proteins fof the US and UI fractions. In this experiment, double-labeling involved first a 24-hour labeling period with 10 µCi [^{3}H]leucine, followed by 8 hours of cell incubation in the absence of radiolabel, and then a 24-hour period of radiolabeling with 1 µCi [^{14}C]leucine. A two-day chase period after the ^{14}C labeling was employed; at this time the total cellular protein had a $^{3}H/^{14}C$ ratio of 3.1 (data not shown). Shown here are the silver-stained US and UI proteins after electrophoresis, with the migration of size markers on the left. The plot on the right shows the $^{3}H/^{14}C$ ratios in 3mm gel slices from the lane of US proteins (lightly shaded symbols) and UI proteins (dark symbols)

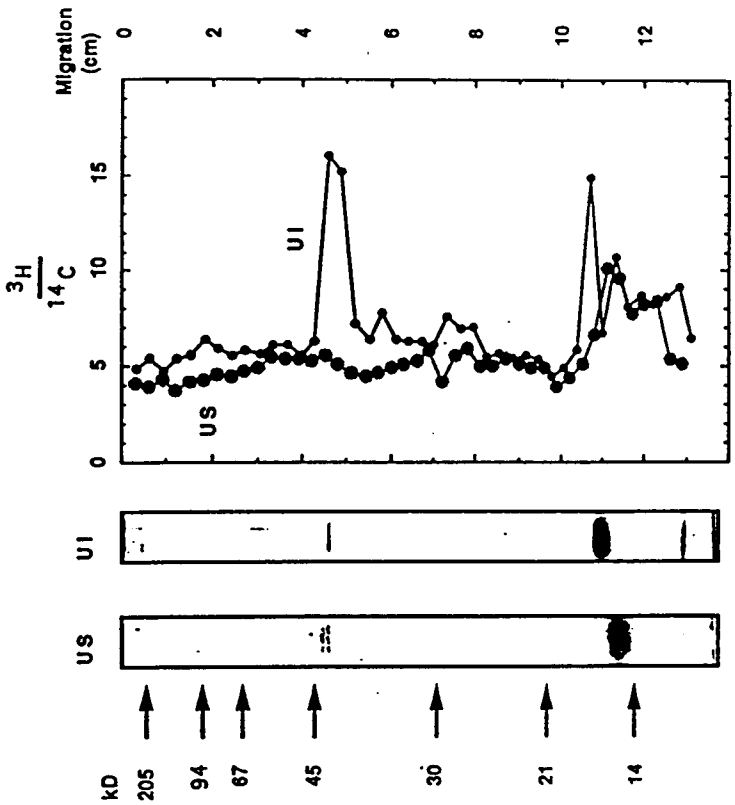

Migration (cm)
0
2
4
6
8
10
12
$^{3}H/^{14}C$
0
5
10
15
UI
US
UI
US
kD
205
94
67
45
30
21
14

Figure A3.3

A3.5. Discussion

These radiolabeling studies show that exponentially dividing PC12 cells synthesize a heterogeneous set of relatively stable proteins that can be isolated according to its insolubility in 6M urea. At least some of these UI proteins persists for two weeks in the cell, near the practical limit for culturing exponentially dividing cells without splitting them. Over three cell-cycle periods are traversed in this period, and so by the end of the period the average cell has inherited proteins that were synthesized three generations previously.

The conclusion that urea-insoluble protein is relatively slow to be cleared from the cell is validated by the internal experimental control supplied by the WS, DS , US and histone-enriched fractions. Thus, on an internally standardized relative scale, there is significance to the slow disappearance of radioisotope from the UI compartment in single-isotope experiments, seen equivalently as the uniquely high $^{3}H/^{14}C$ ratio compartmentation of radioisotope in double-isotope experiments. However, the quantitation of protein half-lives that we have performed is subject to uncertainty since it is well known that radiolabeling experiments can yield inaccurate pictures of protein turnover for reasons of radiolabel reutilization. In addition, isotope ratios obtained by the double-isotope labeling method can only be related to protein half-lives if steady-state conditions ensure that the rate of protein synthesis exactly matches the rate of protein degradation, and the case of exponentially dividing cells clearly deviates from steady-state conditions.

While it is clear that radiolabel is slow to be cleared from the urea-insoluble compartment, it is not obvious how this comes about. The two broad explanations are that 1) proteins from other compartments enter into the UI compartment after long lag periods, adding to the radioactivity of the UI compartment over time even though UI proteins may also be steadily degraded; and 2) newly synthesized protein is immediately targeted for the UI fraction and is inherently stable. The first explanation might be favored for proteins such as tyrosine hydroxylase that are normally soluble but that enter into the UI fraction in small amounts. Also requiring this explanation are proteins that exhibit a higher $^{3}H/^{14}C$ ratio than was nominally used in the labeling experiment, such as in the experiment shown in Figure 3 in which a ~15:1 $^{3}H/^{14}C$ ratio was observed in a ~43 kD protein even though the isotope ratio of the media used for radiolabeling was only 10:1 $^{3}H/^{14}C$. In such a case the lag between protein synthesis and entry into the UI compartment can explain the excess of the ^{3}H radiolabel.

The second explanation, stating that UI-targeted proteins are inherently stable, could account for some of the high $^{3}H/^{14}C$-ratio species seen upon electrophoresis, such as the low molecular weight species that likely correspond to a subfraction of the histones that resist denaturing extraction. In fact, our measurements of slow turnover of the acid-extractable histone fraction of PC12 cells mirror other observations of stable histones (Commerford, et al., 1982; Djondjurov, et al., 1983; Jackson, 1990).

The relative stability of highly-insoluble protein in PC12 cells bears a resemblance to the finding of Rogers and Rechsteiner who found that upon microinjection of structurally defined proteins into cells of the HeLa human epithelial cell line, several proteins that tended to become detergent-insoluble inside the cell were somehow stabilized from proteolysis (Rogers and Rechsteiner, 1988). Hare and Holocher have interpreted that behavior and their own measurements of plasma membranes to suggest that there is a relative stability of nonionic detergent-insoluble proteins due to strong association of such proteins with the cytoskeleton which prevents them from diffusing toward an endocytic degradation (Hare and Holocher, 1994). UI protein may be even more extreme in its inaccessibility to degradation mechanisms. Clearly more has to be known of the physical state of intracellular insoluble protein that may sequester it away from clearance mechanisms.

Compared to most other living cells, mammalian neurons are very long-lived. A problem in aging and in many neurological diseases is the accumulation in and around neurons of insoluble protein derived from relatively soluble precursors (Bordet and Destee, 1992; Braak, et al., 1993; Burkhardt, et al., 1988; De Bernardez-Clark and Georgiou, 1991; Eisen and Calne, 1992; Fifkova and Morales, 1992; Forno, 1986; Funata, et al., 1990; Goldman and Yen, 1986; Hirano, 1994; Meier, et al., 1994; Perry, et al., 1987; Pollanen, et al., 1993; Selkoe, 1994). It is not known if these accumulations are a continuous by-product of cell metabolism, or if there are physiological or pathological triggers for its accumulation. The formation of stable, insoluble protein in the PC12 cell line under culture conditions judged optimal for cell growth favors the

idea that cells continually produce such materials. Unlike neurons, dividing cells probably outgrow the process of protein insolubilization and so may not be adversely affected by it. However, the mechanisms of formation and stabilization of insoluble protein in cultured cells may be similar to those in differentiated neurons.

A3.6. References

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