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Philip N. McFadden

This is the first study to explore the ability of an enzyme to recognize and repair spontaneous age-dependent damage to its own sequence. Protein (D-aspartyl/ L-isoaspartyl) carboxyl methyltransferase (PCM) is known to repair damage that arises from a spontaneous isomerization of aspartyl and asparaginyl residues in other proteins during aging. As PCM contains several conserved aspartyl and asparaginyl residues, this dissertation tested whether PCM can serve as a methyl acceptor in its own methylation reaction.

In investigating the ability of PCM to automethylate, it was discovered that PCM is damaged. The mechanism of this automethylation reaction was determined to be an intermolecular, high affinity, slow turnover reaction and was limited to a subpopulation of damaged PCM molecules, termed αPCM.

Automethylation: A Response to Enzyme Aging

by

Jonathan A. Lindquist

A THESIS

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Jonathan A. Lindquist, Author

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CONTRIBUTION OF AUTHORS

Jonathan Lindquist was involved in the design, experimentation, analysis and writing of each manuscript. Elisabeth Barofsky assisted in the preparation and analysis of samples using matrix-assisted laser desorption/ ionization mass spectrometry. Dr. Philip McFadden was involved in overseeing this work from its onset and in the design, analysis, and writing of each manuscript.

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AUTOMETHYLATION: A RESPONSE TO ENZYME AGING

INTRODUCTION

In 1965, Axelrod and Daly discovered an enzyme in pituitary extracts which they called "methanol forming" enzyme. Seventeen years later, McFadden and Clarke (1982) identified the role of that enzyme in the recognition of altered aspartyl residues within proteins. Since that time, much work has gone into characterizing this enzyme, which has since been renamed protein (D-aspartyl/ L-isoaspartyl) carboxyl methyltransferase (PCM) in recognition of its substrates. PCM activity has been reported in a wide range of organisms from bacteria to mammals and has been identified in virtually every known tissue (Aswad 1995b). Within the cell, PCM activity has been isolated predominantly within the cytosol, but membrane-associated (Boivin, et al. 1993) and nuclear (O'Connor and Germain 1987) activities have been reported. PCM is unique in that it is the only enzyme known to repair protein damage that arises from spontaneous aging reactions.

Proteins perform much of the metabolic, organizational, and structural functions upon which the cell relies for survival. However, proteins are not immortal and are continually subjected to spontaneous reactions that result in damage. Therefore, a cell must continually renew its proteins in order to survive.

Why would cells possess a repair system for protein damage? A likely explanation is that protein synthesis is a costly process from an energetic viewpoint. Protein synthesis requires three ATP equivalents per peptide bond. For the average protein of 50 kDa (417 residues), this would amount to approximately 1250 ATP

equivalents per protein. The degradation of a damaged protein is known to occur in an ATP-dependent manner as well (Zubay 1988, Shakespeare and Buchanan 1976, Stadtman 1992). This process requires 2 ATP equivalents per peptide bond that is hydrolyzed, making the cost of degradation for the average protein approximately 830 ATP equivalents per protein. Together, the net cost of degrading a damaged protein and synthesizing its replacement would be 2080 ATP equivalents. This may not be viewed as a problem for the average cell, but this would be a problem to cells when energy is not abundantly available, such as during periods of starvation or during stationary phase. At these times, the conservation of energy would be essential to a cell's chance of survival. Therefore, an alternative route would be to repair these damaged proteins. From an energetic viewpoint, repair of a protein would be much more efficient. The estimated cost for a biological methylation reaction such as that performed by PCM is only 12 ATP equivalents (Atkinson 1977), a significant saving in comparison to the 2080 ATP equivalents required for the alternative.

Another important consideration in this argument is that not all proteins are readily replaceable. For example, proteins that make up important structural features, such as the cellular architecture or proteins that are involved in the packaging of DNA, may not be readily accessible to the mechanisms of protein degradation. An example of such proteins are histones that have been shown to be extremely long-lived. Other proteins must also persist for the life-time of the organism, such as the crystallins of the eye lens.

There are also cells that cannot synthesize new proteins, such as erythrocytes. Mature erythrocytes lack a nucleus and therefore have no capacity for protein synthesis. To perform their physiological function during their 120 day life-span, erythrocytes must rely upon the total pool of proteins present at the time of maturation. It is this feature that makes erythrocytes the ideal system for studying the mechanisms of cellular maintenance and repair.

Proteins are inherently unstable molecules. During their synthesis, proteins have sites of instability incorporated into them in the form of the amino acids asparagine and aspartate. These residues are the sites of a spontaneous damage reaction that occurs within proteins. This reaction is initiated by attack of the amide nitrogen of the C-terminally neighboring amino acid upon the side-chain carbonyl carbon (see figure A.1). The result of this attack is the formation of a succinimide intermediate (Geiger and Clarke 1987, Stephenson and Clarke 1989). In the case of asparagine, this attack also results in deamidation. The succinimide is an unstable structure that may hydrolyze to yield either L-aspartate or L-isoaspartate. While present, the succinimide may also epimerize to the D-configuration, yielding D-aspartate and D-isoaspartate upon hydrolysis. Since the formation of damage in this manner can lead to multiple products, this reaction is believed to generate a large amount of the heterogeneity observed within proteins (Aswad 1995a).

There are several factors that have been reported to affect the rate of this damage reaction. The most obvious is the primary sequence of the protein, as it is the C-terminally neighboring amino acid that is also involved in this reaction. The rate of

damage has been studied using synthetic peptides (Geiger and Clarke 1987, Stephenson and Clarke 1989, Clarke 1987, Wright 1991, Brennan and Clarke 1995). These results suggest that asparagine has a slightly higher propensity for damage than aspartate. Of the many sequences tested, the fastest sites to undergo damage were Asn-Gly sequences. This was proposed to be due to a lack of steric hindrance, as C-terminal neighboring residues with bulky side chains had much slower rates of damage. Another contributing factor to this reaction is structure, as the rates of deamidation identified in model peptides are generally much faster than the rates determined for the identical sequence in a protein. This is believed to be due to the structural constraints imposed upon a sequence by a protein which limit the possible conformations a given sequence may assume (Clarke 1987, Wright 1991, Brennan and Clarke 1995). However, one potential secondary structure was proposed that possesses the ideal bond angles for a succinimide to form, a type II' beta-hairpin turn, although no such structure has been identified within a protein to date (Clarke 1987). Other factors, such as alkaline pH, elevated temperatures, and high ionic strengths have been shown to have a positive correlation with the rate of damage formation (Clarke 1987, Wright 1991, Brennan and Clarke 1995, Robinson and Rudd 1974). These results suggest that there may be certain environments or conditions that would promote damage within a protein.

As the understanding of this damage reaction has grown, so has the awareness of its presence. Reports of the effects that altered aspartyl residues have had upon the activity of proteins range from complete loss of the biological activity to no effect at all (Wright 1991, Johnson and Aswad 1990b, Teshima, et al. 1995).

It is these damaged molecules that are the substrates for PCM, as well as many proteins that have yet to be identified. PCM has been shown to recognize L-isoaspartyl residues and D-aspartyl residues within peptides and proteins (McFadden and Clarke 1982, Lowenson and Clarke 1992) (see figure A.2). Utilizing S-adenosyl-L-methionine (AdoMet) as a methyl donor, PCM catalyzes the transfer of a methyl group from AdoMet to the free carboxyl residue of the damaged residue, resulting in the formation of a methyl ester linkage. Since this linkage is relatively unstable, it allows reformation of the succinimide intermediate, which releases the methyl group as methanol. The succinimide may then undergo another round of hydrolysis that may then yield either the aspartyl or isoaspartyl products (Ota and Clarke 1990) (see figure A.3).

Incubation of PCM with model L-isoaspartyl peptides and AdoMet resulted in the repair of the majority of these residues to the L-aspartyl configuration (McFadden and Clarke 1987, Johnson, et al. 1987b). Incubation with PCM and AdoMet has also been shown to restore function to damage-inactivated proteins (Johnson, et al. 1987a, Brennan, et al. 1994). These results, along with the identification of proteolytic activities that recognize isoaspartyl peptides (Johnson and Aswad 1990a, Gary and Clarke 1995), argue in favor of the repair-hypothesis for D-aspartyl/L-isoaspartyl methylation.

Since PCM catalyzes an important repair reaction for aging proteins, the question arises as to whether PCM is iteslf subject to aging of a similar type. The sequences of PCM have been reported from several organisms and these sequences show a high degree of amino acid homology (Henzel, et al. 1989, Gilbert, et al. 1988, Ingrosso, et al. 1989, Sato, et al. 1989). It is interesting to note that many potential sites for damage have been

conserved within PCM, including an Asn-Gly sequence that has been shown to be a rapidly damaged sequence in model peptides.

The specific aims of this thesis are:

1. To test if PCM can self-methylate. The ability of PCM to self-methylate will be determined by the identification of [^{3}H-*methyl*]esters incorporated into PCM using [^{3}H-*methyl*]AdoMet as the methyl donor. It is not unreasonable to assume that PCM, like other proteins, becomes damaged with age and can therefore serve as a methyl acceptor. The most direct method to test this question is to look at the enzyme *in vitro* using purified PCM.

2. To identify the sites of damage. Using a combination of proteolytic and chemical digestion methods, the sites of damage will be identified. Several other proteins have been mapped for sites of damage in this manner, so similar examination of PCM should be straight forward.

3. To test the ability of PCM to self-repair. As PCM has been shown to repair damaged peptides upon incubation with AdoMet, similar conditions will be utilized to see if PCM is capable of self-repair. The ability to self-repair will be determined by the inability of PCM to further incorporate [^{3}H-*methyl*] groups from [^{3}H-*methyl*]AdoMet after a putative repair incubation.

4. To identify the ability of PCM to automethylate *in vivo*. Previously, erythrocytes have been used as an *in vivo* system for detecting methyl acceptor proteins and a method to [^{3}H-*methyl*]label the cellular pool of AdoMet has already been

established. The ability to identify *in vivo* automethylated PCM will thus be a matter of isolating PCM away from the bulk of the erythrocyte protein and evaluating the presence of [^{3}H-*methyl*]esters in the enzyme molecule.

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CHAPTER 2

AUTOMETHYLATION OF PROTEIN (D-ASPARTYL/ L-ISOASPARTYL) CARBOXYL METHYLTRANSFERASE, A RESPONSE TO ENZYME AGING

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RATIONALE

Protein (D-aspartyl/ L-isoaspartyl) carboxyl methyltransferase (PCM) is known to catalyze the transfer of a methyl group from the methyl donor S-adenosyl-L-methionine (AdoMet) to the free carboxyl group of altered aspartyl residues in other cellular proteins. Altered aspartyl residues arise in these proteins in an age-dependent manner through a spontaneous damage reaction which occurs at aspartyl and asparaginyl residues. Since PCM is known to possess several conserved aspartyl and asparaginyl residues, the purpose of this chapter is to see if PCM can serve as a substrate for its own methylation reaction.

AUTOMETHYLATION OF PROTEIN (D-ASPARTYL/ L-ISOASPARTYL) CARBOXYL METHYLTRANSFERASE, A RESPONSE TO ENZYME AGING

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A question that is central to understanding the mechanisms of aging and cellular deterioration is whether enzymes involved in recognition and metabolism of spontaneously damaged proteins are themselves damaged and subject to their own activity. We show here by in vitro experiments that protein (D-aspartyl/ L-isoaspartyl) carboxyl methyltransferase (PCM) from bovine erythrocytes does methylate age-dependent amino acid damage in its own sequence. The subpopulation that is methylated, termed the αPCM fraction, appears to be formed through age-dependent deamidation of an asparaginyl site to either an L-isoaspartyl or D-aspartyl site because a) the stoichiometry of automethylation of purified PCM is less than 1%, a value typical of the substoichiometric methylation of many other aged protein substrates, b) αPCM is slightly more acidic than the bulk of PCM, and c) the methyl esterified site in αPCM has the characteristic base-lability of this type of methyl ester. Also, the methyl group is not incorporated into the enzyme as an active site intermediate because the incorporated methyl group is not chased onto substrate protein.

The effect of enzyme dilution on the rate of the automethylation reaction is consistent with methylation occurring between protein molecules, showing that the pool of PCM is autocatalytic even though individual molecules may not be. The automethylation and possible self-repair of the PCM pool has implications for the in vivo efficiency of methylation-dependent protein repair.

KEY WORDS: Protein carboxyl methyltransferase; D-Aspartic acid; L-Isoaspartic acid; Deamidation; Protein repair

INTRODUCTION

Living cells contain enzymes that assist in the refolding, repair or replacement of proteins that have become damaged or denatured by spontaneous aging reactions (Stadtman, 1992; Gething and Sambrook, 1992; Hershko, 1992; Rechsteiner, 1991). In general it is not known whether these enzymes act upon themselves, either intramolecularly or in a pool. Specifically, we are interested in determining whether protein carboxyl methyltransferase (PCM) can recognize and methylate amino acid damage in its own sequence.

PCM transfers the methyl group from S-adenosylmethionine into methyl ester linkages with both D-aspartyl and L-isoaspartyl amino acid residues that are formed by spontaneous aging in a wide range of cytoplasmic and membrane proteins (for reviews see Ota and Clarke, 1990; Johnson and Aswad, 1990; Billingsley, 1990). The function of protein carboxyl methylation of aging protein is not known. It is clear that methyl ester hydrolysis at physiological pH allows a damaged protein site to undergo further covalent rearrangements

to either a "repaired" L-aspartyl residue or to other products that await characterization. Bacterial mutants that lack PCM activity divide normally but do not survive in stationary phase, suggesting that methylation is indeed somehow related to the repair and maintenence of the cell (Li and Clarke, 1992). Automethylation and self-repair of PCM could be important in maintaining the integrity of the pool of spontaneously aging PCM. The following experiments will test the ability of bovine erythrocyte PCM to methylate itself.

EXPERIMENTAL PROCEDURES

Enzyme purification

PCM purification was similar to that described by Gilbert, et al. (1987). Beginning with one liter of freshly drawn bovine blood, the first steps were centrifugation of lysed bovine erythrocytes to remove membranes, and ammonium sulfate precipitation (53% saturation) of the remaining protein. The preparation was then divided into sixteen equal-sized pools that were separately purified by affinity chromatography on S-adenosylhomocysteine-diaminohexyl-Sepharose 4B. The fractions contianing PCM were then pooled and further purified by gel exclusion chromatography on Sephadex G-75, and finally by anion exchange chromatography on Fractogel EMD DEAE 650-S (EM Separations). The procedure resulted in a 3000-fold purification of the enzyme, with a final specific activity of the enzyme exceeding 10,000 units/mg by the standard assay described by Murray and Clarke (1984) in which 1 unit of activity equals 1 pmol of ovalbumin methyl ester formed per minute at pH 6, and in which the weight of PCM was determined by its

binding of colloidal Coomassie Blue within SDS polyacrylamide gels relative to several different standard proteins (bovine serum albumin, bovine erythocyte carbonic anhydrase, bovine trypsinogen, and soybean trypsin inhibitor) as described (Neuhoff, et al.1990). As expected, we observed two major isozymes, PCM I and PCM II, to be resolved in the final purification step. The more acidic PCM II, presumed to be a translation product of an alternatively spliced messenger RNA (Ingrosso, et al., 1991), was used throughout the present study.

Automethylation reactions

Two slightly different [^{3}H]methylation reaction buffers were used to automethylate PCM. The following lists the conditions of the Type A reactions, paired with the Type B conditions in parentheses: 3.28 µM (9.5 µM) [^{3}H-*methyl*]S-adenosylmethionine (15 Ci mmol^{-1}); 28mM (60 mM) sodium citrate buffer; 1.5 mM (1.7 mM) EDTA; 1.5 µg / µl (1.7 µg / µl) each containing the protease inhibitors aprotinin, leupeptin and pepstatin A; 60 µl (30µl) enzyme solution; 81 µl (70 µl) final volume; final pH 6.5 (6.0). The enzyme solution added to both types of reactions contained 20 mM Tris HCl, pH 8.0; 340 mM NaCl; 0.2 mM EDTA; 15 mM 2-mercaptoethanol; 25 µM phenylmethylsulfonyl fluoride. Type B reactions also contained added ovalbumin in some cases. Both Type A and Type B reactions were performed at 37^{o}C.

RESULTS

Detection of automethylated PCM

For these studies, bovine erythrocyte PCM was purified to homogeneity, with the last purification step being anion-exchange chromatography. To test for automethylation, pure PCM was collected in a series of fractions from the anion-exchange column and was then incubated with [^{3}H-*methyl*]S-adenosylmethionine. We then used gel electrophoresis at pH 2.4 to resolve the PCM polypeptide under conditions that would preserve protein methyl esters. The PCM electrophoretic band was first visualized by a protein stain (Figure 2.1A), and then found to contain [^{3}H]radioactivity by autofluorographic exposure of the gel to photographic film (Figure 2.1B). The [^{3}H]radioactivity in the PCM polypeptide was in a [^{3}H]methyl ester linkage, since base-treatment of the electrophoretic band produced volatile [^{3}H]methanol (Figure 2.1C).

Time course measurements show that PCM automethylation plateaues with only a subfraction of PCM molecules becoming methylated (Figure 2.2), indicating that automethylation is limited to a subpopulation of damaged PCM molecules. Even though the reactions were performed at pH 6 to minimize hydrolysis of PCM methyl esters, long time-courses such as this underestimate the size of the subpopulation that is methylated, since spontaneous hydrolysis of methyl esters is a competing reaction as shown by experiments (for example, see below) in which excess unlabeled AdoMet was added midway in a

FIGURE 2.1:

A) Electrophoresis and Coomassie Blue staining of purified PCM following its reaction with [^{3}H-*methyl*]S-adenosylmethionine. Aliquots (60µl) of PCM from anion-exchange chromatography fractions 48 through 56 were [^{3}H]methylated for 41 hours under Type A reaction conditions (see Experimental Procedures). One-half of the reaction mixtures were electrophoresed at pH 2.4 (Fairbanks and Avruch, 1972), and Coomassie staining revealed the electrophoretic bands shown here. The migration of molecular weight standards is indicated on the left. The arrow indicates the expected migration of the PCM polypeptide. B) Autofluorographic detection of [^{3}H]radioactivity. The gel was processed for autofluorography as described (Chamberlain, 1979). Shown here is a 21 day autofluorogram. C) [^{3}H]Protein methyl esters measured in individual gel slices. A pH 2.4 SDS polyacrylamide gel prepared identically as in "A" was divided into individual lanes, and 1 cm slices from the lanes were analyzed for [^{3}H]methanol evolved by base hydrolysis of [^{3}H]protein methyl esters (Chelsky, et al., 1984). The numbers 50 through 54 indicate the anion-exchange fraction. The migration distances of molecular weight standards are indicated at the top of the figure. The arrow shows the expected migration of the PCM polypeptide.

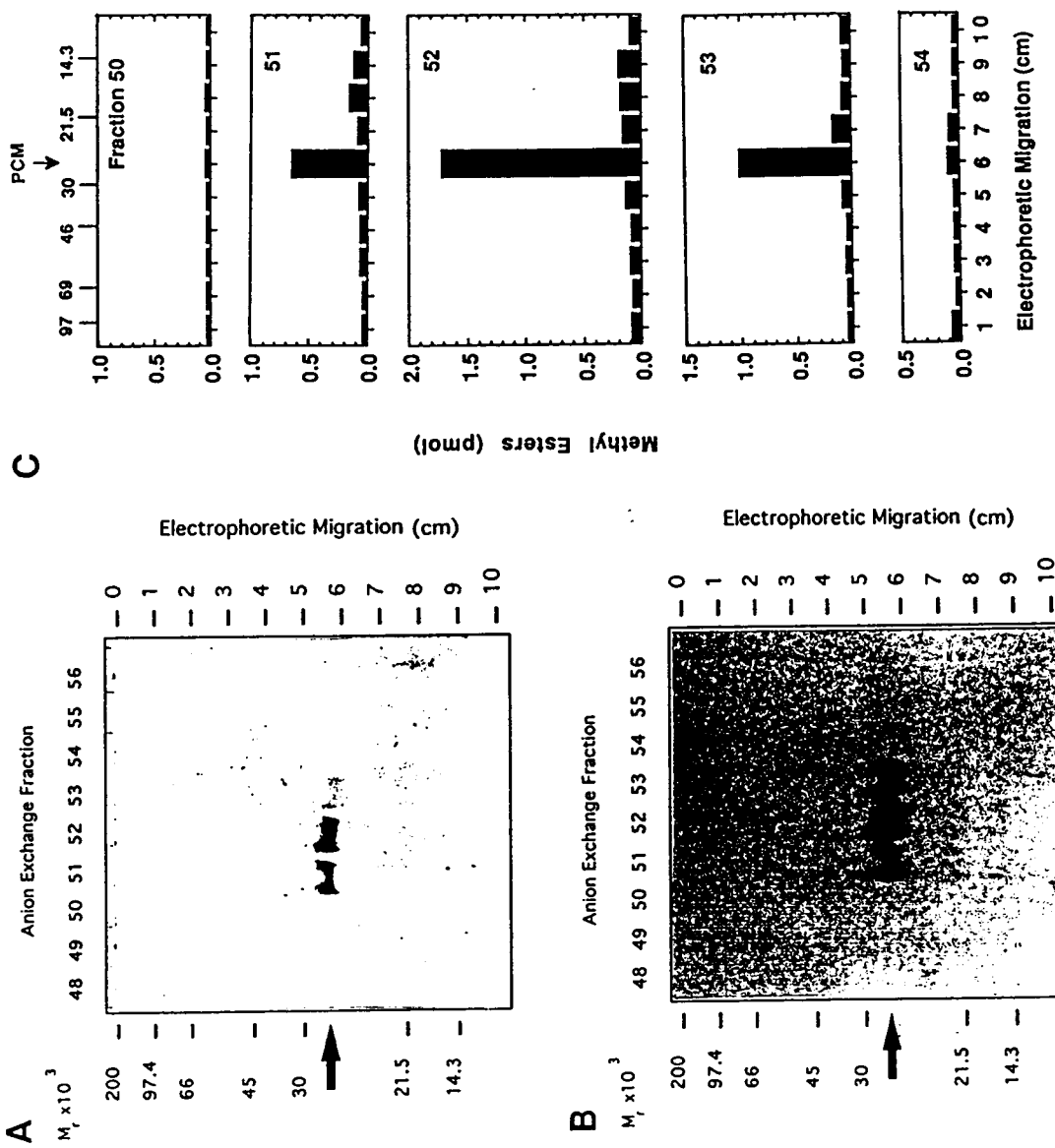
A
B
C
Anion Exchange Fraction
Electrophoretic Migration (cm)
Methyl Esters (pmol)
PCM
Fraction 50
51
52
53
54

FIGURE 2.1

FIGURE 2.2:

Time course of incorporation of methyl esters into the PCM polypeptide. Anion-exchange purified PCM (from a duplicate purification to that used for Figures 2.1 A-C, using bovine erythrocytes from a second animal) was reacted with [^{3}H-methyl]S-adenosylmethionine under Type B conditions (see Experimental Procedures) for the times shown, and then electrophoresed at pH 2.4. The methyl esters incorporated into the electrophoretically separated PCM polypeptide band are shown in fmols (left ordinate) and as a percentage of the total PCM in the reaction mixtures (right ordinate). Error bars are not shown for these single point determinations. The curve shown is an arbitrarily chosen "best fit".

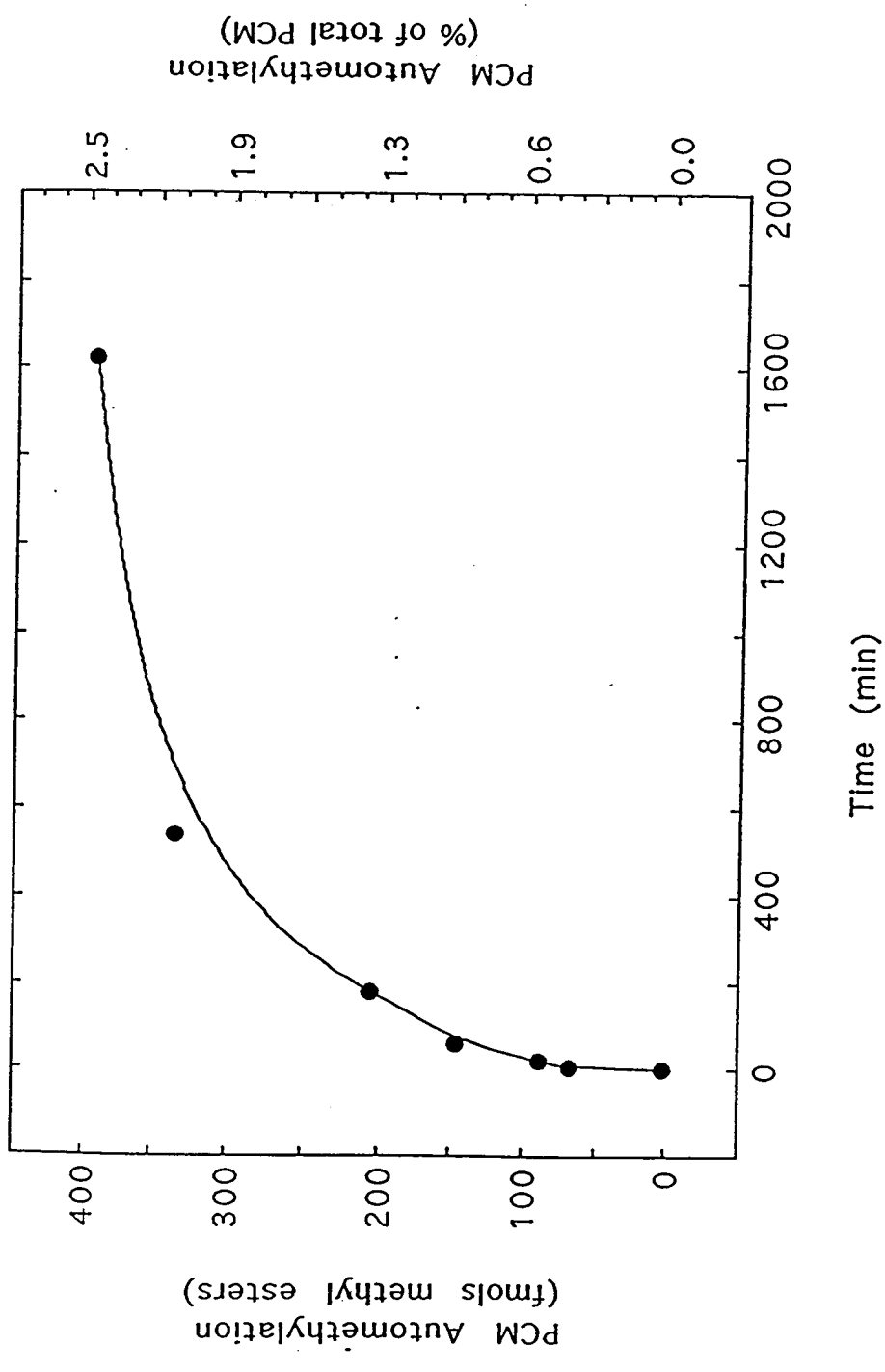
PCM Automethylation
(fmols methyl esters)
400
300
200
100
0
0
400
800
1200
1600
2000
Time (min)
2.5
1.9
1.3
0.6
0.0
PCM Automethylation
(% of total PCM)

FIGURE 2.2

[^{3}H]methylation time-course and the extent of [^{3}H]automethylated PCM was found to slowly decrease.

The subpopulation of PCM that is methylated can be chromatographically enriched as seen by closer scrutiny of the elution behavior of PCM on anion exchange chromatography. For example, calculations based on the data in Figure 2.1C show that the percentages of PCM molecules automethylated in Fractions 51, 52, and 53 were, respectively, 0.3%, 0.8%, and 5.0% of the total protein. PCM which automethylates is therefore more negatively charged and elutes later from the anion-exchange column than does the bulk enzyme. We will use the term αPCM to refer to the subpopulation of PCM molecules that are automethylated. The increased negative charge of αPCM molecules suggests that αPCM is formed by spontaneous deamidation of PCM, consistent with the proposal that the deamidation of L-asparagine residues (neutrally charged) is a major route for producing D-aspartyl and L-isoaspartyl residues (negatively charged) during aging (Aswad, 1984; Murray and Clarke, 1984; reviewed by Wright, 1991).

To verify that the subpopulation of [^{3}H]automethylated protein is in fact PCM and not some unrelated protein, a peak-fraction and a late-eluting fraction of anion-exchange purified PCM were reacted with [^{3}H-*methyl*]S-adenosylmethionine and then resolved by HPLC. Both samples yielded a sharply resolved HPLC fraction as detected by UV absorbance that was confirmed to be PCM both by enzyme activity and immunoreactivity (Figure 2.3A). Quantification of the number of methyl esters per PCM polypeptide then verified the higher content of [^{3}H]methyl esterified αPCM in the late- versus the peak-fraction (0.8% versus 0.1% automethylation; Figure 2.3B). Thus, by independent high

FIGURE 2.3:

High-pressure liquid chromatography of [3H]automethylated PCM. A) HPLC elution profile of UV-absorbing species after [^{3}H]automethylation of PCM. Left chromatogram: An aliquot (60 µl) from an anion-exchange fraction containing the peak of PCM activity was reacted with [^{3}H-*methyl*] S-adenosylmethionine for 18 hours (Type B conditions, see Experimental Procedures) and then injected onto a reversed phase (C4) HPLC column. Elution by acetonitrile gradient yielded the chromatogram shown here of reaction components absorbing at 280 nm. The arrow marks the position of the sharp peak attributable to the PCM polypeptide, the other peaks being evident as background species in similarly analyzed reaction mixtures in which PCM was not present (data not shown). The left inset shows the units of PCM activity surviving the denaturing HPLC conditions in fractions 24-28 in a parallel HPLC analysis of nonradiolabeled PCM (see Experimental Procedures for unit definition). The right inset shows Western blot detection of the PCM polypeptide (horizontal arrow; M_r=27,000) in HPLC fractions 25-27 by specific PCM antiserum and colorimetrically detectable alkaline-phosphatase-linked secondary antibody. A small amount of dimerized PCM (M_r=54,000) was also immunodetectable, with control experiments showing that it was formed after the enzyme incubation, probably during sample lyophilation. Right chromatogram: An aliquot (60 µl) from an anion-exchange fraction immediately following the peak of PCM activity, and hence enriched in αPCM, was similarly reacted with [^{3}H-*methyl*]S-adenosylmethionine. Only a ten-fraction window of the right-hand chromatogram is shown, as the rest of the chromatogram was essentially identical to the 60-fraction chromatogram on the left. B) HPLC elution of [^{3}H]radioactivity. HPLC fractions from the two runs in "A" were assayed for their content of [^{3}H] volatile radioactivity in a vapor-phase assay for protein methyl esters (Chelsky, et al., 1984) (●).

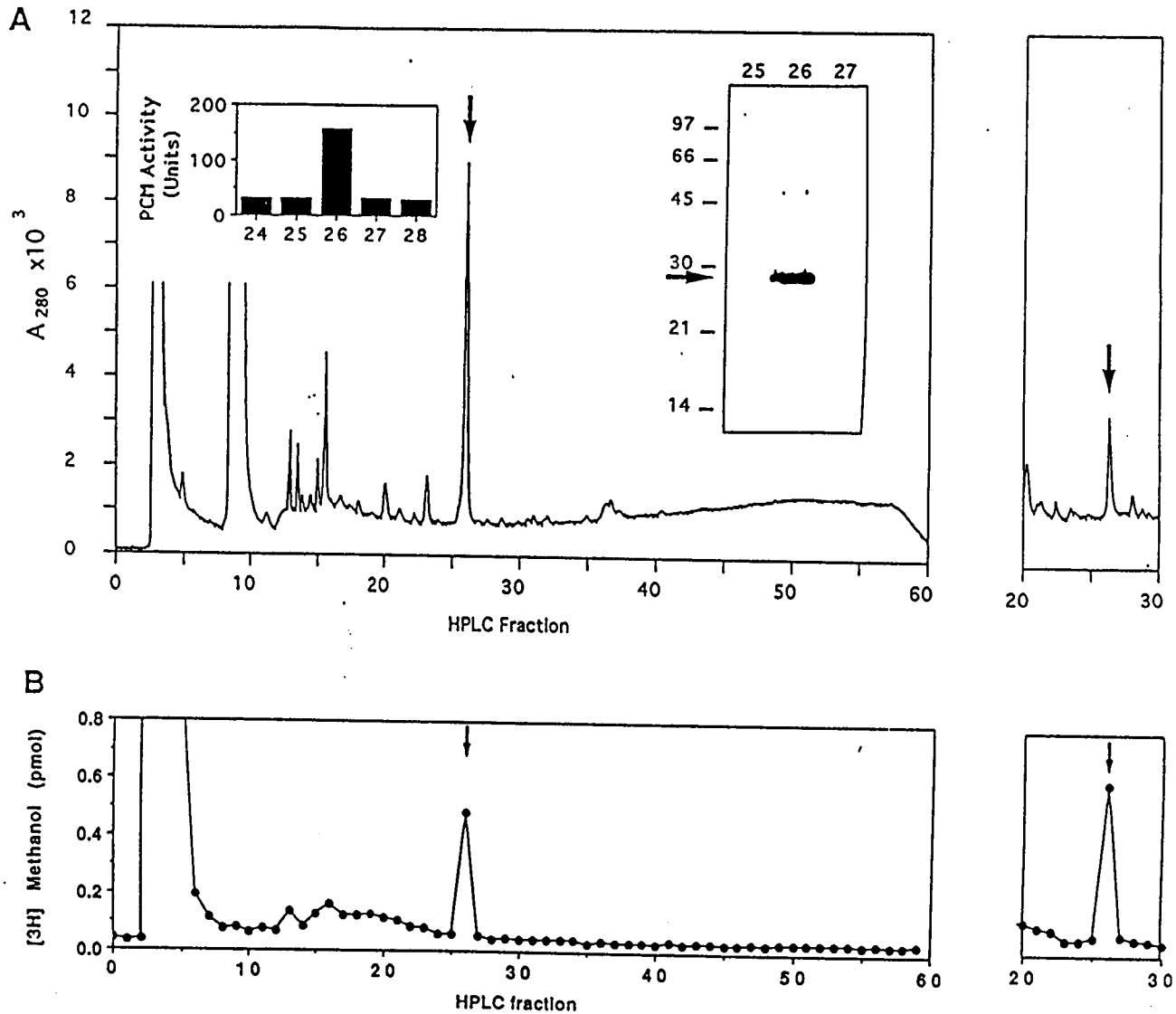
A
12
10
8
6
4
2
0
A_{280} x10 ^{3}
PCM Activity (Units)
200
100
0
24 25 26 27 28
25 26 27
97
66
45
30
21
14
0 10 20 30 40 50 60
HPLC Fraction
20 30
B
[3H] Methanol (pmol)
0.8
0.6
0.4
0.2
0.0
0 10 20 30 40 50 60
HPLC fraction
20 30

FIGURE 2.3

resolution techniques, gel electrophoresis and HPLC, PCM is seen to automethylate its αPCM subpopulation. The αPCM detected by the automethylation reaction is likely to have been formed within the erythrocyte and not during enzyme isolation, enzyme storage, or during the automethylation reaction itself since we have observed no dependence of αPCM content on enzyme storage at 4°C, -20°C or -80°C for up to several months.

Base-lability of the methyl linkage in automethylated PCM

The [^{3}H]radioactivity in the PCM polypeptide appeared to be in a [^{3}H]methyl ester linkage, since base-treatment of the electrophoretic band produced volatile [^{3}H]methanol in the above experimental measurements. [^{3}H]Radioactivity derived by base-treatment of [^{3}H-*methyl*]PCM remained volatile at both extremely acidic and basic pHs showing that neither ionizable [^{3}H]methylamine (Najbauer, et al., 1991) or [^{3}H]methylmercaptan contributed to the volatile radioactivity, and thereby excluding arginine, methionine and cysteine as the methylated site in PCM. Additionally, the hydrolysis of PCM methyl esters to [^{3}H]methanol at varying pH was measured as described (Terwilliger and Clarke, 1981). The PCM methyl esters were found to hydrolyze with a $t_{1/2}$ of less than 30 minutes at pH 8.5 and 37°C; a highly base-labile behavior clearly characteristic of the D-aspartyl and/or L-isoaspartyl class of methyl esters and not of esters of glutamic acid residues or of the C-terminal carboxyl group. The [^{3}H]methyl esters in the PCM polypeptide thus are incorporated into D-aspartyl and/or L-isoaspartyl residues, establishing that PCM methylates its own sequence by the same chemical linkage used in the methylation of other age-damaged proteins.

Evidence against the methyl ester being an active site intermediate

One possible explanation was that upon binding [^{3}H-*methyl*]AdoMet, the methyl group is transferred to PCM in a methyl ester linkage as a covalent intermediate involved in enzyme catalysis, and that this active-site methyl group remains bound to the enzyme in the absence of a protein substrate. To test this possibility PCM was incubated with [^{3}H-*methyl*]AdoMet for a period of time (60 minutes) sufficient to methylate 0.5% of the PCM molecules. At this time, addition of excess unlabeled AdoMet and excess ovalbumin, a model aged protein substrate, did not result in a chasing of [^{3}H]methyl esters off of the PCM polypeptide in the next 60 minutes (Figure 2.4, left lane). The [^{3}H]methylation of ovalbumin that occurred despite the large isotopic dilution of AdoMet is fully in agreement with expectations based on the known stoichiometry of ovalbumin methylation (~5%; McFadden, 1983; Lowenson and Clarke, 1991) and on the AdoMet specific activity achieved in the experiment. Addition of unlabeled AdoMet and no ovalbumin for the 60 minute chase period led to fewer [^{3}H]methyl esters in PCM than did the chase with ovalbumin (Figure 2.4, center lane), showing that, if anything, ovalbumin slightly stabilizes the methyl esters in PCM. A control addition of buffers with neither ovalbumin or AdoMet at 60 minutes shows that the final level of PCM [^{3}H]automethylation was identical to that attained with additions of ovalbumin and unlabeled AdoMet (Figure 2.4, right lane). Thus, there is no evidence that methyl esters in PCM can be subsequently transferred to another protein.

FIGURE 2.4.

Gel electrophoresis and autofluorography of [^{3}H-*methyl*]esterified PCM after incubation in the presence of a protein substrate. Three samples of PCM (1.5 μM; containing 2% αPCM) were incubated (Type B conditions) with [3H-methyl]S-adenosylmethionine for 60 minutes. At this time additions one of the following additions was made: Left lane, 5 μl ovalbumin (2 mg ml-1 in 0.2 M sodium citrtae, pH 6) together with 5 μl of AdoMet (5 mM in 10 mM HCl); Center lane, 5 μl of pH 6 citatrate buffer together with 5 μl of AdoMet in 10 mM HCl; Right lane, 5 μl of citrate buffer together with 5 μl of 10 mM HCl. Incubations were continued for an additional 60 minutes at 37°C, after which the samples were electrophoresed on a pH 2.4 SDS polyacrylamide gel that was processed for autofluorography. Shown here is a 16 day autofluorogram. The migration distances of protein molecular weight standards are indicated, including the migration distance of ovalbumin at 45,000. The migration of the PCM polypeptide is indicated by the arrow.

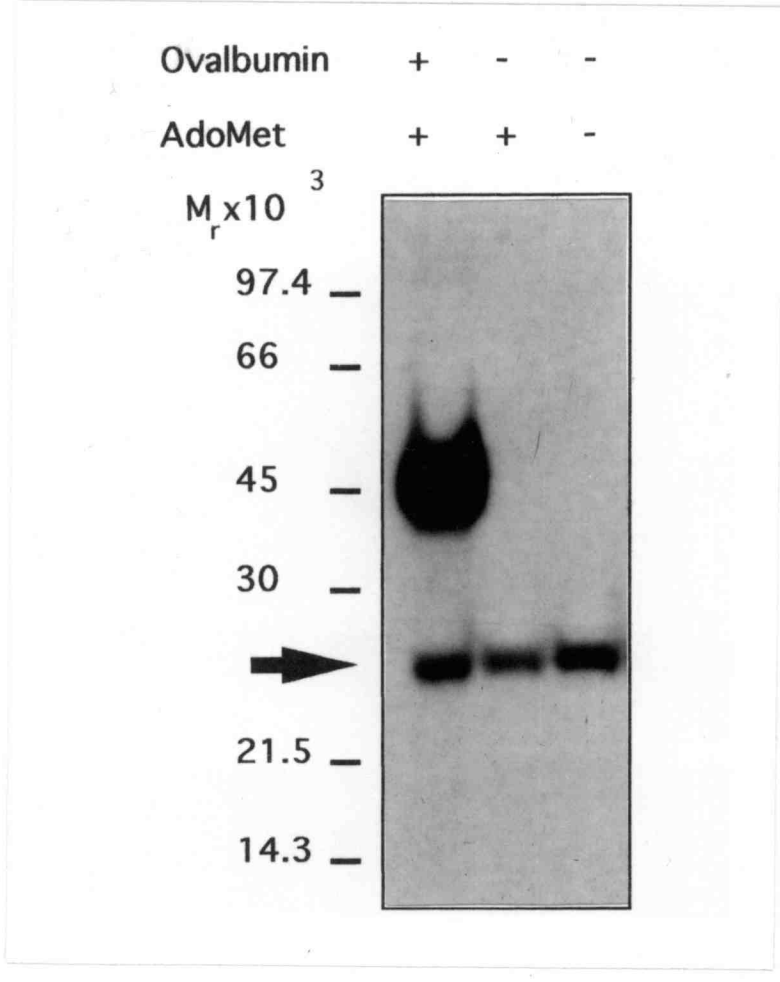

FIGURE 2.4

Dilution behavior of PCM automethylation

We next tested whether PCM automethylation is an intramolecular or intermolecular reaction. Since dilution of the enzyme would have different effects in these alternative cases, the initial velocity of the [^{3}H]automethylation reaction was measured as a function of enzyme dilution (Figure 2.5). When plotted as the percentage of PCM that is automethylated per unit time, the case of an intramolecular automethylation reaction would have yielded a horizontal line since enzyme dilution would not affect the percentage of enzyme molecules that are automethylated. However, the rate of automethylation of PCM varied with enzyme dilution, consistent with an intermolecular methylation reaction occurring between PCM molecules.

DISCUSSION

This work demonstrates the automethylation of protein carboxylmethyltransferase, and has interesting implications as an example of an enzyme pool that acts upon its own aged-damaged structures. Methyl esterification of aging proteins is a well-established process in living cells, and many proteins have been shown to be methylated at L-isoaspartate and D-aspartate sites. Automethylation of PCM thereby shows that the enzyme is susceptible to the same type of protein damage that affects its substrates.

The intermolecular nature of the automethylation reaction suggests that methylation of αPCM is mechanistically similar to the methylation of other age-damaged protein molecules, with an active PCM molecule methylating a damaged PCM molecule. However,

FIGURE 2.5:

The effect of dilution on the rate of PCM automethylation. The initial rate of PCM automethylation (percent of the total PCM methylated per minute) was measured as a function of enzyme dilution under Type B reaction conditions. The data points represent three experimental measurements of a sample of highly purified PCM that was obtained as a late-eluting fraction from anion exchange chromatography, and was measured to contain about 9% (+- 1%) αPCM. The reaction mixtures contained various volumes of purified PCM with the remaining volumes being replaced with buffer containing identical salts as in the purified PCM solution so that the final reactions volumes were 70 µl at the enzyme concentrations shown here. Reactions were quenched after either 20 minutes (■, □), or after 40 minutes (●). Incorporation of [^{3}H]methyl esters into PCM was measured after pH 2.4 SDS polyacrylamide gel electrophoresis, with determination of [^{3}H]methanol released from the excised gel slice containing the Coomassie Blue-stained PCM polypeptide. The curves shown were generated using identical constants obtained from linear and non-linear least squares fitting. An assumed error of up to 20% in the data yields only a very small variation in the value of K_s (less than 5%) (Segel 1975).

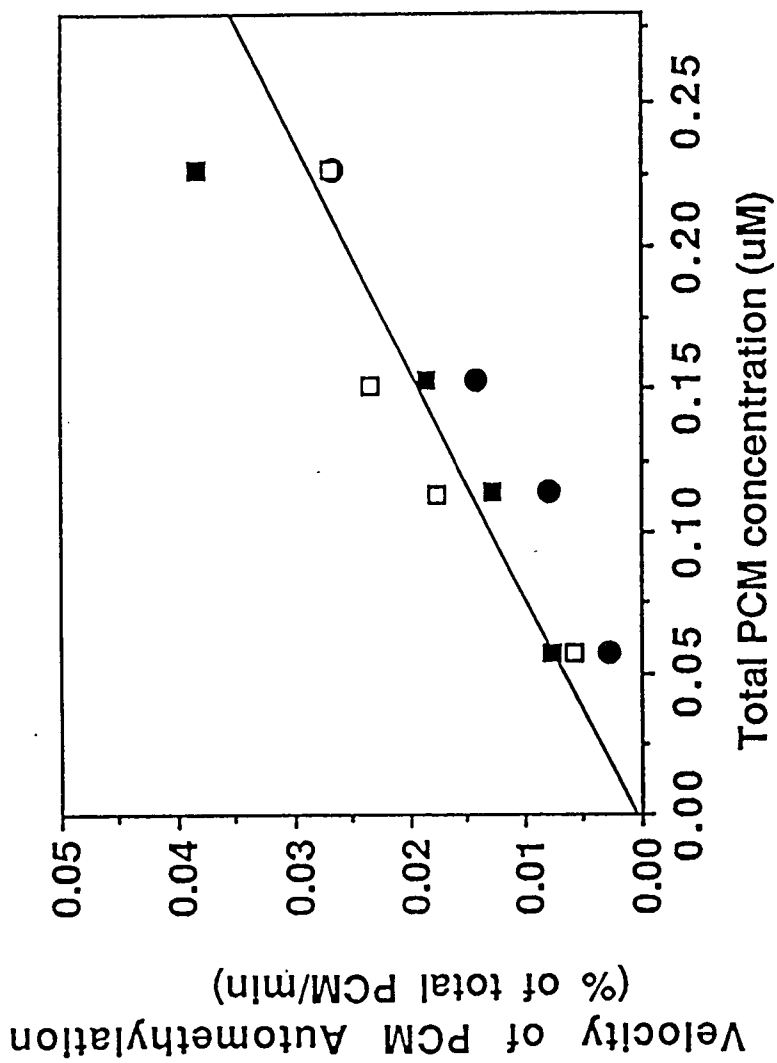
Velocity of PCM Automethylation
(% of total PCM/min)
0.05
0.04
0.03
0.02
0.01
0.00
0.00
0.05
0.10
0.15
0.20
0.25
Total PCM concentration (uM)

FIGURE 2.5

depending on the function of methylation, a self-methylating pool of enzyme could allow for feedback effects that do not exist for the methylation of other proteins. For example, if methylation leads to the self-repair of the PCM pool, then the automethylation reaction could be viewed as a process that determines the efficiency with which all other aging proteins can be repaired by PCM. Such "repair of the repair system" might have added importance in a terminally differentiated cell such as the erythrocyte where PCM is not manufactured de novo.

The precise amino acid sites at which PCM is damaged and automethylated are under investigation in our laboratory. Deamidation of a single asparagine residue would cause a shift in the isoelectric point of PCM from approximately 6.5 to approximately 6.3. This calculation is based on simple pK_a values for functional groups with no interaction between functional groups, an acetylated N-terminus (Ingrosso, et al., 1989), and no other charge modifications occurring in the sequence of PCM. This increased negative charge could account for the enrichment of αPCM in the late-eluting enzyme fractions from the high-resolution anion exchange column we employed in purifying PCM. There are between five and seven asparagines in the PCM primary sequence from several mammalian species (Ingrosso, et al., 1989; Henzel, et al., 1989; Sato, et al., 1989; Romanik, et al., 1992). Asn-Gly sequences are known to be particularly prone to deamidation, so a clear candidate for a site of automethylation is the Asn-Gly that is found near the N-terminus in the primary sequence of each of these sequences. It will be interesting to learn whether the homologous site in bovine erythrocyte PCM, upon spontaneous deamidation and aspartyl isomerization, provides a site for enzyme automethylation. Asparagines adjacent to amino acids other than

glycine are also subject to deamidation at rates that probably depend on the three-dimensional structure of a protein (Clarke, 1987; see Wright, 1991), and so the other asparagines in PCM are also potentially sites of spontaneous deamidation and automethylation. It is also possible that spontaneous isomerization of L-aspartic acid to L-isoaspartic acid or D-aspartic acid provides the automethylation site, though in this case a coincident mechanism for making the PCM molecule more negatively charged would have to be postulated.

Our data do not distinguish whether the age-damaged subpopulation of αPCM molecules are themselves active catalysts in methyl group transfer. This will remain an open question until αPCM can be further purified for a direct test of its activity. It may be difficult to purify αPCM to homogeneity using anion exchange chromatography since in addition to PCM molecules that are methylated at either L-isoaspartyl or D-aspartyl sites, a population of PCM molecules with normal L-aspartyl sites is expected to arise by spontaneous deamidation (and perhaps by repair to L-aspartyl sites). These postulated normal L-aspartyl PCM molecules, with the same charge as αPCM molecules, are probably not substrates in an automethylation since there has never been any evidence for L-aspartyl methylation in other substrates. Immunoaffinity affinity protocols based on monoclonal antibodies against L-isoaspartyl residues (Lehrman, et al., 1992) open an interesting avenue to purification of pure αPCM.

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CHAPTER 3

A DAMAGED SUBPOPULATION OF PROTEIN (D-ASPARTYL/ L-ISOASPARTYL) CARBOXYL METHYLTRANSFERASE IS METHYLATED BY A HIGH AFFINITY, LOW-TURNOVER REACTION

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RATIONALE

Protein (D-aspartyl/ L-isoaspartyl) carboxyl methyltransferase (PCM) was shown to be capable of incorporating a methyl group into its own sequence in a manner consistent with its repair reaction. This automethylation reaction has been shown to be limited to a subpopulation of damaged enzyme molecules, termed αPCM. In establishing the mechanism of the automethylation reaction to be intermolecular, some unusual behavior was observed within these molecules as they automethylate. The purpose of this chapter is to analyze the strange kinetic behavior of the automethylation reaction.

A DAMAGED SUBPOPULATION OF PROTEIN (D-ASPARTYL/ L-ISOASPARTYL) CARBOXYL METHYLTRANSFERASE IS METHYLATED BY A HIGH AFFINITY, LOW-TURNOVER REACTION.

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KEYWORDS: Protein methylation/ Enzyme kinetics/ Aging

SUMMARY

Pools of protein D-aspartyl/L-isoaspartyl carboxylmethyltransferase (PCM) are self-methylated on a subpopulation of age-damaged enzyme molecules termed the αPCM fraction. The specific rate of αPCM methylation plateaus at high concentrations of total PCM, indicative of a saturating reaction. The assumption of a rapid equilibrium in the interaction between αPCM and active PCM has allowed the derivation of a rate equation that lends a good theoretical fit to the concentration-dependence of the self-methylation reaction. These kinetics show that PCM has a higher affinity for αPCM than for most other age-damaged protein substrates, which by itself would suggest that the methylation and repair of age-damaged PCM takes priority over the other activities of PCM. However the kinetic turnover number for αPCM is very low, indicating that the binding

of αPCM to PCM results in nearly a dead-end complex. The function of PCM self-methylation is thus mysterious since at face value these kinetic constants suggest that kinetic preoccupation with αPCM could lower the effectiveness of the PCM toward the methylation and repair of other damaged proteins.

INTRODUCTION

To address the question of self-recognition of protein damage by an enzyme, we tested whether a mammalian enzyme involved in aging metabolism, protein carboxyl methyltransferase (PCM), can recognize and methylate amino acid damage in its own sequence. PCM is known to transfer the methyl group from S-adenosylmethionine into methyl ester linkages with D-aspartyl and L-isoaspartyl amino acid residues formed by spontaneous epimerization and deamidation reactions in a wide range of cytoplasmic and membrane proteins (Ota and Clarke, 1990; Johnson and Aswad, 1990). As reported (Lindquist and McFadden, 1994), a subpopulation of purified PCM from bovine erythrocytes, termed the αPCM fraction, does in fact become methylated upon incubation with [^{3}H-*methyl*]S-adenosylmethionine. αPCM molecules make up approximately 1% of the total PCM population. Such a low stoichiometry of methylation is typical for the methylation of aged protein substrates since proteins isolated from living tissues typically contain substoichiometric levels of D-aspartyl and L-isoaspartyl residues. The purpose of the following note is to consider the unusual kinetics that arise in the self-modification of a small subpopulation of enzyme molecules.

RESULTS AND DISCUSSION

Since we have not yet been able to purify αPCM away from the bulk of PCM, the primary tool for investigating the kinetics of αPCM methylation has been to test the rate of αPCM methylation as a function of the total concentration of PCM. In these experiments, the specific rate of αPCM methylation per unit time (e.g. methylated αPCM molecules per total PCM molecules per minute) clearly increases as the total concentration of PCM is increased. This argues convincingly against an intramolecular mechanism in which αPCM molecules bind S-adenosylmethionine and transfer a methyl group to their own polypeptide sequence. Instead, the dependence of the reaction rate on PCM concentration indicates that more than a single PCM molecule is involved in methylation of an αPCM molecule. The simplest interpretation of these studies is that an αPCM molecule is methylated by a second PCM molecule.

Detailed measurements show that the increase in αPCM methylation with total PCM concentration is nonlinear, with the specific rate of αPCM methylation tending toward a plateau at high concentrations of total PCM. While this plateauing behavior is indicative of a saturating reaction, conventional approaches of enzyme kinetics cannot be easily applied since the substrate, αPCM, is vastly exceeded in its concentration by active PCM. However, the assumption of a rapid equilibrium in the interaction between αPCM and active PCM has allowed the derivation of a rate law that lends a good theoretical fit to our dilution experiments.

Derivation of a kinetic model describing αPCM methylation

At any dilution tested, the total pool of methylatable αPCM is assumed to be a constant fraction, α, of the total enzyme pool

$[\alpha PCM]_{tot} = \alpha[PCM]_{tot}$. (1)

It is assumed that methylation of αPCM occurs within a reversibly formed enzyme-substrate complex, PCM * αPCM, with the rate of methylation in the absence of any significant reverse reaction being given by

$v = k_p [PCM * \alpha PCM]$. (2)

k_p in the above equation is the rate constant for product formation, i.e. the turnover number for catalysis, whose value we wish to measure for comparison to other measured turnover numbers for PCM. The concentration of uncomplexed αPCM is given by

$[\alpha PCM] = [\alpha PCM]_{tot} - [PCM * \alpha PCM]$. (3)

Since α is a small number ($\alpha << 1$), the concentration of unbound active enzyme is assumed to be negligibly changed by the formation of the enzyme-substrate complex, giving the expression for free enzyme

$[PCM] = [PCM]_{tot} - [PCM * \alpha PCM] \cong [PCM]_{tot}$. (4)

Since the formation of product is trivially slow, due to the small size of k_p as will ultimately be determined, an expression for the rate of αPCM methylation at any enzyme dilution can be derived using the assumption of a rapid equilibrium between free enzyme, free substrate and the enzyme-substrate complex. The expression for that dissociation constant is

$K_S = [\alpha PCM]\,[PCM] / [PCM * \alpha PCM]$. (5)

Algebraic manipulation of the above five equations yields the following expression for the rate of αPCM methylation,

$v = \alpha k_p ([PCM]_{tot})^2 / (K_s + [PCM]_{tot})$, (6).

Equivalently, the following equation for a hyperbola yields the specific rate, v', of αPCM methylation as a function of the total PCM concentration

$v' = v/[PCM]_{tot} = \alpha k_p [PCM]_{tot} / (K_s + [PCM]_{tot})$ (7).

Using the above relationships, k_p ($0.0095 min^{-1}$) and K_S (0.5 μM) were calculated from experimental data for v, α, and $[PCM]_{tot}$ by curve-fitting.

The kinetic model derived above implicitly chooses a case in which αPCM molecules are capable of methylating other αPCM molecules in the relatively rare bimolecular encounters between two αPCM molecules. If instead it is assumed that αPCM molecules are inactive as catalysts because they are damaged, the derived k_p for αPCM methylation changes by an immeasurably small margin, toward a lower value than discussed given here.

Implications of αPCM methylation

The values for K_S and k_p point to a specialized reaction unlike other PCM methylation reactions. The turnover number for αPCM methylation, k_p, is one- to two-orders of magnitude lower than for the methylation of any other polypeptide by PCM (Lowenson and Clarke, 1991), suggestive of a rather stable enzyme-substrate complex

that yields methylated αPCM only very slowly. A turnover number this low allows the assumption to be made that the dissociation constant for the complex, K_s, calculated to be 0.50 μM, is essentially equal to the K_m (Michaelis constant) for the methylation of αPCM by active enzyme. A K_m of 0.50 μM is among the lowest for any known reaction catalyzed by PCM (Ota and Clarke, 1990; Lowenson and Clarke, 1991), signifying a high-affinity reaction for αPCM methylation. Given the PCM concentration in the living cell of about 5 μM, the measured K_s for αPCM methylation indicate that more than 90 in 100 cellular αPCM molecules could be found in a reversible complex with active PCM, with little competition from other substrate proteins due to their generally higher K_m values. However, given the low k_p for αPCM methylation, fewer than 1 in 100 αPCM molecules would become methylated each minute. This combination of high affinity and low turnover suggests that as more αPCM is formed by spontaneous aging, the enzyme could conceivably become self-occupied by its slow self-methylation reaction, interfering with the methylation and further metabolic processing of other age-damaged proteins.

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CHAPTER 4

DETERMINATION OF THE AUTOMETHYLATION SITES IN BOVINE ERYTHROCYTE PROTEIN (D-ASPARTYL/L-ISOASPARTYL) CARBOXYL METHYLTRANSFERASE

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RATIONALE

Protein (D-aspartyl/ L-isoaspartyl) carboxyl methyltransferase (PCM) was shown to be damaged in a manner consistent with the presence of altered aspartyl residues. The sites of altered aspartyl formation have been determined in several other proteins using a combination of proteolytic enzymes and chemical cleavage techniques. The purpose of this chapter is to apply these techniques to identify the sites of altered aspartyl residues within PCM.

DETERMINATION OF THE AUTOMETHYLATION SITES IN BOVINE ERYTHROCYTE PROTEIN (D-ASPARTYL/L-ISOASPARTYL) CARBOXYL METHYLTRANSFERASE

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Bovine erythrocyte protein (D-aspartyl/ L-isoaspartyl) carboxyl methyltransferase was shown to possess a subpopulation of damaged molecules that were susceptible to methylation (Lindquist, J.A. and McFadden, P.N. (1994) *J. Prot. Chem.* 13, 23-30.), termed the αPCM fraction. Using a variety of enzymatic and/ or chemical methods in combination with matrix-assisted laser desorption/ ionization (MALDI) mass spectrometry, two sites of automethylation were identified within the PCM molecule, aspartate-217 located near the C-terminus of the protein and asparagine-188. The identification of not one, but two sites of automethylation, suggests that αPCM is not a homogeneous population of damaged PCM molecules, but rather a subpopulation of molecules with a hierarchy of damage sites (α_1, α_2, α_3,...). The effects of this damage upon the activity of PCM are not yet known.

INTRODUCTION

The processes of spontaneous damage and its repair have recently become a focus for researchers interested in the causes of aging, cell death, and certain disease pathologies (Stadtman 1992, Stadtman 1988, Roher, et al. 1993). Of the many forms of spontaneous damage identified in proteins, several are known to occur in an age-dependent manner. Whether the age-dependent increase in protein damage is the result of an increase in the rate of damage or merely the accumulation of damaged products has yet to be determined. One such age-dependent process is the spontaneous deamidation of asparagine or the isomerization and racemization of aspartate that leads to the formation of L-isoaspartyl and D-aspartyl residues in proteins. The mechanism leading to this damage has been well studied (Robinson and Rudd 1974, Clarke 1987, Wright 1991) and awareness of its existence and the possible consequences has grown significantly in the past few years. One interesting aspect of this damage reaction, which makes it perhaps even more intriguing, is the existence of a putative repair system, the enzyme protein (D-aspartyl/ L-isoaspartyl) carboxyl methyltransferase (PCM) [EC 2.1.1.77]. The repair reaction catalyzed by PCM has also been well characterized in recent years (McFadden and Clarke 1987, Johnson, et al. 1987a, Johnson, et al. 1987b, Brennan, et al. 1994). Since protein damage is spontaneous, an important question to address is, "What is the effect of damage upon the repair systems themselves?" Within its primary sequence (Gilbert, et al. 1988), PCM contains many conserved aspartyl and asparaginyl residues. Therefore, it is not unreasonable to assume that PCM becomes damaged like other proteins and can serve as a substrate for its own methylation reaction. Recently, this

question was addressed by showing that PCM is indeed damaged and is capable of automethylating; [^{3}H] methyl groups are incorporated into its own sequence in a manner consistent with its repair reaction (Lindquist and McFadden 1994a). In characterizing this reaction, it was discovered that automethylation was limited to a subpopulation of enzyme molecules that are damaged (1-2% PCM $_{Total}$). The reaction of PCM with these molecules showed very interesting kinetic properties: a high affinity interaction (Km = 0.5 μM) with very slow turn-over (k_p = 0.0095 min.$^{-1}$) (McFadden and Lindquist 1994b). But, which site(s) in the PCM molecule undergoes this type of damage? To address these questions, a combination of radiolabelling and peptide mapping methodologies (Ota, et al. 1987, Ota and Clarke 1989, Potter, et al. 1993) were utilized to isolate the site(s) of damage within the PCM molecule.

EXPERIMENTAL METHODS

PCM purification

PCM was purified following the method of Gilbert, et al. (1988) with some modification as previously published Lindquist and McFadden (1994a). Briefly, 1L of bovine blood was centrifuged at 3,000 rpm (1400 x g) for 10 minutes to pellet the erythrocytes and the plasma aspirated off. Erythrocytes were washed 4 times in 10 mM sodium phosphate/ 150 mM NaCl, pH 7.4 with care taken to aspirate off the buffy coat. Packed erythrocytes were then lysed in 10 volumes of 5 mM sodium phosphate/ 5 mM disodium ethylenediaminetetraacetate (EDTA)/ 10%(v/v) glycerol/ 25 μM

phenylmethylsulphonyl fluoride (PMSF)/ 15 mM 2-mercaptoethanol, pH 8.0 (Buffer A) and frozen in liquid N_2. After thawing, on ice, membranes were pelleted by centrifugation at 10,000 rpm (10,000 x g) for 90 minutes and the supernatant (or cytosol) carefully removed to avoid membrane contamination. The PCM containing fraction was then precipitated in ammonium sulfate (53% saturation) and removed by centrifugation at 11,000 rpm (12,000 x g) for 60 minutes, the pellet containing the PCM had a slightly red appearance. The pellet was resuspended by gentle dounce homogenization in Buffer A followed by dialysis. PCM was then purified by affinity chromatography on S-adenosylhomocysteine-diaminohexyl-Sepharose 4B, followed by a second ammonium sulfate precipitation (55% saturation), gel exclusion chromatography on a Sephadex G-75-120 column, and then finally by FPLC on a Fractogel EMD DEAE 650-S (anion exchange) column. The enzyme eluted from the column in a linear salt gradient in three 2 ml fractions. A final purification factor of 5500-fold was achieved with a specific activity measured at 16,500 units/ mg using the assay method of Murray and Clarke (1 unit equals 1 pmol of methyl groups transferred per minute to ovalbumin at pH 6.0).

Automethylation reactions

Typically, a 90 µl aliquot of FPLC purified PCM, taken from one of the three FPLC fractions containing 40, 730, or 162 pmols of enzyme respectively, was added to 63 µl of 0.2 M sodium citrate buffer, pH 6.0. To which, 2 µl of a protease inhibitor cocktail containing leupeptin, pepstatin A, aprotinin, PMSF, and EDTA was added to prevent possible proteolysis during the incubation. The reaction was prepared on ice, and

initiated by the addition of 2 µl of [^{3}H-*methyl*]AdoMet [15 Ci/mmol]. The reaction was incubated at 37°C for the length of time indicated in the figure legend. Reactions were stopped by freezing in liquid N_2 and [^{3}H]automethylated PCM purified by reversed phase HPLC.

HPLC purification

High pressure liquid chromatography (HPLC) was performed using a deionized (dI) water/ acetonitrile gradient system. Solvent A is dI H_2O/ 0.1%(v/v) TFA [Sigma]. Solvent B is 90%(v/v) acetonitrile [Burdick & Jackson Brand] /9.9%(v/v) dI H_2O/ 0.1%(v/v) TFA. Protein purification was performed using a Vydac C_4 column [particle size 5 µ, 4.6 mm x 250 mm] and component elution monitored at 280 nm. Peptide purification was performed using a Dynamax C_{18} column [particle size 5 µ, 4.6 mm x 250 mm] with elution monitored at 214 nm.

Trypsin digestion

HPLC purified [^{3}H] automethylated PCM was lyophilized to dryness, resuspended in 50 µl of 0.2 M sodium citrate pH 6.0 and digested with TPCK-treated Trypsin (1:10) [Sigma] (2 mg/ml in 1 mM HCl, freshly prepared) for 40 minutes at 37°C. Tryptic fragments were then purified by reversed phase HPLC.

Cyanogen bromide cleavage

HPLC purified [^{3}H] automethylated PCM was lyophilized to dryness, resuspended in 90 µl 70% formic acid and reacted under N_2 for 18 hours at room temperature with 1 µmol of CNBr [Sigma]. Reactions were stored in the dark under nitrogen and allowed to digest overnight at room temperature. CNBr fragments were then purified by reversed phase HPLC.

Mass Spectrometry

Mass determinations were made using a matrix-assisted laser desorption/ ionization (MALDI) mass spectrometer with an accelerating voltage of +24 kV (Jensen, et al. 1993). Lyophilized samples were resuspended in 5 µl 0.1% TFA and mixed 1:1 to 1:5 with a matrix of 4-hydroxy-α-cyano-cinnamic acid (HCCA). The samples were applied to the sample introduction probe, precrystallized, wiped, and reapplied for fast crystallization (Jensen, et al. 19xx). Samples were then rinsed briefly in dI H_2O and inserted into the vacuum. Mass spectra were obtained from 30 laser pulses with a Spectra-Physics Nd:YAG laser at 355 nm.

Assays for radioactivity

One-tenth of each HPLC fraction was assayed for base-volatilized radioactivity by pipetting the sample into a glass inner vial containing an equal volume of 0.1 M NaOH. The inner vials were then placed into 20 ml scintillation vials containing 4 mls of xylene

based liquid scintillation cocktail [Scintiverse E, Fisher] and capped. Samples were allowed to equilibrate for several hours at room temperature before counting. The efficiency of capture of base volatilized methanol was determined by measuring the recovery of [^{14}C]methanol standards equilibrated under identical conditions.

RESULTS

[^{3}H]automethylated PCM was eluted from a reversed phase C_4 HPLC column at approximately 25 minutes as a single, sharp, radiolabelled peak as expected from previous experience. Following digestion with trypsin, [^{3}H]automethylated PCM was resolved using reversed phase HPLC into three peaks that eluted at 5 minutes, 37 minutes, and 39 minutes respectively (see Figure 4.1). The material eluting from the column at 5 minutes was that of [^{3}H]methanol generated from hydrolysis of [^{3}H-*methyl*]esters. This material consists only of volatile radioactivity that was lost when the material was lyophilized to dryness. The 37 minutes and 39 minute peaks both contain base-volatile [^{3}H] radioactivity, characteristic of compounds containing methyl ester linkages. These fractions also correspond to UV detectable material on the HPLC chromatogram. They were submitted for mass spectral analysis. Preliminary data (not shown) revealed several mass peaks associated with these samples. These fractions were re-injected onto the HPLC, to confirm their retention time of elution and to increase their purity. The mass spectral analysis of the repurified material yielded simplified spectra. The material eluting at 37 minutes yielded a spectrum (Figure 4.2) containing a peak at 1964.5 Da which corresponds, by mass, to a 17 amino acid peptide [residues 204-220]

FIGURE 4.1:

Base-volatile [^{3}H] radioactivity profile associate with reversed phase HPLC separation of [^{3}H-*methyl*] esterified peptides derived from tryptic digestion of [^{3}H]automethylated PCM. Following the [^{3}H]automethylation reaction, [^{3}H]automethylated PCM was purified on a Vydac protein C_4 column. The purified [^{3}H]automethylated PCM was lyophilized and then resuspended in 0.2 M sodium citrate pH 6.0 and digested with TPCK-treated trypsin (1:10) for 40 minutes at 37°C. Tryptic fragments were then purified by reversed phase HPLC over a Dynamax C_{18} column in a gradient of +1% acetonitrile per minute at a flow rate of 1.0 ml min.$^{-1}$. One-tenth of each fraction was assayed for base-volatilized radioactivity by adding an equal volume of 0.1 M NaOH to hydrolyze the [^{3}H-*methyl*]esters and allowing the samples to equilibrate for several hours at room temperature. The efficiency of vaporization was determined by measuring the recovery of [^{14}C]methanol standards equilibrated under identical conditions. The peaks corresponding to the tryptic fragments T-1 and T-2 are indicated.

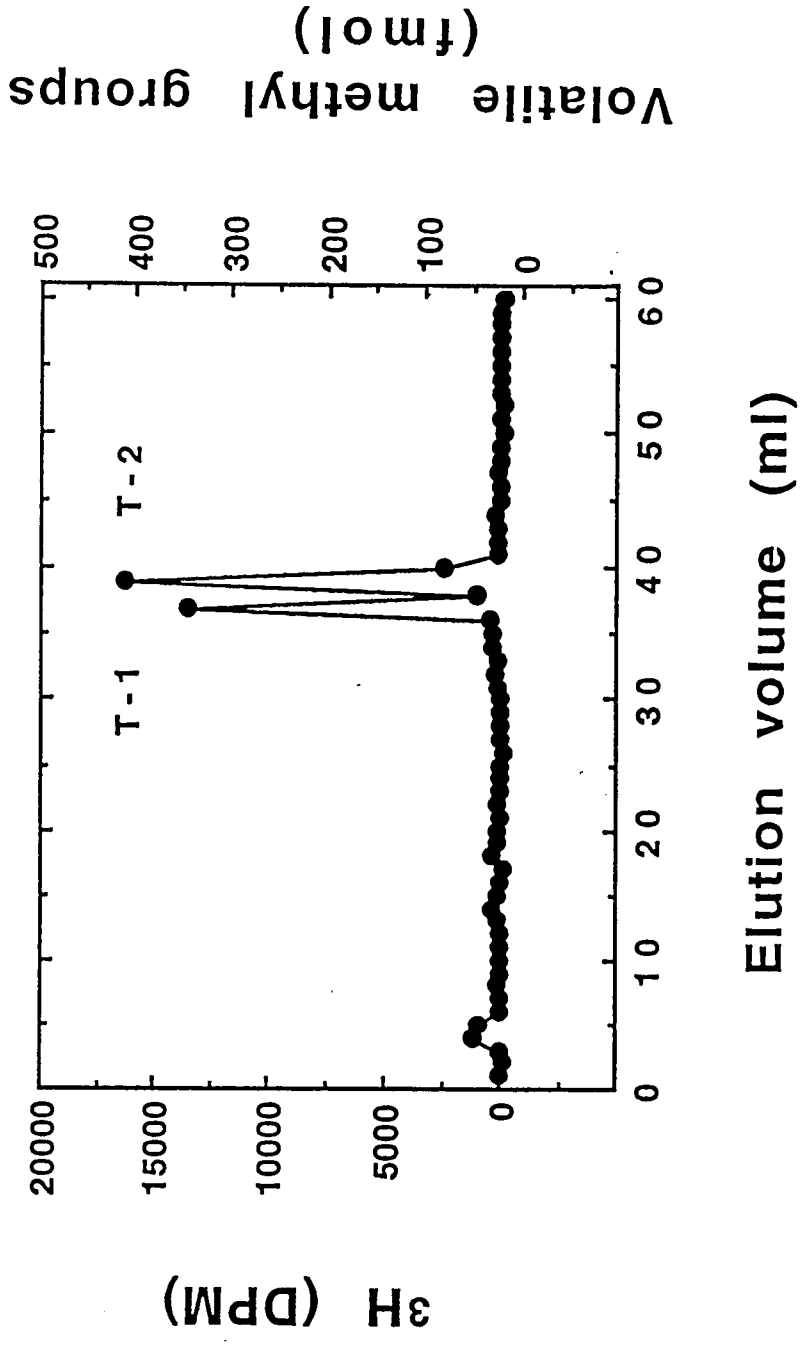
Volatile methyl groups
(fmol)
500
400
300
200
100
0
T-2
T-1
20000
15000
10000
5000
0
3H (DPM)
0
10
20
30
40
50
60
Elution volume (ml)

FIGURE 4.1

FIGURE 4.2:

Mass spectrum obtained using a matrix-assisted laser desorption/ ionization (MALDI) mass spectrometer from the analysis of the tryptic peptide T-1. The mass spectrometer was operated with an accelerating voltage of +24 kV. The lyophilized peptide was resuspended in 0.1% TFA water to an approximate concentration of 100 μM and diluted 1:5 in a matrix of 4-hydroxy-α-cyano-cinnamic acid (HCCA). The spectrum shown is the result of 30 laser pulses.

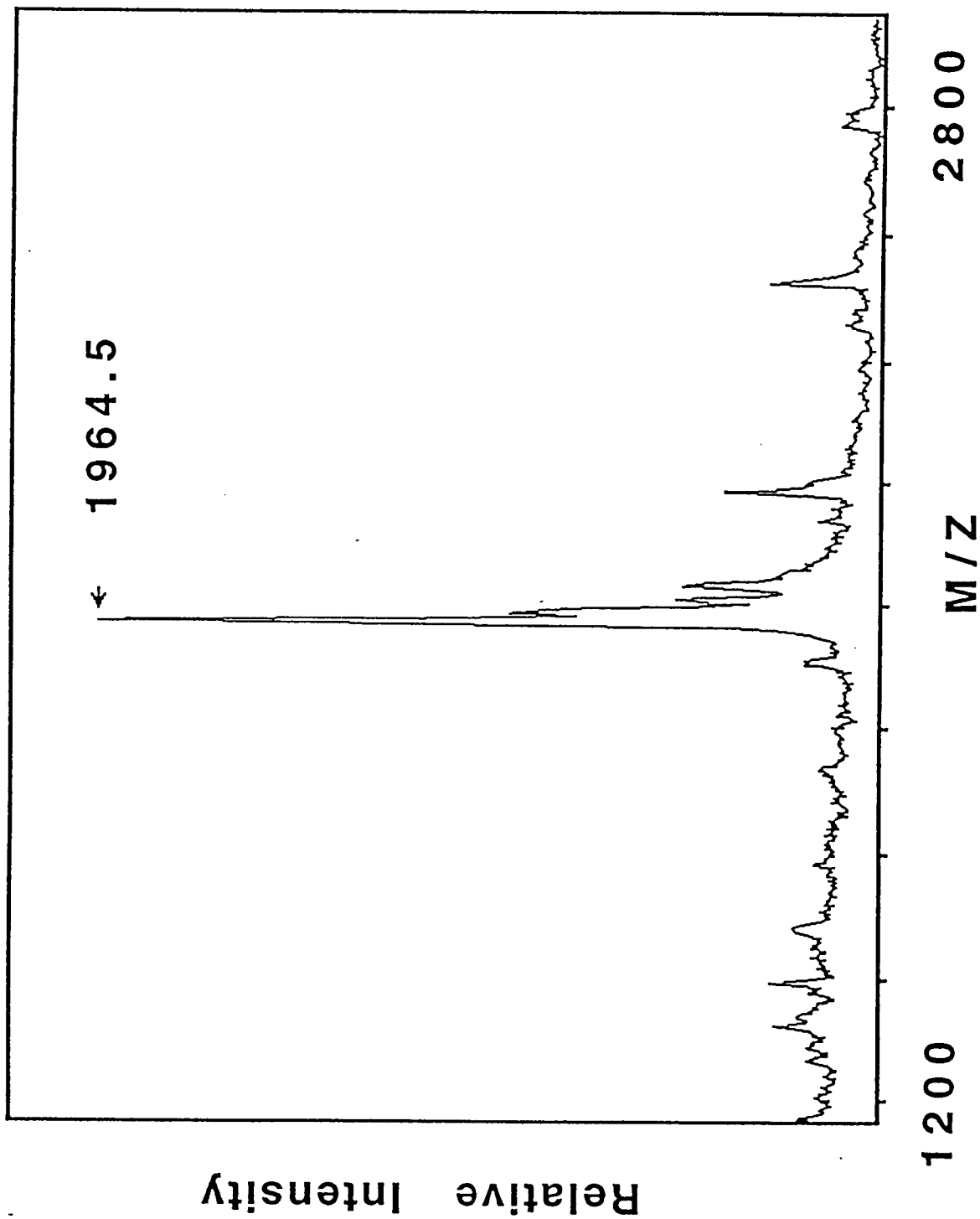

FIGURE 4.2

FIGURE 4.3:

Mass spectrum obtained using a MALDI mass spectrometer from the analysis of the tryptic peptide T-2.

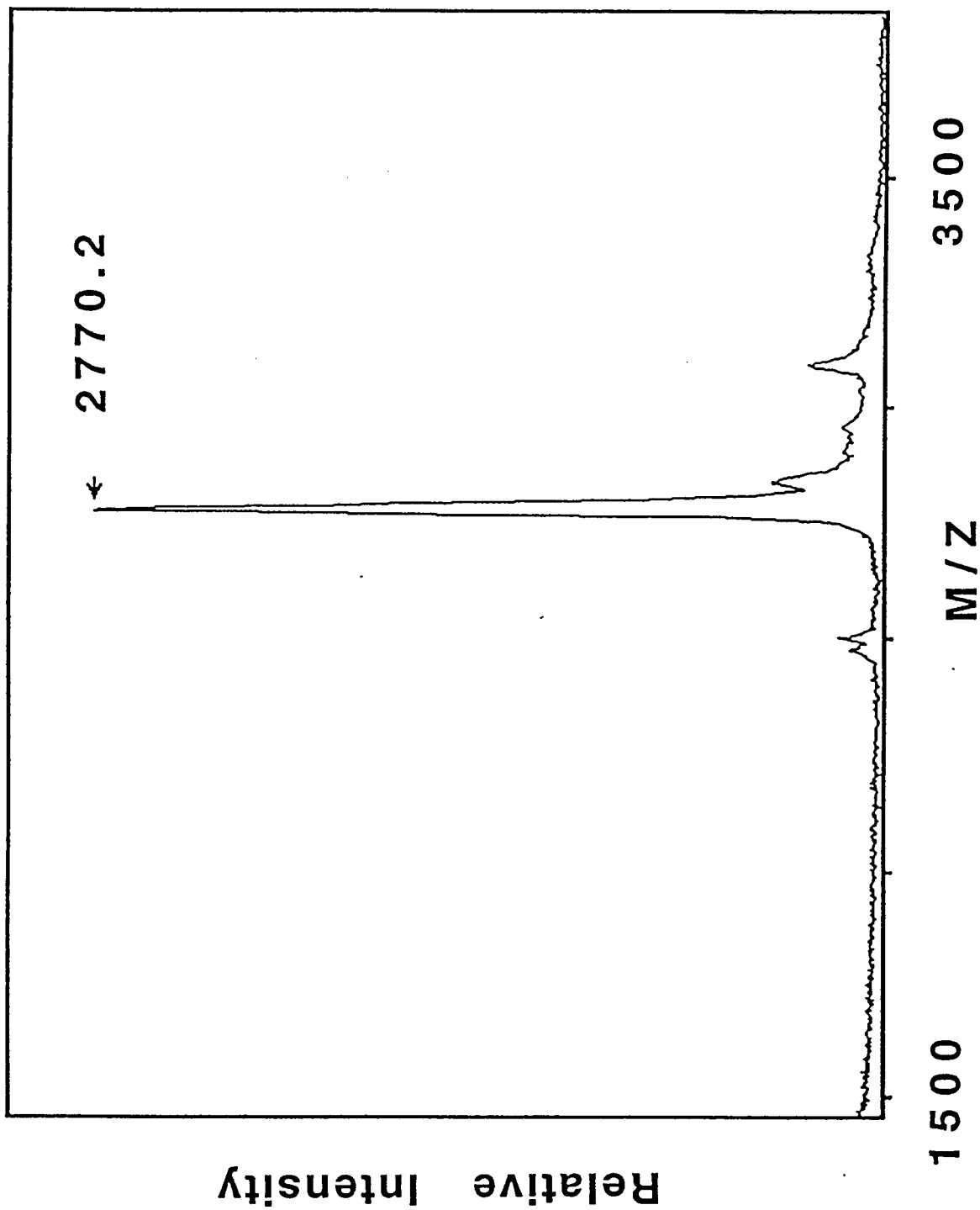

FIGURE 4.3

FIGURE 4.4:

Base-volatile [^{3}H] radioactivity profile of reversed phase HPLC purification of [^{3}H-*methyl*] esterified peptides derived by cyanogen bromide cleavage of [^{3}H] automethylated PCM. PCM was [^{3}H] automethylated for 1 hr and purified on a Vydac protein C_4 column, lyophilized, and then resuspended in 70% formic acid and reacted under N_2 for 18 hrs at room temperature with 1 μmol of CNBr [Sigma]. Reactions were stored in the dark under nitrogen and allowed to digest overnight at room temperature. CNBr fragments were then purified by reversed phase HPLC over a Dynamax C_{18} column in a gradient of +1% acetonitrile per minute at a flow rate of 1.0 ml min.$^{-1}$. One-tenth of each fraction was assayed for base-volatilized radioactivity as described in "Assays for radioactivity". The peaks corresponding to the cyanogen bromide fragments C-1 and C-2 are indicated.

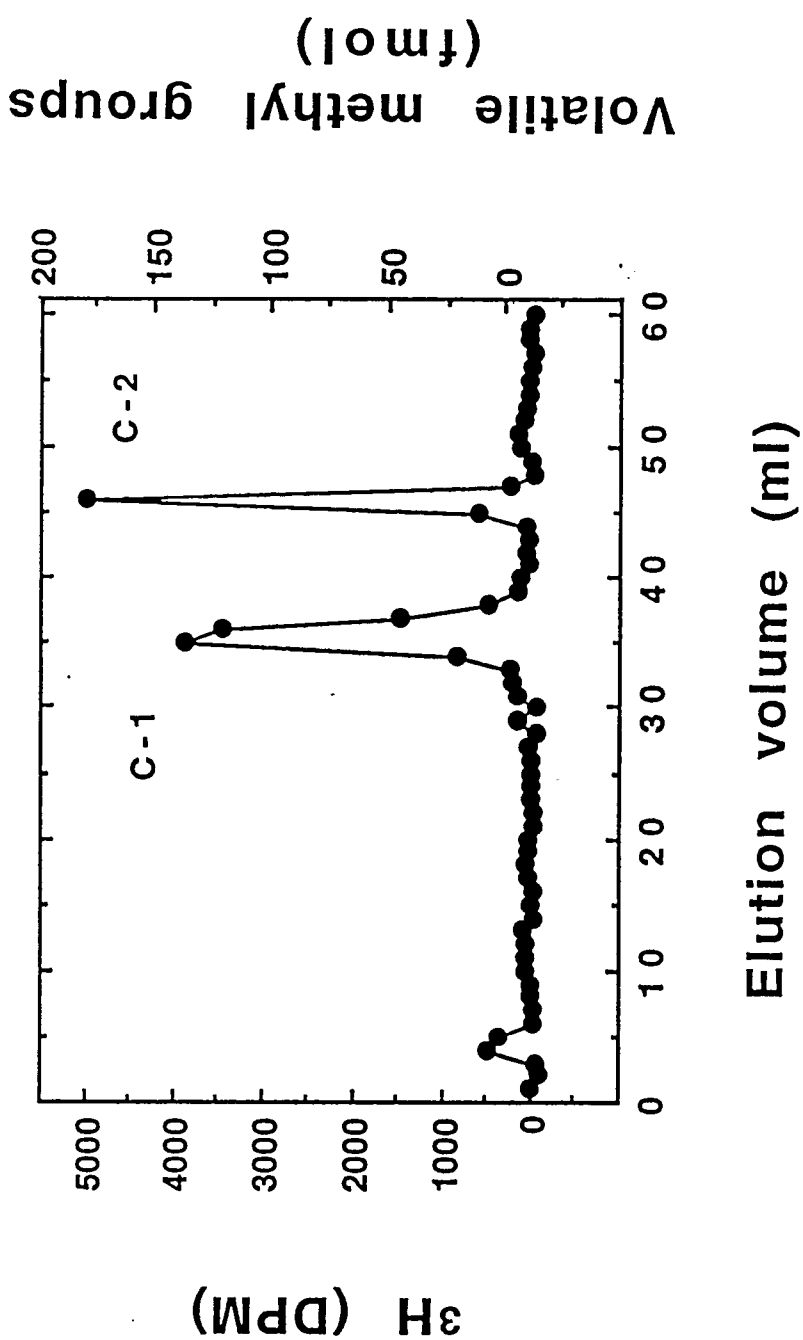
Volatile methyl groups
(fmol)
200
150
100
50
0
C-2
C-1
0
10
20
30
40
50
60
Elution volume (ml)
3H (DPM)
5000
4000
3000
2000
1000
0

FIGURE 4.4

FIGURE 4.5:

The mass spectrum obtained using a MALDI mass spectrometer from the analysis of the CNBr fragment C-1.

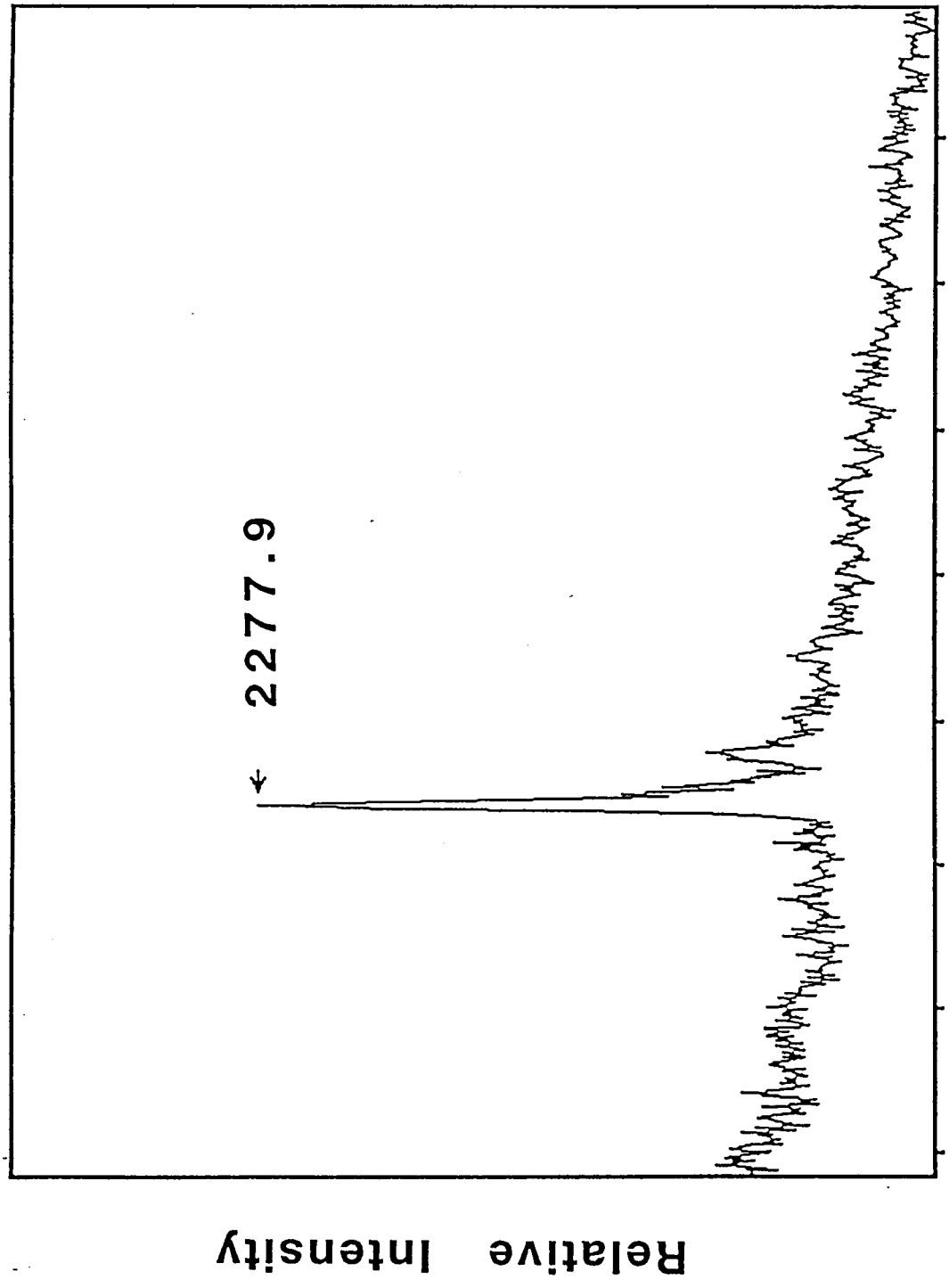

FIGURE 4.5

derived from the C-terminal portion of the protein and contains a single Asx residue as a possible site of damage and ester incorporation. The material eluting at 39 minutes yielded a spectrum (Figure 4.3) with a peak at 2770.2 Da that corresponded to a 26 amino acid peptide [residues 178-203] containing multiple Asx residues, requiring further information to identify the specific amino acid that was modified and thus the need for further analysis.

It was previously shown that the extent of automethylation of PCM plateaued after approximately 24 hrs (Lindquist and McFadden 1994a) suggesting that automethylation was limited to a subpopulation of PCM molecules. However, varying the length of the automethylation reaction or the pool of anion exchange FPLC purified PCM yielded no observable differences in the number or proportion of sites detected (data not shown).

In order to gather further information as to the specific sites of automethylation, [^{3}H]automethylated PCM was digested with cyanogen bromide. The cyanogen bromide digest was then resolved using reversed-phase HPLC yielding several distinct peaks as shown in Figure 4.4. Again, the material eluting at 4 to 5 minutes possesses the characteristics of methanol. The material eluting at 35 minutes and 46 minutes respectively, both contained UV absorbing material and were re-chromatographed before submitting for mass spectral analysis. The material eluting at 35 minutes yielded the spectrum shown in Figure 4.5. A single peak of 2277.9 Da was detected that corresponds to a 19 amino acid peptide containing the C-terminal portion of the protein [residues 209-227]. This fragment contains two Asp sites, one of which had been identified by tryptic

digestion. The peak eluting at 46 minutes yielded the spectrum shown in Figure 4.6. This spectrum contains a large peak at 4516.5 Da that corresponds by mass to a 46 amino acid peptide [residues 145-190] containing multiple Asx residues, one of which overlaps with a site determined by tryptic digestion. A second peak at 2258.0 Da corresponds to the doubly charged ion of the same peptide and the smaller peak at 3091 Da is an unassignable contaminant.

In order to verify the tentative assignments made so far, [^{3}H]automethylated PCM was sequentially digested using both cyanogen bromide and trypsin. Subsequent digestion of the cyanogen bromide peptides, C-1 and C-2, with trypsin, following their initial purification by reversed phase HPLC, produced the volatile [^{3}H] radioactivity profiles shown in Figure 4.7 and Figure 4.8. The [^{3}H] radioactivity profile for tryptic digestion of the cyanogen bromide fragment C-1 is shown in Figure 4.7. The material eluting at 4 to 5 minutes corresponds to [^{3}H]methanol. The material eluting at 15 minutes and 29 minutes was collected and analyzed using mass spectrometry. The 15 minute fraction yielded two mass peaks of 576 Da and 806 Da. The 576 Da peak corresponds by mass to a tetrapeptide QWSR [MW 575.6 Da] derived from the parent CNBr peak, while the 806 Da peak corresponds to an autodigestion fragment of trypsin (T7) [MW 805 Da] (Spectrum not shown). The 29 minute fraction, CT-1, consists of a single mass peak of 1362.9 Da (Figure 4.9) that corresponds, by mass, to a 12 amino acid peptide [residues 209-220] derived from the C-terminal region of the protein, which contains a single aspartic acid residue as the only possible site of damage. This sequence is also what one would expect for subcleavage of the peptide determined by cyanogen

FIGURE 4.6:

The mass spectrum obtained using a MALDI mass spectrometer from the analysis of the CNBr fragment C-2.

FIGURE 4.6

FIGURE 4.7:

Base-volatile [^{3}H] radioactivity profile of reversed phase HPLC resolution of [^{3}H] methyl esterified peptides derived by tryptic digestion of the HPLC purified CNBr fragment C-1. PCM was [^{3}H]automethylated for 16 hrs and purified on a Vydac protein C_4 column, lyophilized, and then digested with cyanogen bromide as described in "Cyanogen bromide cleavage". CNBr fragments were then purified and the fractions corresponding to the elution of each of the two peaks of radioactivity at 35 minutes (C-1) and 46 minutes (C-2) were pooled. This panel shows the [^{3}H] radioactivity profile for tryptic digestion of the CNBr fragment C-1. The peak corresponding to the subfragment CT-1 is indicated.

3000
2000
1000
0
CT-1
80
60
40
20
0
0
10
20
30
40
50
3H (DPM)
Volatile methyl groups (fmol)
Elution volume (ml)

FIGURE 4.7

FIGURE 4.8:

The base-volatile [^{3}H] radioactivity profile for tryptic digestion of the CNBr fragment C-2. The peak corresponding to the subfragment CT-2 is indicated.

CT-2
^{3}H (DPM)
Volatile methyl groups (fmol)
Elution volume (ml)
10000
8000
6000
4000
2000
0
300
250
200
150
100
50
0
0
10
20
30
40
50
60

FIGURE 4.8

FIGURE 4.9:

Mass spectrum obtained using a MALDI mass spectrometer from the analysis of the subfragment CT-1.

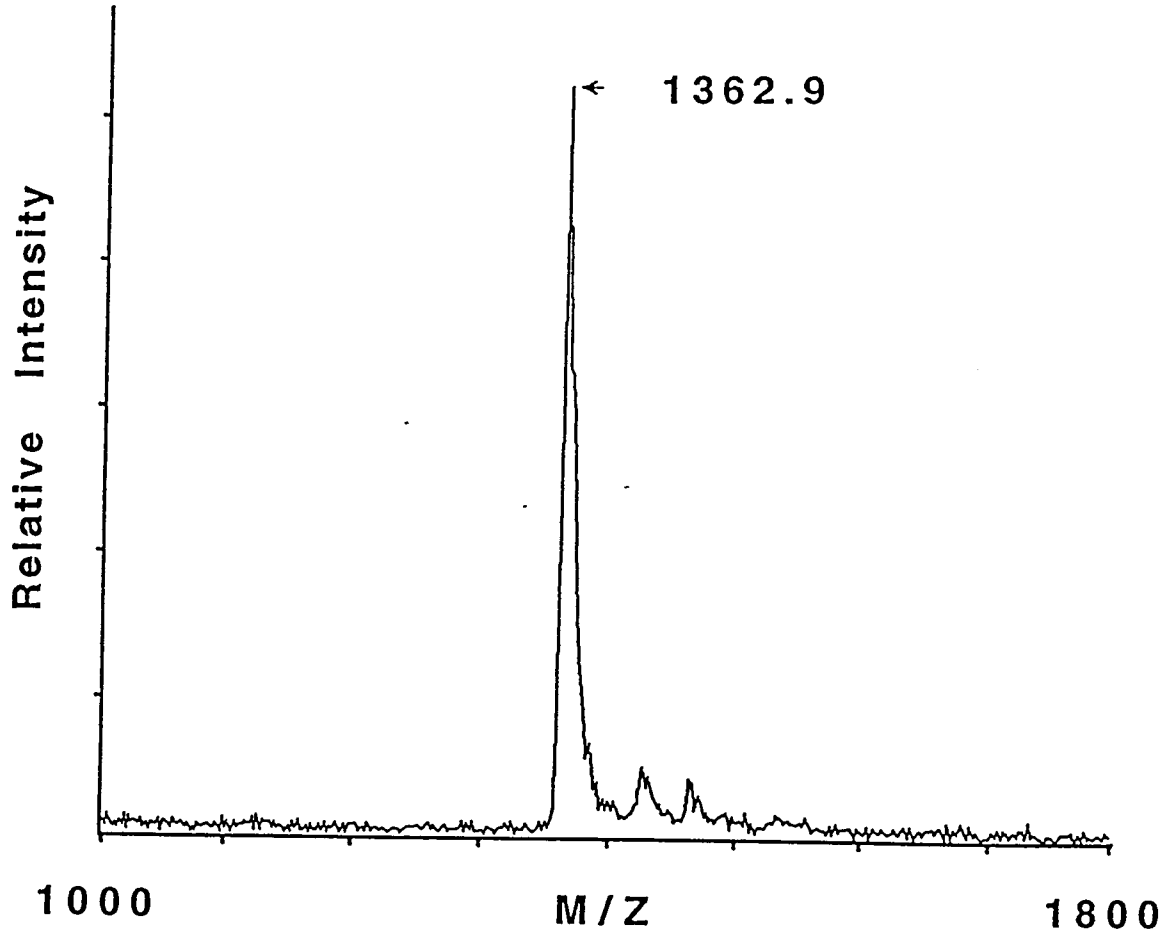

FIGURE 4.9

bromide digest and overlaps with the peptide determined independently by trypsin digestion. Together, these data positively identify Asp-217 as a site of automethylation.

The [^{3}H] radioactivity profile for tryptic digestion of the cyanogen bromide fragment C-2 is shown in Figure 4.8. Mass spectral analysis of the 33 minute fraction, CT-2, yielded no detectable material. Analysis of the 32 minute fraction yielded the spectrum shown in Figure 4.10. A single mass peak of 1218.9 Da was detected that corresponds to a 13 aminoacid peptide [residues 178-190] containing a single asparagine residue as the only possible site of damage. This peptide is also what one would expect for subcleavage of the peptide determined by cyanogen bromide digest which overlaps with the peptide determined independently by trypsin digestion. The smaller mass peak of 1283.6 Da corresponds to the adduction of a single Cu ion from the matrix to the parent mass peak. Smaller mass peaks were detected at 681 Da and 742 Da respectively. The peaks correspond to solvent contaminants as they are present in every sample submitted and are not due to the matrix. Together, these data positively identify Asn-188 as a site of automethylation.

DISCUSSION

In order to determine the sites of automethylation, a combination of proteolytic and/or chemical methods was used to digest [^{3}H] automethylated PCM. Mass spectral analysis of the proteolytic fragments allowed the identification of two damaged sites, Asn_{188} and Asp_{217}, which PCM is capable of recognizing and automethylating (Figure 4.11). Asn_{188} was identified as the only Asx residue shared between fragments isolated

Figure 4.10:

Mass spectrum obtained using a MALDI mass spectrometer from the analysis of the subfragment CT-2.

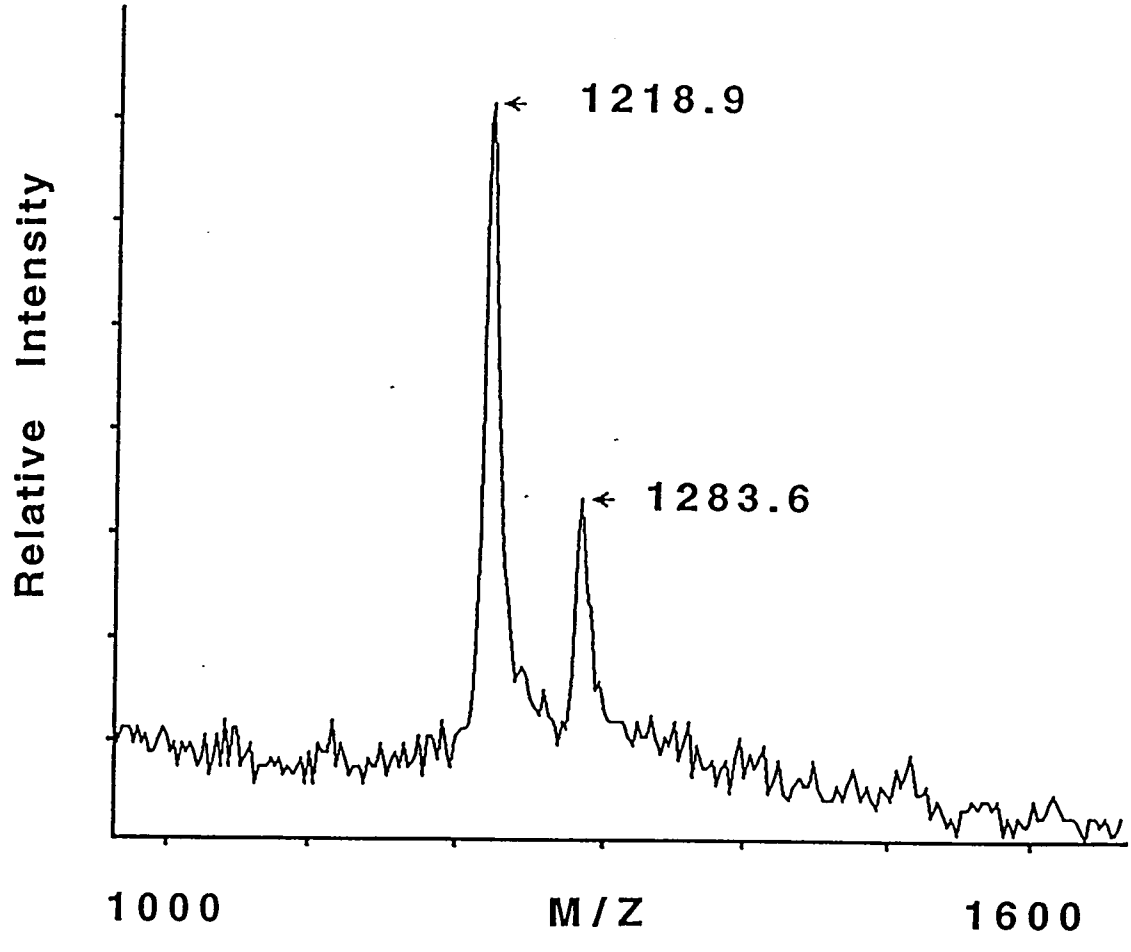

FIGURE 4.10

FIGURE 4.11:

Primary sequence of bovine protein (D-aspartyl/ L-isoaspartyl) carboxyl methyltransferase isozyme II. The fragments identified by tryptic digestion and/or chemical cleavage of [^{3}H]automethylated PCM are indicated. The cyanogen bromide fragments C-1 and C-2 are indicated by the black bars. The tryptic fragments T-1 and T-2 are indicated by the gray bars and the subfragments CT-1 and CT-2 are indicated by the white bars.

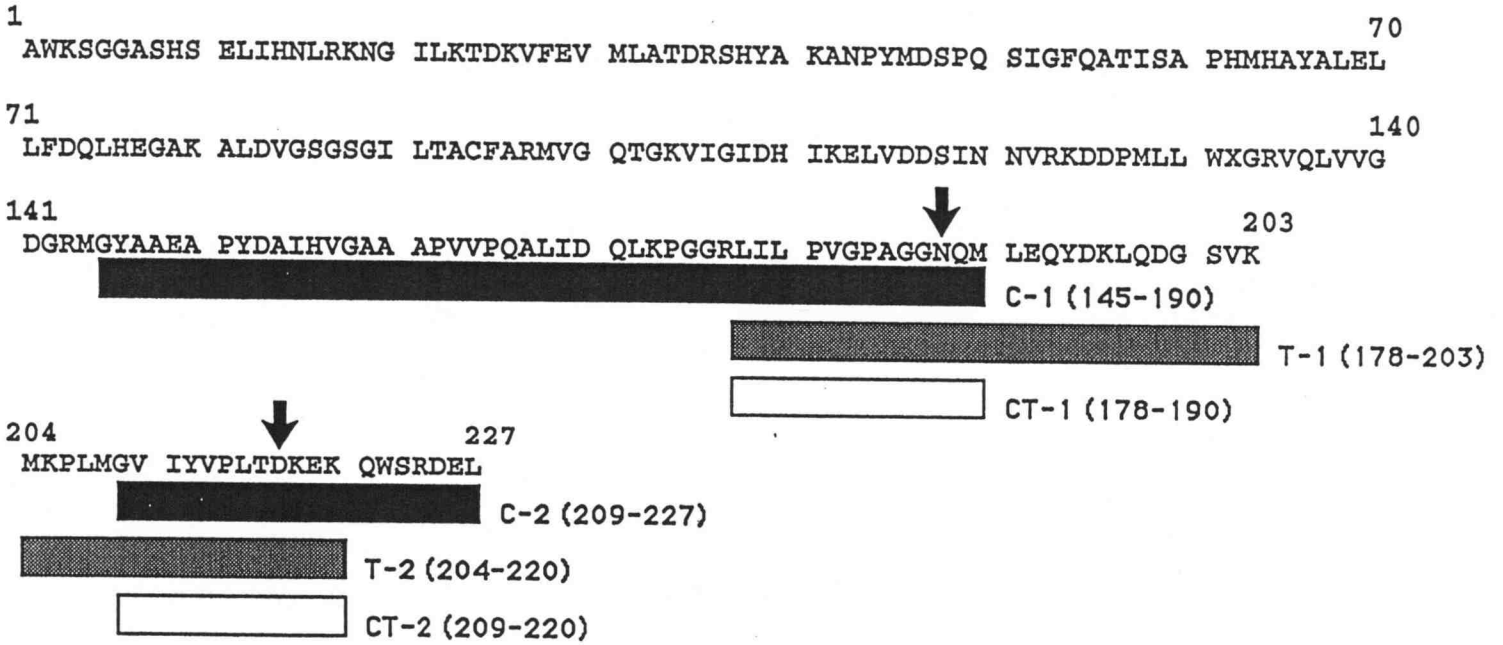
1
AWKSGGASHS ELIHNLRKNG ILKTDKVFEV MLATDRSHYA KANPYMDSPQ SIGFQATISA PHMHAYALEL
70
71
LFDQLHEGAK ALDVGSGSGI LTACFARMVG QTGKVIGIDH IKELVDDSIN NVRKDDPMLL WXGRVQLVVG
140
141
DGRMGYAAEA PYDAIHVGAA APVVPQALID QLKPGGRLIL PVGPAGGNQM LEQYDKLQDG SVK
203
C-1 (145-190)
T-1 (178-203)
CT-1 (178-190)
204
MKPLMGV IYVPLTDKEK QWSRDEL
227
C-2 (209-227)
T-2 (204-220)
CT-2 (209-220)

FIGURE 4.11

from the interior of the enzyme and confirmed by double-digestion. Asp_{217} was identified as the single Asx residue of overlap between the C-terminally derived fragments and this result was also confirmed utilizing double-digestion.

Analysis of the sequence of the tryptic fragments T-1 and T-2 showed that digestion had apparently not gone to completion, as both fragments contained tryptic sites that had not been cleaved. The initial hypothesis was that perhaps the acidic nature of the cleavage reaction had altered the specificity of trypsin, such that these sites were not cleaved. The answer to this question was obtained from the work of Gilbert, et al. 1988. The sequence of bovine erythrocyte PCM was determined from protein sequencing of tryptic peptides obtained under normal cleavage conditions. Scrutiny of these tryptic peptides revealed that Gilbert had observed the identical cleavage pattern seen here. These results suggest that the resistance of these sites to digestion with trypsin involves factors inherent to the structure of PCM.

As automethylation is a sub-stoichiometric reaction, the ability to identify fragments from [^{3}H]automethylated PCM relied upon the elution of the methylated species with the identical unmethylated peptide fragment. For the two species to coelute, the methyl group could not significantly contribute to the overall hydrophobicity of the peptide. In the case of many of the larger peptide fragments, this was not believed to be of concern. However, this became a problem in the identification of the fragment CT-2, as the methylated species eluted at 33 minutes. Mass spectral analysis of this fraction yielded no detectable material. Analysis of the crude digest (data not shown) showed the presence of the undigested parent peptide at 4516 kDa and the expected cleavage

products at 3317 kDa and 1218 kDa respectively. As a methyl group is predicted to shift the elution of a peptide towards the more hydrophobic phase, the fractions preceding the elution of the methylated species were analyzed. Fraction 32 yielded the mass spectrum shown in Figure 4.10. This analysis identified the smaller of the two cleavage products predicted, which also contained the residue of overlap previously identified by comparison of the tryptic fragment T-2 and the cyanogen bromide fragment C-2.

The identification of two damaged sites within PCM supported an earlier observation made during the purification of PCM that damage had made the enzyme more acidic and thus it bound more tightly to the anion exchange resin. This observation is consistent with the more acidic nature of altered aspartyl residues. The most obvious explanation is the conversion of an amide to a carboxylate observed in the deamidation of asparagine. However, the slightly more acidic pK_a of the α-carboxylate in comparison with that of the β- may also contribute to this effect. The presence of two damaged sites led to the hypothesis that αPCM is not a homogeneous population of molecules, but rather a diverse population with a hierarchy of damaged sites (α_1, α_2, α_3,...).

While damage has been shown to lead to the inactivation of other proteins, there is no evidence yet to determine the effect of this damage upon PCM. In identifying the sequence of human PCM, Ingrosso, et al. compared this sequence with the sequences of other known AdoMet binding proteins. From this comparison, they proposed three regions of the enzyme that could be involved in the binding of AdoMet. Region I contains residues 81 through 97. This region contains the motif "Gly-Ser-Gly-Ser-Gly" that has been proposed as a nucleotide binding motif and is proposed to interact by

hydrogen bonding with the ribose (Aitken 1990). Region II and III contain residues 151-157 and 171-180 respectively. However, no function was proposed for these regions. In a separate effort to identify the catalytic site of PCM, Paik and Kim (1993) used photoaffinity labeling to identify active site residues. They identified a peptide [residues 113-121] containing two adjacent aspartyl residues which they proposed to be involved in neutralizing the charge of the sulfonium ion in AdoMet. Since the damaged sites identified, Asn_{188} and Asp_{217}, are not located in any of the regions proposed to be involved with AdoMet binding, it is possible that damage to these sites will not affect this activity.

For damaged sites to be accessible to the repair reaction, they must be located on the solvent accessible surface of the protein. Since catalytic sites are believed to be located within the interior of proteins, it is likely that these residues are not involved in the catalytic mechanism and it is also likely that they would not affect the binding of peptide substrates. However, the effects that damage will have upon the overall three-dimensional structure of the enzyme cannot be determined because the 3-D structure is unknown. Although it is possible that damage to these sites could perturb structure in such a way that activity might be affected.

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CHAPTER 5

SELF-REPAIR OF L-ISOASPARTYL/D-ASPARTYL PROTEIN CARBOXYL METHYLTRANSFERASE: "ENZYME, HEAL THYSELF!"

Jonathan A. Lindquist and Philip N. McFadden

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RATIONALE

Protein (D-aspartyl/ L-isoaspartyl) carboxyl methyltransferase (PCM) was shown to be damaged in a manner consistent with the presence of altered aspartyl residues. Two sites of automethylation were localized within the sequence of PCM, Asn_{188} and Asp_{217}. The purpose of this chapter is to assess the ability of PCM for self-repair at these damaged sites.

SELF-REPAIR OF L-ISOASPARTYL/D-ASPARTYL PROTEIN CARBOXYL METHYLTRANSFERASE: "ENZYME, HEAL THYSELF!"

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The enzyme protein (D-aspartyl /L-isoaspartyl)carboxyl methyltransferase has been shown to be damaged in a manner consistent with the presence of altered aspartyl residues, and that the location this damage can be mapped to two sites within the protein, Asn_{188} and Asp_{217}. In investigating the ability of the enzyme to repair these sites of damage, we have found that the methyl esters formed at these two sites are relatively stable ($t_{1/2}$ = 39.2 hrs), resulting in a slow rate of repair which is exceeded by the rate of damage at these sites. Therefore, net self-repair does not appear to occur at these sites under the experimental conditions tested. A second class of methylated sites with more rapid ester turnover ($t_{1/2}$ = 3.4 hrs) was also detected. This latter class represents self-repairable damage within the enzyme pool.

INTRODUCTION

Proteins are inherently unstable molecules. During their synthesis, sites of instability are incorporated in the form of the amino acids aspartate and asparagine. These amino acids are susceptible to a spontaneous form of damage, which results from the attack of the amide nitrogen of the C-terminally neighboring amino acid upon the carbonyl carbon atom. This attack results in the formation of a succinimide, which may then hydrolyze to yield several products: L-aspartate, D-aspartate, L-isoaspartate, and D-isoaspartate. The factors affecting the rate of this damage (primary sequence, pH, temperature, ionic strength, etc.) have been studied in the past few years and the nature of this reaction is coming to be understand. Currently, this reaction is believed to account for much of the heterogeneity observed in proteins, as it may lead to the formation of several different products at any given sites of damage.

The predominant product of this damage reaction is the formation of an abnormal amino acid: L-isoaspartate. Isoaspartyl residues have been identified in many proteins and are responsible for a decrease or loss of biological activity in the majority of these instances. Cells appear to possess a system that is capable of repairing this damage, the enzyme protein (D-aspartyl/L-isoaspartyl) carboxyl methyltransferase (PCM). In studies with model peptides, PCM has been shown to repair most of these damaged sites to L-aspartate (Johnson et al. 1987b, McFadden and Clarke 1987) and recent evidence suggests that this repair may have the potential to restore activity to damage-inactivated proteins (Brennan, et al.1994, Johnson et al. 1987a).

The identification of altered aspartyl residues within PCM has also been recently identified (Lindquist and McFadden 1994a) and mapped to two sites within the protein, Asn_{188} and Asp_{217} (chapter 4). Since PCM has been shown to repair other damaged molecules, we were interested to see if it is capable of self-repair.

Maintenance of the repair systems is essential for proper function of the cell. Damage to a repair system that results in a reduced efficiency of repair may lead to a rapid accumulation of damaged proteins within the cell. Indiscriminant damage throughout the cell may be responsible for the reduction in activity of other systems, essential for cell survival, which could then result in cell death.

EXPERIMENTAL METHODS

Protein purification

Bovine erythrocyte protein (D-aspartyl/L-isoaspartyl) carboxyl methyltransferase isozyme II was purified according to the method of Gilbert, et al. (1987) with some modifications (Lindquist and McFadden 1994a). Briefly, 1 L of bovine blood was centrifuged at 1400 x g for 10 minutes to pellet the erythrocytes and the serum component was removed. The erythrocytes were carefully washed four times with phosphate buffer saline to remove contaminants and the buffy coat. Following the washing steps, the erythrocytes were lysed in a hypotonic phosphate buffer by freeze/thaw in liquid nitrogen. The lysed erythrocytes were centrifuged at 10,000 x g for 90 minutes to pellet the membranes. The resulting cytosol was carefully removed, so as

to prevent contamination from the membrane pellet. This material was then ammonium sulfate precipitated (53%) to remove most of the hemoglobin. The pellet was resuspended by dounce homogenization and dialyzed to remove the ammonium sulfate. This material was then separated into sixteen aliquots and purified by affinity chromatography on a S-adenosyl-L-homocysteine-Sepharose 4B affinity resin (Paik and Kim 1980). The affinity purified material was pooled and ammonium sulfate precipitated (55%) a second time. The pellet was again resuspended by homogenization and divided into four aliquots which were further purified by size exclusion chromatography on a Sephadex G-75-120 resin. The gel filtration purified material was pooled and divided into seven aliquots that were further purified using fast protein liquid chromatography (FPLC) on an anion exchange resin [Fractogel EMD DEAE 650 (S)]. This material is later referred to as FPLC-R1 through R7 depending upon which of the seven aliquots were used. PCM isozyme I was located in the flow through, while PCM isozyme II, which bound to the resin, was eluted from the column using a linear salt gradient from 0 to 2 M NaCl. PCM isozyme II eluted in a single peak and was collected as three 2 ml fractions which are designated as f15, f16, or f 17. This material was aliquoted, frozen in liquid nitrogen, and stored at -80°C. The fractions were studied separately, to identify differences due to slight charge heterogeneity in PCM.

Repair reactions

Purified bovine erythrocyte protein D-aspartyl/L-isoaspartyl carboxyl methyltransferase isozyme II (90 µl, in 20 mM Tris-HCl /0.34M NaCl / 0.2 mM EDTA/

10%(v/v) glycerol pH 8.0 with 25 μM phenylmethylsulfonyl fluoride (PMSF) and 15 mM 2-mercaptoethanol) was incubated with 50 μl 0.1 M NaH_2PO_4 pH 7.8 and 15 μl of either 1 mM AdoMet (in 1 mM HCl) or 1 mM HCl for 24 hrs at 37°C.

[^{3}H]Repair reactions

[^{3}H] repair reactions were performed as described above with the following modification. Before addition of the enzyme, the reaction containing 50 μl 0.1 M NaH_2PO_4 pH 7.8, 15 μl of 1 mM AdoMet (in 1 mM HCl), and 30 μl of S-adenosyl-L-[^{3}H-*methyl*]methionine [15 Ci/mmol, NEN] was lyophilized just to the point of dryness and resuspended with 65 μl deionized (dI) H_2O to minimize oxidation of the sample.

HPLC purification of PCM

Following the repair reaction, PCM was re-isolated by reversed phase high performance liquid chromatography (HPLC) in a water/acetonitrile gradient system. Solvent A is dI H_2O/ 0.1%(v/v) TFA [Sigma]. Solvent B is 90%(v/v) acetonitrile [Burdick & Jackson UV grade]/ 9.9%(v/v) dI H_2O/ 0.1%(v/v) TFA. Repair reaction mixtures were injected onto a Vydac Protein C_4 column [5 μ particle size, 4.6 mm x 250 mm] with component elution monitored at 280 nm. The elution position of PCM in this system had been previously determined (Lindquist and McFadden 1994a) by both immunoreactivity and activity assays. The PCM peak was collected and lyophilized to

dryness in a speed-vac, to remove the HPLC solvents so that the sample could be proteolytically digested (see below).

Proteolytic digestion

Following lyophilization, the enzyme was resuspended in 50 µl 0.2 M sodium citrate buffer pH 6.0 and 5 µl of trypsin (1 mg/ml in 1 mM HCl, diluted immediately prior to use from a 10 mg/ml stock solution to minimize autodigestion) [TPCK treated, Sigma] was added. Proteolytic digestion was allowed to proceed for 40 minutes at 37°C before quenching with the addition of 2 µl of 100 mM PMSF (in isopropanol).

[^{3}H]Methylation reactions

Following quenching of the protease, 5 µl of S-adenosyl-L-[^{3}H-*methyl*]methionine [15 Ci/mmol, NEN], 1 µl of 1 mM AdoMet (in 1 mM HCl), and 10 µl of PCM [80 pmol] were added. Methylation reactions were carried out for 40 minutes at 30°C (Johnson, B.A. and Aswad, D.W. (1991) Anal. Biochem. 192, 384-391.). Reactions were quenched on ice and immediately injected onto the HPLC.

Peptide purification

Tryptic peptides were separated by reversed phase HPLC on a Dynamax C_{18} column [5 µ bead size, 4.6 mm x 250 mm] using a linear gradient of 0-70% solvent B

with component elution monitored at 214 nm. Fractions were collected at 1 minute intervals and an aliquot from each was assayed for base volatile radioactivity.

Radioactivity assay

An aliquot (500 µl) of each fraction was pipetted into a glass inner vial [15 mm x 45 mm, Kimble] containing 100 µl of 1 M NaOH. These vials were placed inside of 20 ml scintillation vials containing 4 mls of xylene based liquid scintillation cocktail [Scintiverse E, Fisher] and capped. Vials were allowed to equilibrate before counting and the degree of equilibration monitored using [^{14}C]methanol standards.

Time course of demethylation

PCM was methylated for 24 hrs (as described above under "[^{3}H]repair reactions") with 20 µM [^{3}H-*methyl*]AdoMet [10,000 dpm/pmol]. After 24 hrs of incubation, an excess of non-radioactive AdoMet was added to bring the final concentration to 0.5 mM [400 dpm/pmol] and the incubation continued. Following the addition of AdoMet, time points were taken and immediately purified by reversed phase HPLC. An aliquot of each sample (10%) was analyzed for its total [^{3}H]methyl ester content and the remaining portion digested with trypsin. The digestion mixtures were resolved as described under "peptide purification". The HPLC fractions eluting from 31 to 45 minutes were analyzed for their [^{3}H]methyl ester content as these constitute the major damaged sites within PCM (chapter on sites).

RESULTS AND DISCUSSION

In order to establish the basal level of damage within the enzyme, PCM which had not been previously incubated at 37°C was proteolytically digested with trypsin. The trypic fragments were then [^{3}H]methylated and resolved using reversed phase HPLC (Figure 5.1). The material eluting at 4-6 minutes, 12 minutes, and 17 minutes is due to either methanol derived during the repair reaction or to the unincorporated radiolabel and/or its degradation products, as these peaks have been previously shown to be insensitive to digestion with pronase and were observed in control incubations without PCM (data not shown). The larger peaks at 37 minutes and 39 minutes are the sites of automethylation, T-1 and T-2 (chapter 4). The smaller peaks eluting at 20 minutes, 23 minutes, 27 minutes, 30 minutes, 32 minutes, 34 minutes, 42 minutes and 48 minutes are "cryptic sites" of damage that cannot be methylated in intact PCM (see chapter 7).

In order to test the effects of incubation, PCM was incubated in the absence of AdoMet and then proteolytically digested. The fragments obtained from digestion were then [^{3}H]methylated and resolved by reversed-phase HPLC (Figure 5.2). A 2-fold increase in damage is seen at several sites, but this increase is most noticeable in the peaks corresponding to T-1 and T-2. This increase suggests that these sites undergo damage during the incubation.

In order to assess the ability of the enzyme for self-repair, incubation of PCM in the presence of AdoMet was performed. Analysis of the [^{3}H]methylated peptides derived from proteolytic digestion of the repair reaction demonstrated a decrease in the

FIGURE 5.1:

[^{3}H] Methylation of tryptic PCM fragments. Fragments derived from tryptic digestion of PCM (629 pmol) were [^{3}H] methylated and separated on a C18 column using reversed phase HPLC. Peptides were eluted with a linear gradient of 0 to 70% solvent B in 70 minutes. An aliquot of each fraction was assayed for base volatile radioactivity.

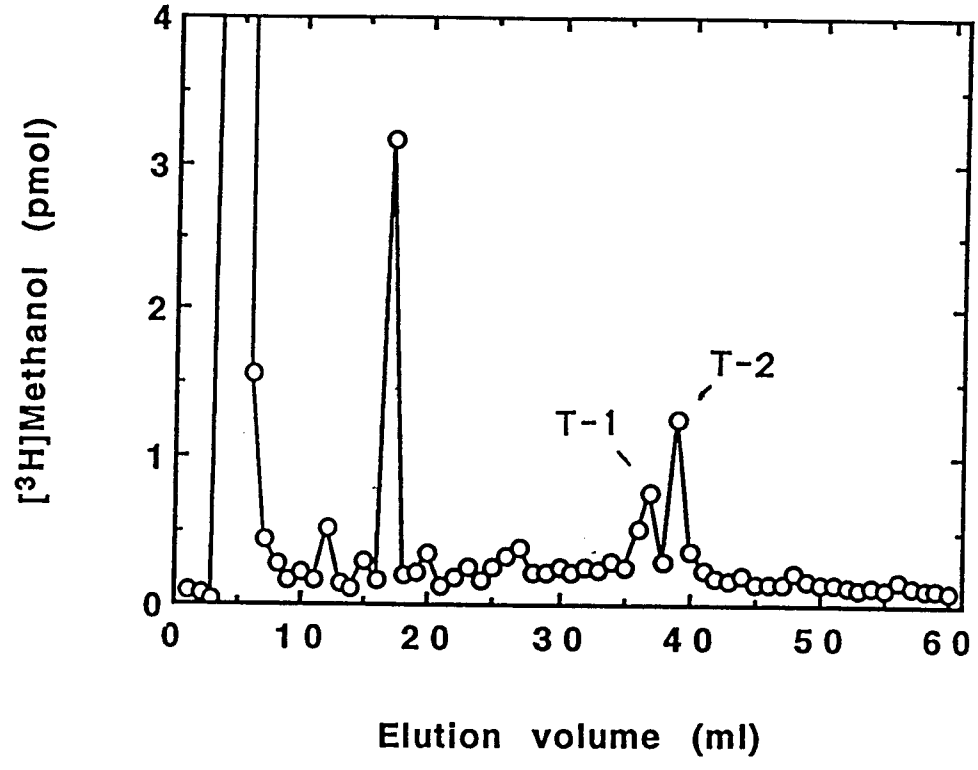
[³H]Methanol (pmol)
4
3
2
1
0
T-1
T-2
0
10
20
30
40
50
60
Elution volume (ml)

FIGURE 5.1

FIGURE 5.2:

[^{3}H] Methylation of tryptic PCM fragments derived from proteolytic digestion of PCM (643 pmol) following a 24 hr incubation at pH 7.8 in the absence of AdoMet.

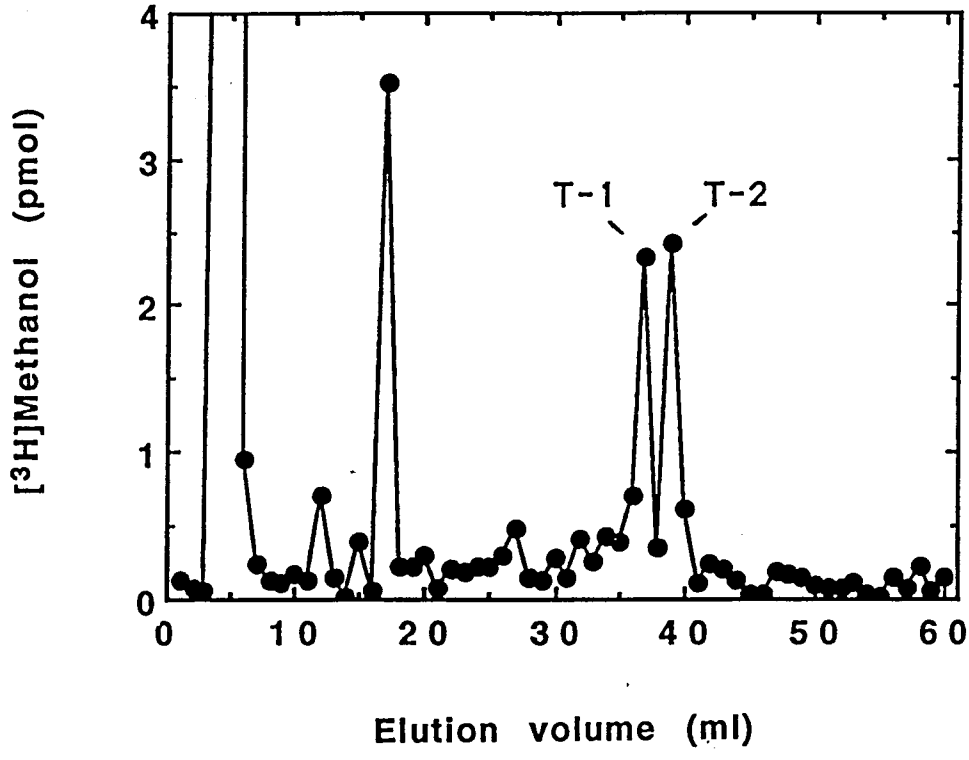
[3H]Methanol (pmol)
4
3
2
1
0
T-1
T-2
0
10
20
30
40
50
60
Elution volume (ml)

FIGURE 5.2

availability of the T-1 and T-2 sites to [^{3}H]methylation following tryptic digestion (Figure 5.3). The decrease in [^{3}H]methylation is either the result of repair or the masking of these sites with non-radioactive methyl groups.

In order to determine if the unavailability of the sites, T-1 and T-2, is due to masking by methyl groups or because of repair, PCM was incubated under repair conditions in the presence of [^{3}H]AdoMet. Figure 5.4 shows that the sites in the T-1 and T-2 fragments retained [^{3}H]methyl esters, arguing in favor of the alternative, that masking of these sites with methyl groups was responsible for their decrease. The presence of methyl groups that are retained at physiological pH suggests that the methyl groups are quite stable. Interestingly, PCM incubated in the presence of [^{3}H]AdoMet shows the same level of damage as enzyme incubated without AdoMet. This result shows that the enzyme is capable of methylating these damaged sites as quickly as they are formed.

To compare the extent of unrepaired damage under these conditions, a histogram comparing the peaks, T-1 and T-2, is presented (Figure 5.5). Condition 1 establishes the basal level of damage in the PCM fraction prior to incubation to be approximately 0.0023 and 0.0029 mol/mol of methyl groups per T-1 and T-2 site, respectively. A 24 hr incubation of the samples without AdoMet (condition 2) shows that the stoichiometry of methylation at both of these sites has almost doubled. The masked sites (condition 3) that were revealed by the incubation with [^{3}H]AdoMet (condition 4) appear to be of equal magnitude to the samples incubated without AdoMet (condition 2). These resluts suggest that these sites are sensitive to damage and that PCM is capable of recognizing

FIGURE 5.3:

[^{3}H] Methylation of tryptic PCM fragments. Prior to proteolytic digestion, PCM (626 pmol) was incubated for 24 hrs at pH 7.8 in the presence of ~100 μM AdoMet. Following this incubation, fragments derived from proteolytic digestion of PCM were [^{3}H] methylated and separated by reversed phase HPLC.

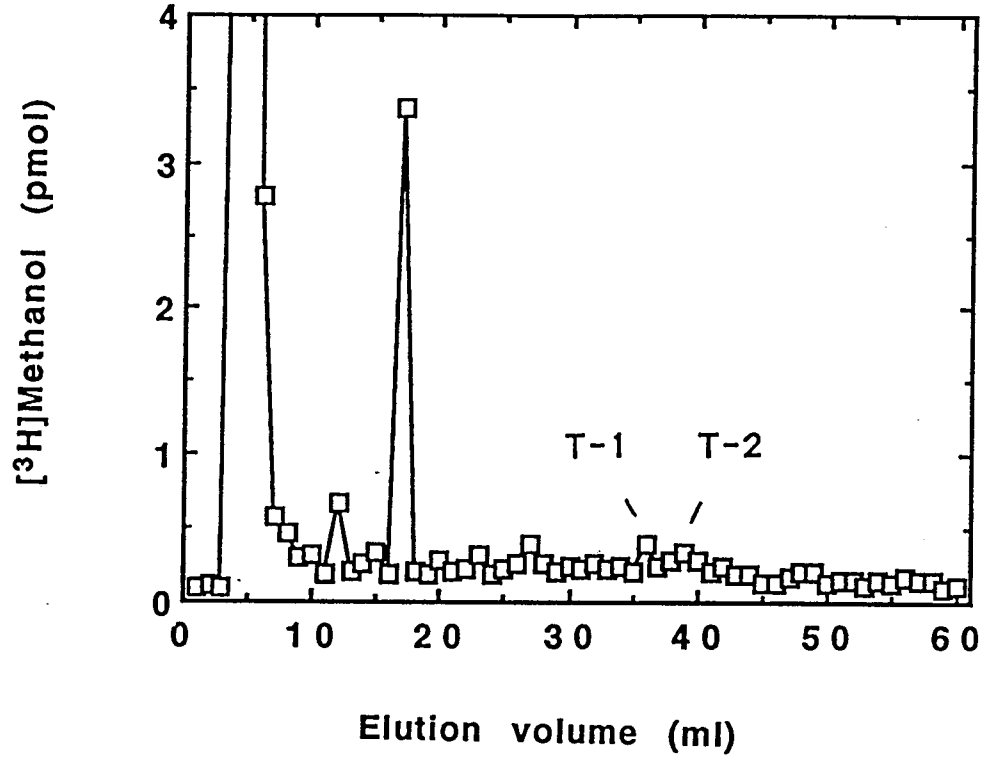
[3H]Methanol (pmol)
4
3
2
1
0
T-1
T-2
0
10
20
30
40
50
60
Elution volume (ml)

FIGURE 5.3

FIGURE 5.4:

Tryptic fragments of PCM following a 24 hr incubation at pH 7.8 in the presence of 100 μM [^{3}H-*methyl*] AdoMet (2,550 dpm/pmol).

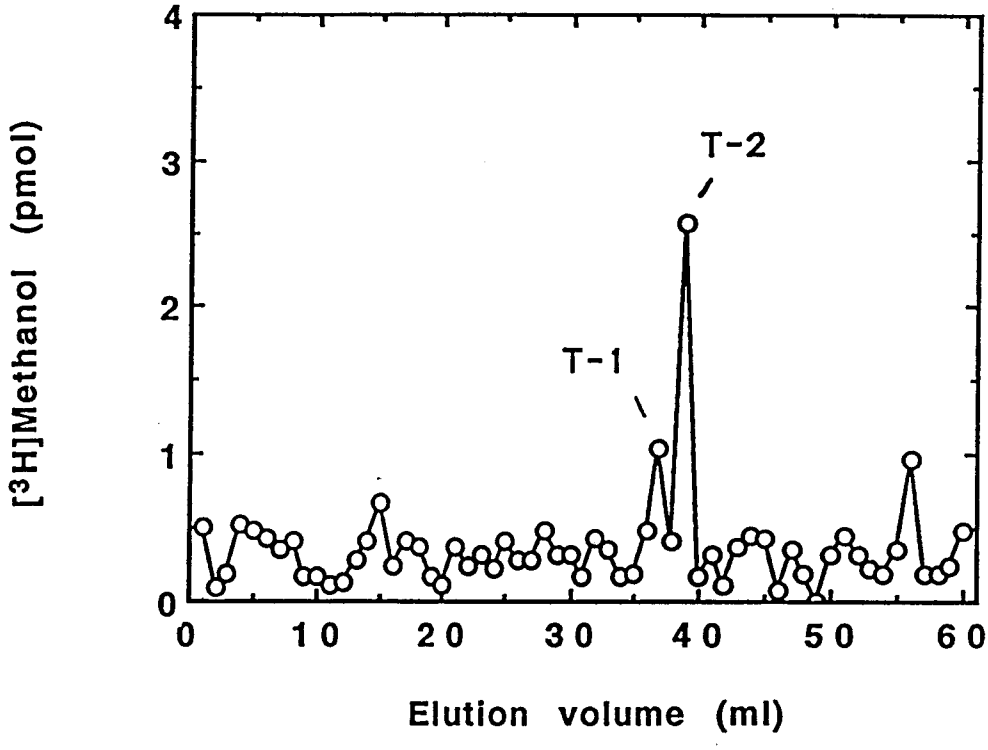
[³H]Methanol (pmol)
4
3
2
1
0
T-2
T-1
0
10
20
30
40
50
60
Elution volume (ml)

FIGURE 5.4

FIGURE 5.5:

Histograms comparing the stoichiometry of methylation for the tryptic fragments T-1 and T-2 is shown above. Condition 1, PCM with no prior incubation (Figure 5.1). Condition 2, PCM incubated in the absence of AdoMet (Figure 5.2). Condition 3, PCM incubated with AdoMet (Figure 5.3). Condition 4, PCM incubated with [^{3}H]AdoMet (Figure 5.4).

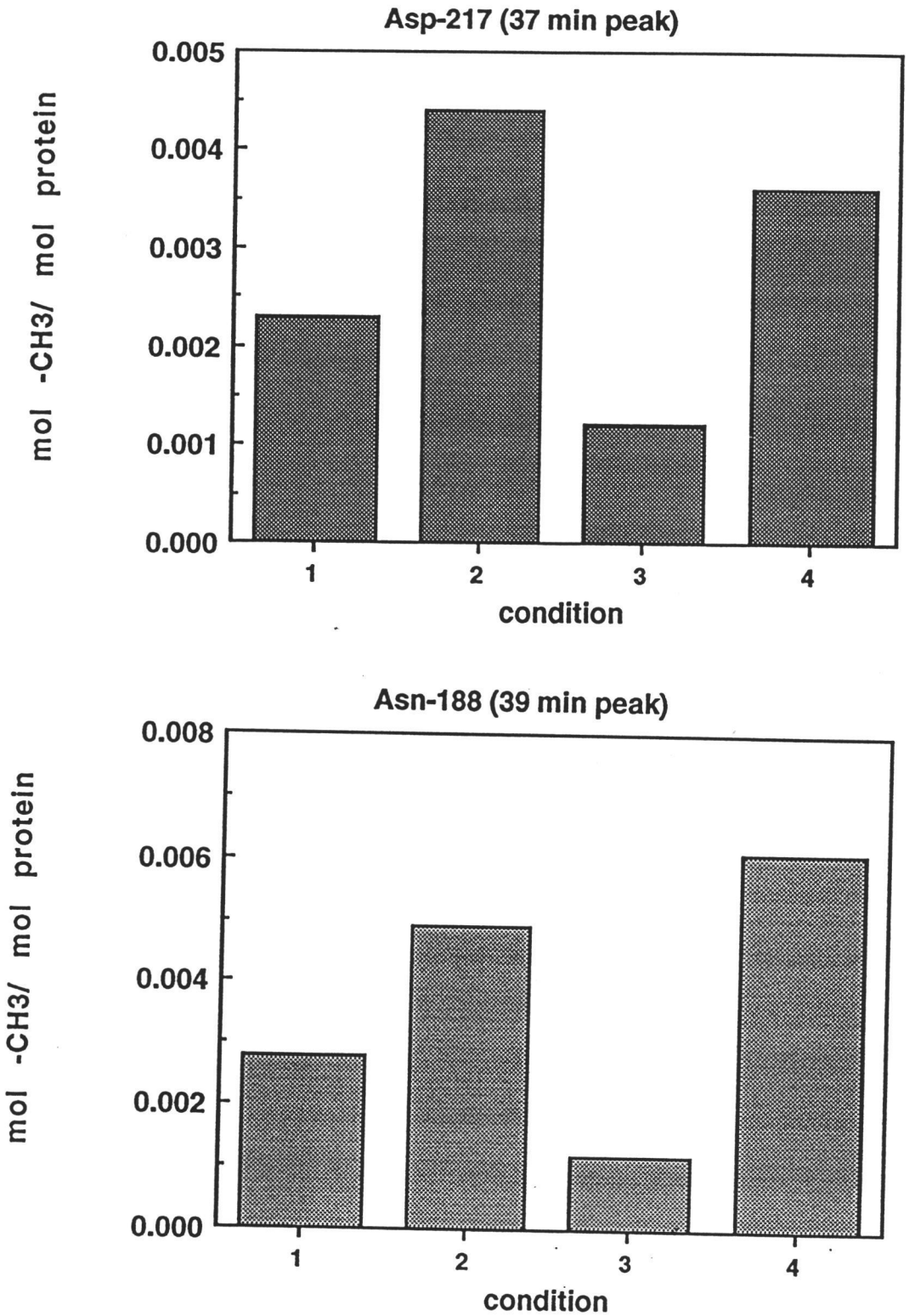
Asp-217 (37 min peak)
mol -CH3/ mol protein
0.005
0.004
0.003
0.002
0.001
0.000
1
2
3
4
condition
Asn-188 (39 min peak)
mol -CH3/ mol protein
0.008
0.006
0.004
0.002
0.000
1
2
3
4
condition

FIGURE 5.5

and methylating this damage as it occurs. However, the results also show that these methyl groups are stable at physiological pH and do not measurably turn over in a 24 hr time frame. Since methylation of the damaged site is the first step in repair, it can be assumed that these sites are repaired upon demethylation. However, no repair was observed due to the slow turnover of esters at T-1 and T-2 in the 24 hr incubation. Since the stoichiometry of methylation doubles with 24 hrs of incubation, while no repair has occurred, under these conditions the rate of damage exceeds the rate of repair. The consequence of an imbalance in these rates would be the accumulation of damage at these sites.

As an independent measure of ester stability, a time course was performed. After allowing a sufficient time for PCM to [^{3}H]automethylate, an excess of non-radioactive label was added and time points collected. Figure 5.6 shows the curve obtained from the analysis of these time points for their [^{3}H] radioactivity. The curve obtained does not appear to be a simple exponential decay, which is to be expected due to the presence of multiple damage sites within the enzyme, but a plot of ln DPM vs. time yields lines with a slope ($-k_d$) of -0.2050 hr^{-1} and -0.0177 hr^{-1}. The values for k_d can be converted into an average off rate for the methyl esters of $t_{1/2}$ = 3.4 hrs for the fast sites and 39.2 hrs for the slow sites. In comparison to the half-lives determined for [^{3}H] methylated erythrocyte membrane proteins (Terwilliger, T.C. and Clarke, S. 1981), the slow sites are approximately 10-fold slower than any previously observed.

Tryptic digestion of the time points resolved by HPLC (Figure 5.7) shows that T-1 and T-2 belong to the slow class of methyl esters, as the radioactivity associated with

FIGURE 5.6:

Time course of methyl group turnover. Following a 24 incubation at pH 7.8 in the presence of 20 μM [^{3}H-*methyl*] AdoMet [10,000 dpm/pmol], an excess of cold AdoMet was added to bring the final concentration to 0.5 mM. To determine the off rate of the methyl esters, aliquots of the repair reaction were analyzed for their base-volatile [^{3}H]methyl ester content at various times following the addition. Error bars are not shown for these single point determinations. A linear fit was chosen as an expontential curve did not appear to fit these data.

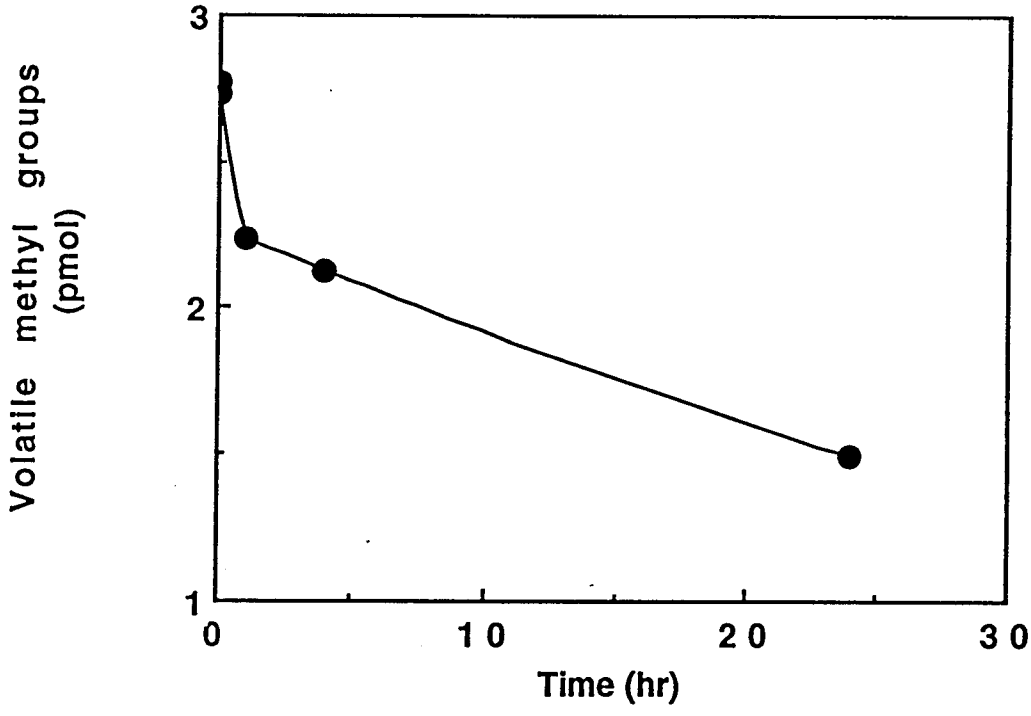

FIGURE 5.6

FIGURE 5.7:

Volatile [^{3}H]radioactivity profile of tryptic digested time points. A. Zero time of chase with non-radioactive AdoMet B. 1 hr of chase. C. 4 hr of chase. D. 24 hr of chase.

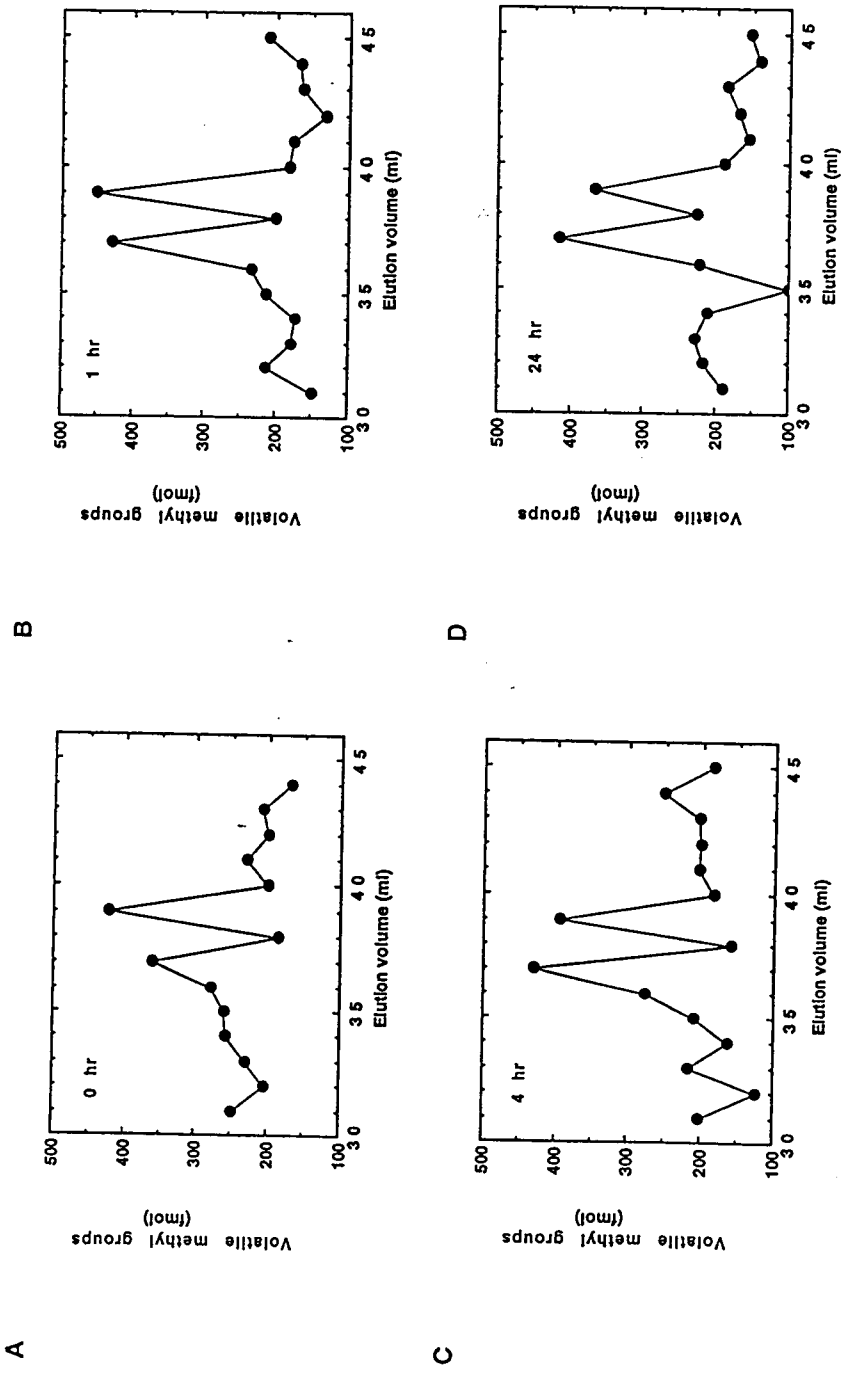
A
B
C
D
0 hr
1 hr
4 hr
24 hr
Volatile methyl groups (fmol)
Elution volume (ml)
500
400
300
200
100
3 0
3 5
4 0
4 5

FIGURE 5.7

each site was stable, supporting the conclusion that half-lives of over 24 hrs are involved. The sites possessing short half-lives have not been identified, but are interesting because they are turned over rapidly enough to allow self-repair in a 24 hr time frame. These sites are presumably so unstable as to be lost during site mapping by trypsin fragmentation. Quantification of the number of sites with fast and slow half-lives (Figure 5.6) indicates that as many as half of the automethylated sites are turned over rapidly.

While PCM appears capable of repairing damage at the "fast" sites, the inability of PCM to maintain the T-1 and T-2 sites reflects an inherent inefficiency in this repair system. The accumulation of unrepaired damage within PCM may lead to a loss of activity, as the presence of altered aspartyl residues has been shown to result in a loss of biological cativity in other proteins (Teshima 1995). Inefficiency in the repair system, resulting from a loss of activity in the repair enzymes, may lead to the accumulation of altered aspartyl damage in other systems throughout the cell.

A theoretical phenomenon, termed an error catastrophe or error cascade, has been proposed as a mechanism for intracellular aging, but evidence in its support has been elusive (Arking 1991, Dice 1993). Inefficient mechanisms for the repair or degradation of damaged proteins have been proposed to be the cause of a cellular error cascade. The failure of PCM to efficiently self-repair may be the first example of an inefficient repair mechanism and may also provide a system in which to test the possibility of a cellular error catastrophe..

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CHAPTER 6

INCORPORATION OF TWO ^{18}O ATOMS INTO A PEPTIDE DURING ISOASPARTYL REPAIR REVEALS REPEATED PASSAGE THROUGH A SUCCINIMIDE INTERMEDIATE.

Jonathan A. Lindquist and Philip N. McFadden

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RATIONALE

Protein (D-aspartyl/ L-isoaspartyl) carboxyl methyltransferase (PCM) has been shown to repair the majority of the L-isoaspartyl residues in model peptides to L-aspartyl residues. The purpose of this chapter is to measure the efficiency of repair by following the incorporation of $H_2{}^{18}O$ into the model peptide isotetragastrin [Trp-Met-isoAsp-Phe-NH_2] during a repair reaction.

INCORPORATION OF TWO ^{18}O ATOMS INTO A PEPTIDE DURING ISOASPARTYL REPAIR REVEALS REPEATED PASSAGE THROUGH A SUCCINIMIDE INTERMEDIATE.

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To study the mechanism of protein carboxyl methyltransferase-driven repair of age-damaged sites in polypeptides, a model L-isoaspartyl peptide, L-isotetragastrin, was enzymatically repaired to normal L-tetragastrin in the presence of ^{18}O-enriched water. By this design, the enrichment of ^{18}O atoms in the peptide would reflect the number of cycles through a hydrolyzable succinimide intermediate during formation of the repaired product. Mass determinations by FAB mass spectrometry, revealed that the repaired peptide contained two ^{18}O atoms incorporated, demonstrating that more than a single cycle of methylation and demethylation is necessary to ensure stoichiometric repair.

ABBREVIATIONS

HPLC, high pressure liquid chromatography, FAB, fast atom bombardment, TFA, trifluoroacetic acid, PCM, protein D-aspartyl/L-isoaspartyl carboxyl methyltransferase, L-

Normal, [L-Asp^3]tetragastrin; L-Iso, [L-isoAsp^3]tetragastrin; D-Normal, [D-Asp^3]tetragastrin; D-Iso, [D-isoAsp^3]tetragastrin.

KEY WORDS

Protein D-aspartyl/L-isoaspartyl carboxyl methyltransferase; protein repair; HPLC; aging; fast-atom bombardment mass spectrometry

INTRODUCTION

Spontaneous damage of amino acids in aging proteins not only has the potential to disrupt the structure and function of proteins, but may also create abnormal structures that are not degradable by cellular mechanisms of protein turnover. Protein carboxyl methylation has been proposed to rid the cell of two abnormal amino acids that commonly form in aging proteins, D-aspartic acid and L-isoaspartic acid (Johnson and Aswad, 1990; Ota and Clarke, 1990). These damaged sites are formed spontaneously through reactions of asparagine deamidation and aspartic acid cyclization that involve formation of a succinimide intermediate (Figure 6.1). Epimerization and hydrolysis of the succinimide at either the α- or β-carboxyl group can result in the formation of L-aspartic acid, D-aspartic acid, L-isoaspartic, and D-isoaspartic acid sites (Wright, 1991).

Protein D-aspartyl/L-isoaspartyl carboxyl methyltransferase (PCM) is known to transfer a methyl group from S-adenosyl-L-methionine (AdoMet) into a methyl ester linkage with D-aspartyl (McFadden and Clarke, 1982) and L-isoaspartyl sites (Aswad,

FIGURE 6.1:

The succinimide intermediate in the formation of isomeric aspartyl structures, with the α- and β-carboxyl groups designated. The asterisks indicate sites at which ^{18}O incorporation would occur if the succinimide is hydrolyzed in [^{18}O]water.

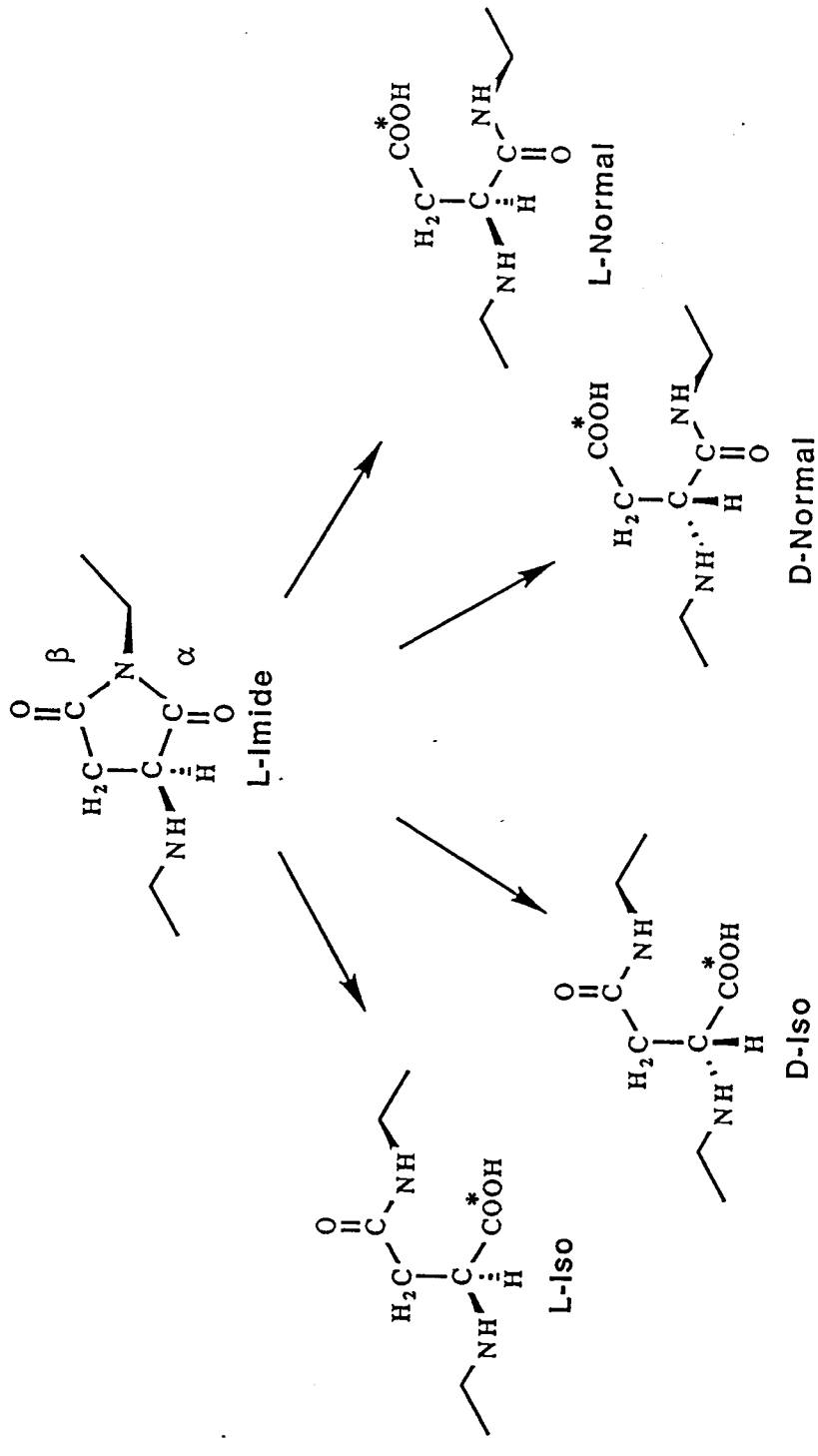

FIGURE 6.1

1984; Murray and Clarke, 1984). Restoration to a normal L-aspartic acid can in principle occur by demethylation via formation of a succinimide intermediate, since epimerization and/or hydrolysis at the β-carboxyl group of the succinimide then provides the opportunity to produce a normal L-aspartic acid site. However, such a pathway should also produce abortive rounds of methylation and demethylation in which succinimide hydrolysis results in an abnormal D-aspartyl, L-isoaspartyl, or D-isoaspartyl configuration. Of these, L-isoaspartic acid and D-aspartic acid can be methylated once again, giving further opportunities for repair to L-aspartic acid. Eventually, after repeated rounds, there should be a large enrichment in the L-aspartic acid configuration, as well as a by-product of D-isoaspartic acid which is not known to be methylated by PCM. Kinetic measurements of repair reactions using model age-damaged peptides and purified PCM have broadly supported this scheme (Johnson, et al., 1987a; Johnson, et al., 1987b; McFadden and Clarke, 1987), even though the abortive repair steps in the proposal have not been directly observed by tracer studies.

To directly test for abortive repair events we have now measured L-isoaspartyl repair in ^{18}O enriched water, allowing ^{18}O to be incorporated into either the α or β carboxyl of the aspartyl residue during succinimide hydrolysis (Figure 6.1). Double-incorporation of ^{18}O would occur only if a peptide had been methylated, cyclized to a succinimide, and hydrolyzed as a succinimide at least twice during the course of an enzymatic incubation. Previously, ^{18}O incorporation has been used to identify C-terminal proteolytic fragments (Rose, K. *et al.*, 1988), sites of N-glycosylation in proteins (Gonzalez, J. *et al.*, 1992), and the mechanism of N-glycosidic cleavage (Kaartinen, V. *et*

al., 1992), and so we expected that by utilizing analogous analytical approaches including fast-atom bombardment mass spectrometry we would be able probe for the presence of doubly ^{18}O-labeled peptides.

EXPERIMENTAL PROCEDURES

Materials

Tetragastrin·HCl was from Sigma. AdoMet·HSO_4 was from Boehringer-Mannheim. [^{18}O]Water (43 atom %) was from ICON (Mt. Marion, N.Y.). Glycerol (Ultrapure, US Biochemicals) and sodium phosphate (Sigma) were of grades that were certified by their manufacturers to be uncontaminated by detectable protease activity.

Peptide synthesis and purification

[L-isoAsp^3]Tetragastrin was prepared as described (McFadden and Clarke, 1986). Briefly, tetragastrin·HCl (12.6 μmols; L-Trp-L-Met-L-Asp-L-Phe-NH_2 ·HCl) was methyl esterified at aspartate by reacting it at 25 mM in methanol / 0.1 N HCl for 18 hrs at 23°C in the dark. The mixture was then diluted 10-fold with water, frozen, and lyophilized. The peptide ester was then dissolved in aqueous 0.1 M ammonium acetate pH 9.5, to a final concentration of 1 mM, and allowed to de-esterify for 20 hrs at 23°C. The peptide, now a mixture of isomers, was recovered by lyophilization, and the [L-isoAsp^3]tetragastrin was purified by gradient reversed-phase HPLC on a Dynamax C_{18}

column (5 μ bead size, 4.6mm x 25 mm; Rainin) with peptide detection at 214 nm (ε_{214} = 35,000 M^{-1} cm^{-1}). The peptide was recovered in a volatile solvent (0.1% v/v trifluoroacetic acid/ acetonitrile). The identification of [L-isoAsp^3]tetragastrin was verified by assay for its enzymatic carboxyl methylation (Murray and Clarke, 1984). A contaminant of [D-isoAsp^3]tetragastrin of about 5% is presumed to have been formed by epimerization at the aspartyl site during the de-esterification reaction (McFadden and Clarke, 1986).

Enzyme purification

Protein D-aspartyl/L-isoaspartyl carboxyl methyltransferase (PCM) was purified from bovine erythrocytes by a published method (Gilbert, et al., 1988) with modifications as described (Lindquist and McFadden, 1994a). The procedure resulted in a 5500-fold purification of the enzyme, with a final specific activity of 16,500 units/mg by the standard assay described (Murray and Clarke, 1984) in which 1 unit of activity equals 1 pmol of ovalbumin methyl esters formed per minute at pH 6. The isozyme used in this study was the more acidic of the two isozymes (PCM II), a translation product of an alternatively spliced messenger RNA (Ingrosso, et al., 1991; MacLaren, et al., 1992). Lyophilization of the enzyme prior to its use in repair reactions lowered its activity (V_{max}) by half.

Repair reactions

Reaction compositions were similar to those of McFadden and Clarke (1987), but were assembled with three separate stock solutions of reactants that were each prepared in ^{18}O water. Solution 1: [L-isoAsp^3]tetragastrin (1.4 nmols in HPLC solvent) and AdoMet (15 nmols in 10 mM HCl) were mixed together, lyophilized, and then dissolved in 23 µl [^{18}O]water (43 atom %). Solution 2: Sodium phosphate buffer (0.1 M pH 7.8, 66 µl) was lyophilized and re-dissolved in 23 µl ^{18}O water. Solution 3: PCM (20 µl of a 7.2 µM solution) and 10 µl glycerol were mixed together, lyophilized, and then redissolved in 10 µl of ^{18}O water. The three stock solutions were then mixed together on ice to give 66 µl final volume, and then incubated at 37°C for 0 to 48 hrs in capped 0.5 ml polypropylene tubes. Polypropylene centrifuge tubes were sterilized beforehand by autoclaving to minimize the presence of active proteolytic contaminants. A control reaction was prepared identically, but used normal [L-Asp^3]tetragastrin in place of [L-isoAsp^3]tetragastrin. Reactions were stopped by freezing in liquid nitrogen, with storage at -20°C for 1-3 days. Peptides were purified from the reaction mixtures by HPLC as described under peptide purification. HPLC fractions were lyophilized and resuspended with 10 µl 0.1% trifluoroacetic acid. Duplicate trials of the above procedures were performed on separate days with freshly prepared stock solutions.

Mass spectrometry

Peptides were analyzed by positive ion FAB mass spectrometry on a Kratos MS-50 operated by Brian Arbogast (Department of Agricultural Chemistry, Oregon State University). An aliquot (2-3 μl) of a peptide in solvent A was suspended in a matrix of 0.01 M oxalic acid in thioglycerol/glycerol (2:1) and applied to the probe tip. Samples were ionized by xenon bombardment, with the accelerating voltage at 8 kV. The detector was operated at 25 kV, with scans of 30 seconds through the mass range 100 to 1000 Daltons at a resolution of 1100 and 10% valley definition for raw data collection. Peak intensities were determined by integration, using the DS90 software (Kratos). To take into account the observed background oscillation which results in a higher background peak at every mass (Visentini et al. 1991), several background peaks above and below the field of interest were selected and their intensities averaged. The averaged background for odd or even mass peaks was then subtracted before calculating percent differences in peak intensities from those of standard tetragastrin.

RESULTS

Peptide repair in the presence of ^{18}O water

[L-isoAsp^3]Tetragastrin (L-Iso) was subjected to PCM-catalyzed repair in the presence of ^{18}O water. The assembly of the reactants was modified from previously established reaction conditions to allow replacement of ordinary water with

[^{18}O]enriched water. This relied on the fortunate ability of PCM activity to survive through lyophilization and rehydration in [^{18}O]water.

HPLC analysis (Figure 6.2) showed that after a 48 h repair reaction approximately 40% of the peptide retained the structure of the starting peptide, L-Iso. The other 60% of the peptide was converted to alternate structures, including [D-isoAsp^3]tetragastrin (D-Iso) which eluted as a distinct yet unresolved peak after L-Iso, and an unresolved mixture of [D-Asp^3]tetragastrin (D-Normal) and [L-Asp^3] tetragastrin (L-Normal). Because of the poor chromatographic resolution of D- and L- peptide forms, the following mass spectral analyses were of the pooled mixtures, [D+L]Iso, and [D+L]Normal.

FAB mass spectrometry of [D+L]Iso from the 48 hr repair reaction showed a major species of 597 Da, corresponding to the M+1 ion (Figure 6.3A) expected for peptide with no ^{18}O incorporated. The [D+L]Normal mixture from the repair reaction also contained a 597 Da M+1 ion as the predominant ion (Figure 6.3B). Ions of 599 Da and 601 Da, corresponding to the M+3 and M+5 ions expected of singly and doubly incorporated ^{18}O, were present in both the [D+L]Iso and [D+L]Normal mixtures. To allow later analysis of the M+3 and M+5 ions attributed to chemical exchange of ^{18}O into peptides without the involvement of PCM, a control reaction was performed in which an equivalent weight of L-normal tetragastrin was reacted with ^{18}O water, PCM, and AdoMet; after HPLC purification, the mass spectrum of this control peptide was obtained (Figure 6.3C). This reaction showed that no ^{18}O was incorporated into the peptide in the absence of a repair reaction. Also, to establish how much of the M+3 and M+5 ions were merely background resulting from naturally occurring heavy isotopes we measured the mass spectrum of an unreacted standard of L-normal tetragastrin (Figure

FIGURE 6.2:

HPLC analysis of peptides from ^{18}O repair reaction mixtures that initially contained 1.4 nmols of [L-isoAsp^3]tetragastrin.

A) ^{18}O repair reaction analyzed after zero time of incubation. The aliquot that was analyzed represented 0.42 nmol of initial [L-isoAsp^3]tetragastrin.

B) ^{18}O repair reaction analyzed after a 48 hr incubation. The aliquot that was analyzed represented 1.4 nmol of initial [L-isoAsp^3]tetragastrin.

The scale for detection at 214 nm is shown by the vertical bars. The horizontal bars indicate [D+L isoAsp^3]tetragastrin and [D+L Asp^3]tetragastrin that were collected as pools for mass spectrometry. Also shown are the elution positions of the methyl ester and succinimide intermediates in the repair reaction.

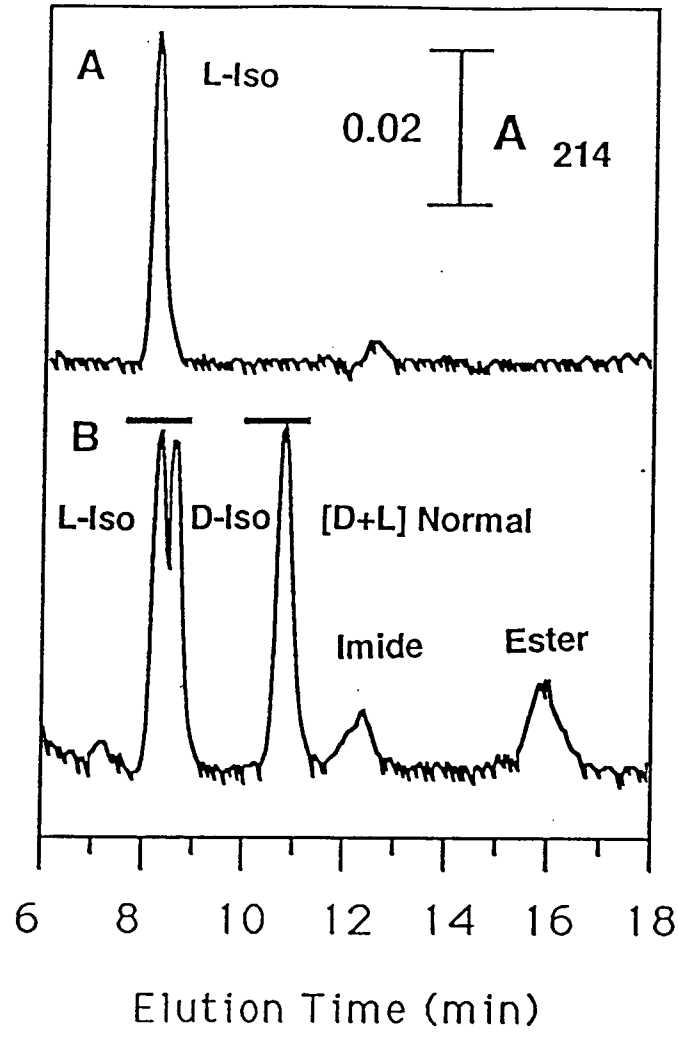
A
L-Iso
0.02
A 214
B
L-Iso
D-Iso
[D+L] Normal
Imide
Ester
6
8
10
12
14
16
18
Elution Time (min)

FIGURE 6.2

FIGURE 6.3:

FAB-mass spectra of HPLC purified peptides showing the relative intensity of ions at masses between 580 and 610 Da.

A) [D+L isoAsp^3]Tetragastrin from an ^{18}O repair reaction.

B) [D+L Asp^3]Tetragastrin from an ^{18}O repair reaction.

C) Tetragastrin from a control ^{18}O repair reaction in which only normal tetragastrin was initially present.

D) The spectrum of standard tetragastrin that had not been incubated in any manner.

The 0,1, and 2 refer to the masses expected for incorporation of zero, one, and two ^{18}O atoms into a tetragastrin polypeptide. A second trial of these experiments produced similar spectra.

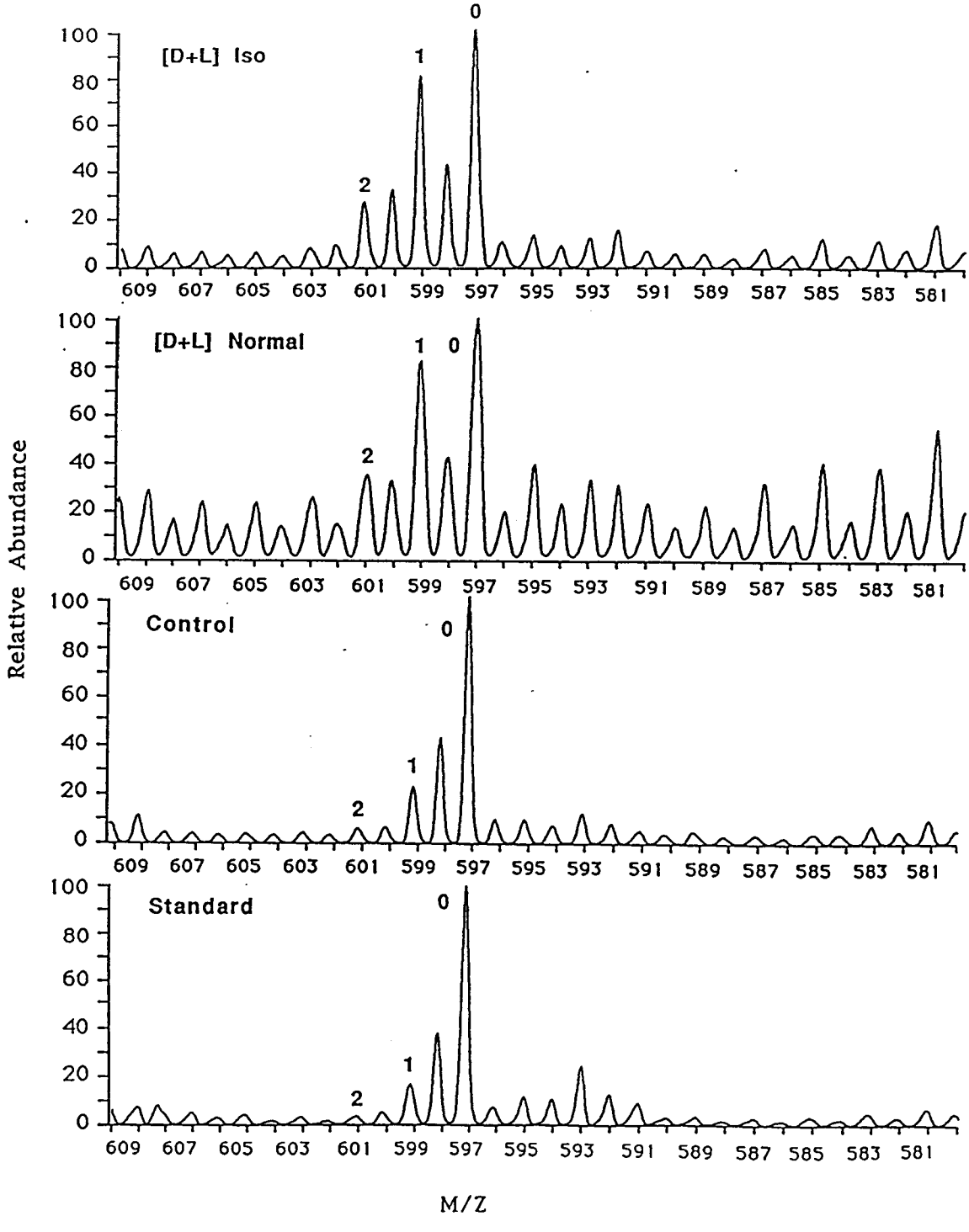
[D+L] Iso
[D+L] Normal
Control
Standard
Relative Abundance
M/Z
100
80
60
40
20
0
609 607 605 603 601 599 597 595 593 591 589 587 585 583 581
0
1
2

FIGURE 6.3

6.3D), finding an essentially perfect match in relative peak intensities to expectations based on the natural abundances of C, N, O and H isotopes in tetragastrin (66.3% M+1, 7.9% M+3, and 0.2% M+5).

To correct for this contribution of the naturally occurring isotopes, the relative intensities of ions from standard tetragastrin were subtracted from those of peptides from repair reactions. For convenience, these corrected ion intensities are shown as their percent differences from the intensities of standard L-Normal ions of 597 to 602 Da. (Figure 6.4, unshaded bars). Ion mass distributions were clearly affected by the ^{18}O repair reactions. This can be seen first in the spectrum for [D+L]Normal from the repair reaction, which deviated considerably from the standard spectrum, with the M+1 (597) ion being lowered in relative intensity by 29%, and the M+3 (599) and M+5 (601) ions being 21% and 8% more intense, respectively (Figure 6.4A, unshaded bars). A similar situation was observed for the ions of [D+L]Iso from the repair reaction (Figure 6.4B, unshaded bars). In contrast, the ions of L-Normal from the control reaction deviated little from standard L-Normal tetragastrin that had not been incubated in any manner (Figure 6.4C).

To determine the distribution of ^{18}O incorporation that produced the above spectra, theoretical spectra of unlabeled peptide, mono-^{18}O peptide, and di-^{18}O peptide were added together in various proportions until good subjective fits to the experimental spectra were obtained. The shaded bars in Figure 6.4 show what were judged to be the best computational fits to the experimental spectra. The best fit to the [D+L]Normal spectrum showed that unlabeled peptide, mono-^{18}O peptide, and di-^{18}O peptide were present in the approximate proportion 59: 36: 5 (Figure 6.4A, shaded bars). For

FIGURE 6.4:

Ion intensities in the M/Z range of 597 to 602, shown as percent differences from standard tetragastrin. Open bars are experimental data, with the error bars indicating the range of duplicate measurements. Solid bars are theoretical fits as described in the text.

A) [D+L Asp^3]tetragastrin from ^{18}O repair reactions (open bars). The theoretical fit (solid bars) is for the ratio 59: 36: 5, unlabled / monolabeled/ doubly labeled peptide.

B) [D+L $isoAsp^3$]tetragastrin from ^{18}O repair reactions (open bars). The theoretical fit (solid bars) is for the ratio 57: 36:7, unlabled / monolabeled/ doubly labeled peptide.

C) Tetragastrin from a control incubation in which normal L-tetragastrin was exposed to conditions of the ^{18}O repair reaction.

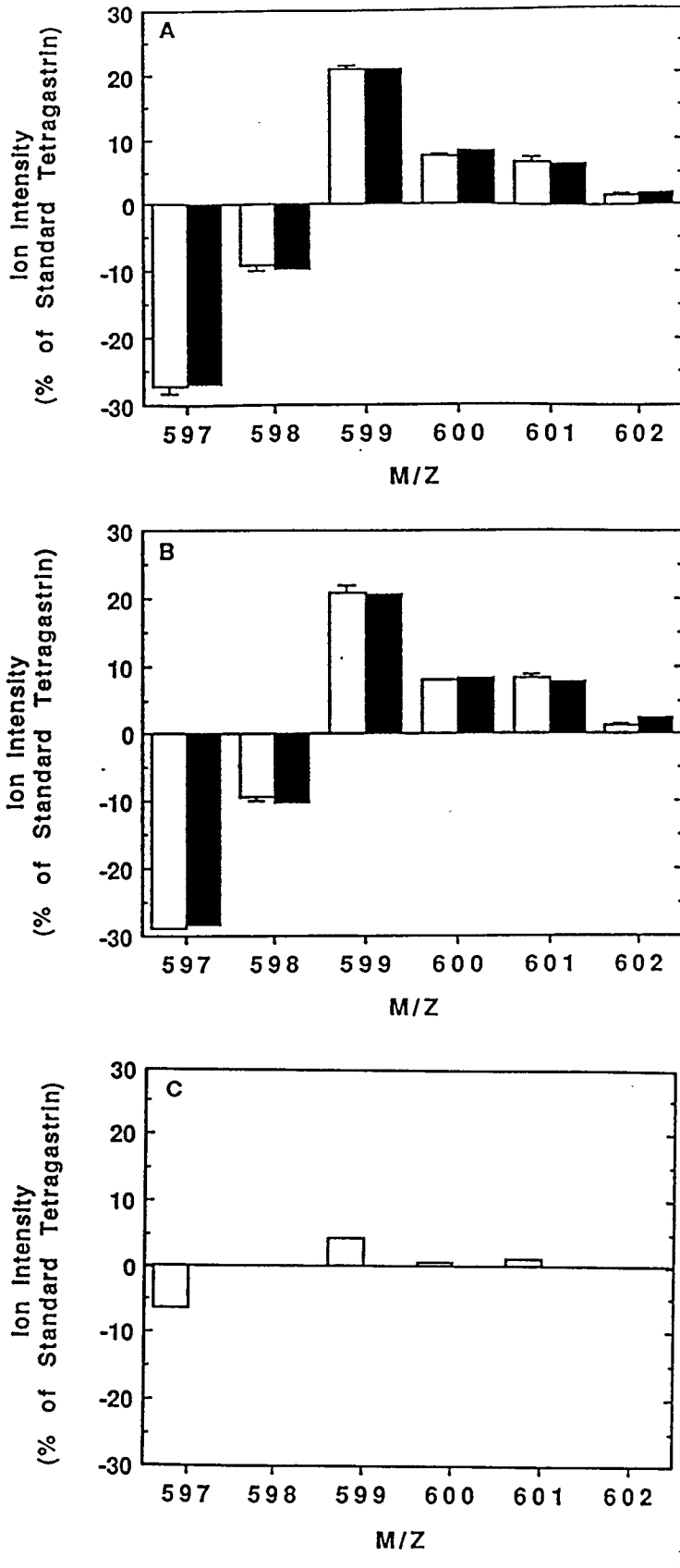
A
B
C
Ion Intensity
(% of Standard Tetragastrin)
30
20
10
0
-10
-20
-30
597
598
599
600
601
602
M/Z

FIGURE 6.4

[D+L]Iso from the repair reaction, the proportion was found to be approximately 57: 36: 7 (Figure 6.4B, shaded bars). Thus, both the [D+L]Normal and [D+L]Iso from ^{18}O repair reactions contained peptide molecules that had been doubly labeled with ^{18}O.

Kinetic model of ^{18}O incorporation during peptide repair

To model the events of the repair reaction in [^{18}O]water, a kinetic model was developed for ^{18}O incorporation into L-Iso during enzymatic methylation, non-enzymatic demethylation to a succinimide, and epimerization and hydrolysis of the succinimide to yield the variously ^{18}O-labeled forms of L-Iso, D-Iso, L-Normal, and D-Normal. (Figure 6.5). Our nomenclature uses a pair of subscripts (e.g. 0,0) to indicate the number of ^{18}O atoms incorporated into the α- and β-carboxyl groups, respectively. The kinetic constants for the enzymatic and nonenzymatic steps had been elucidated earlier (McFadden and Clarke 1987) and are listed in the Table. We assume a) that L-Iso is the only peptide that can be methylated by PCM, with the K_m and V_{max} as listed, b) that L-succinimide forms by a first-order demethylation reaction, c) that L-succinimide epimerizes to D-succinimide by a first-order reaction that is essentially irreversible on the time-scale of the study, d) and that first-order hydrolysis of D- and L-succinimides is four-times as rapid at the α-carboxyl group than at the β-carboxyl group, as shown in numerous experimental cases (Clarke et al. 1992). The model also includes the assumption of ^{18}O incorporation (43 atom %) into α- and β-carboxyls during succinimide hydrolysis. An important restriction of this model is that at most a single

FIGURE 6.5:

Kinetic model for ^{18}O incorporation during peptide repair. Enzymatic and nonenzymatic reactions assumed to be of significance in ^{18}O repair reactions. Reaction rate constants k_1 through k_7 are described in Table I. The isomeric forms of tetragastrin include L-Iso, D-Iso, L-Normal, and D-Normal. The post-script "α, β" symbolizes the number of ^{18}O atoms incorporated in the α- and β-carboxyl groups of the peptide aspartic acid. It is assumed that there is no isotope effect upon any reaction rate. Dashed arrows indicate the two steps that for simplicity of calculation were assumed to occur at negligible rates: Firstly, since the K_m for methylation of D-Normal by PCM is 840-fold higher than the K_m for L-Iso, it is assumed that the only species that is methylated is L-Iso. Secondly, is assumed that epimerization of L-Succinimide (L-Imide) to D-Succinimide (D-Imide) occurs at a significant rate, but that the reverse reaction (D-Imide to L-Imide) can be ignored throughout the time course because of the low initial concentration of D-Imide.

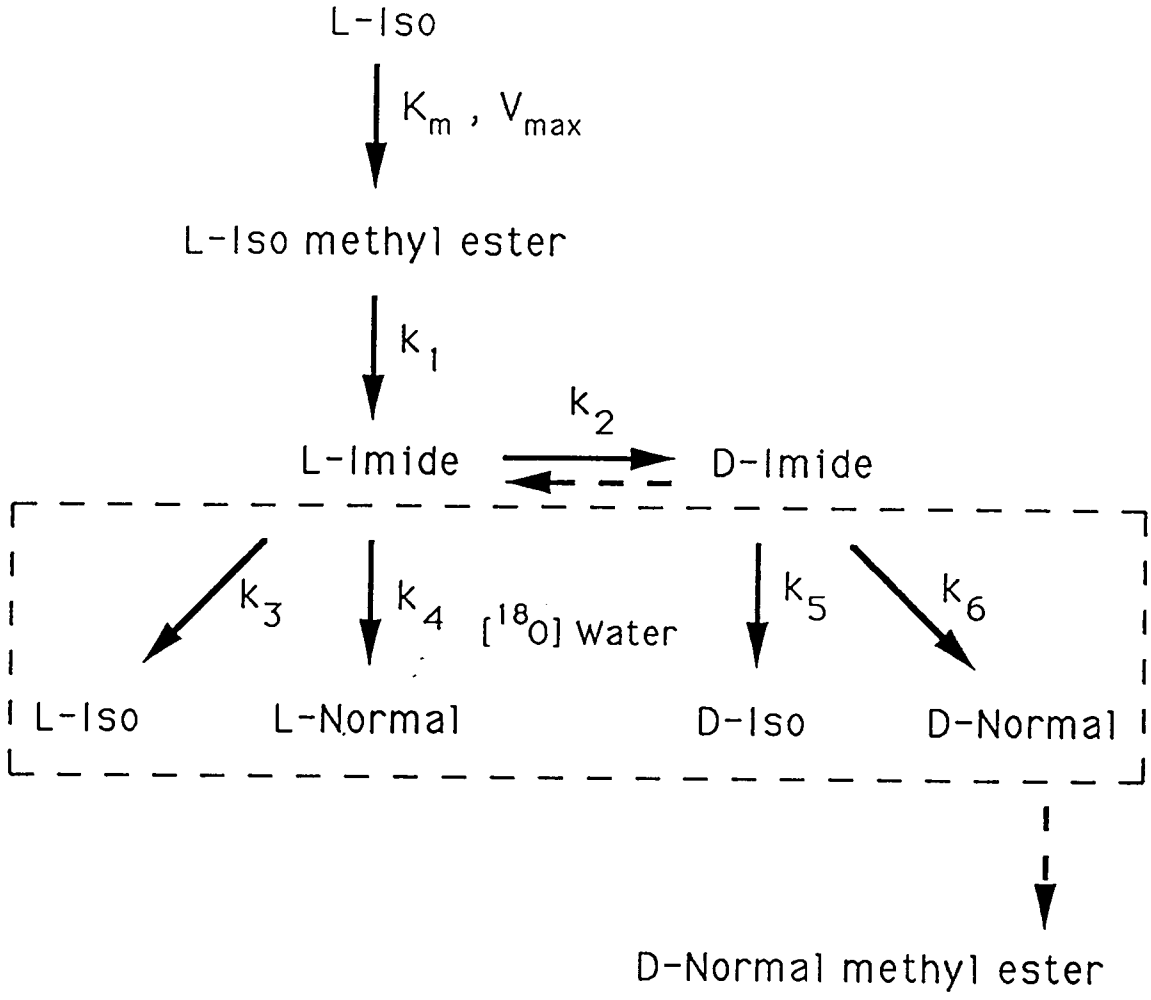
L-Iso
K_m , V_{max}
L-Iso methyl ester
k_1
k_2
L-Imide
D-Imide
k_3
k_4
[^{18}O] Water
k_5
k_6
L-Iso
L-Normal
D-Iso
D-Normal
D-Normal methyl ester

FIGURE 6.5

^{18}O atom can be incorporated in the β-carboxyl group since the first hydrolytic event at a β-carboxyl yields L-Normal or D-Normal, neither of which can be methylated or transformed to other species under the assumptions of the model. Both the α- and β-carboxyls must be ^{18}O-labeled, then, in order for L- or D-Normal peptides to contain two ^{18}O atoms.

To test how this model compared to the experimental repair reactions, the formation of [D+L]Iso species and [D+L]Normal species with 0, 1 or 2 atoms of ^{18}O was calculated as a function of time (Figure 6.6). From this modeled time course, the distribution of ^{18}O labeled peptides at about 1600 minutes yielded peptide mass distributions (57: 38: 5:, unlabeled Iso/ monolabeled Iso/ doubly-labeled Iso; and 51: 44: 5, unlabeled Normal/ monolabeled Normal/ doubly-labeled Normal) that were most similar to those observed in the actual ^{18}O repair reactions. This kinetic model further calculates that by this time about 1.9 hydrolyses of a [D+L]Succinimide structure would have occurred for each L-Iso originally present in the reaction mixture.

DISCUSSION

In this study, enzymatically repaired peptides were detected with two ^{18}O atoms from [^{18}O]water, substantiating the prediction that repeated hydrolysis of a succinimide intermediate takes place in the in vitro repair of L-isoaspartyl peptides. Succinimides typically hydrolyze to a proportion of 0.8 isoaspartyl-product and 0.2 normal aspartyl-product. Given this efficiency, and for the moment ignoring epimerization, the following

FIGURE 6.6:

Modeled time course for peptide repair in the presence of [^{18}O]water. The relative abundance of [D+L]Iso and [D+L]Normal, containing 0, 1 or 2 atoms of ^{18}O, are shown as a function of time. The curves were generated using the model proposed in Figure 5 and the kinetic constants inthe Table.

TABLE

Kinetic constants used to model peptide repair in the presence of [^{18}O]water.

Rate Constant	Value[1]
V_{max}	0.4 μM min^{-1}
K_m	3.0 μM
k_1	1.7 x 10^{-2} min^{-1}
k_2	6 x 10^{-4} min-1
k_3	4.8 x 10^{-3} min^{-1}
k_4	1.2 x 10^{-3} min^{-1}
k_5	4.8 x 10^{-3} min^{-1}
k_6	1.2 x 10^{-3} min^{-1}

[1]Values of these constants were determined previously (McFadden and Clarke, 1987).

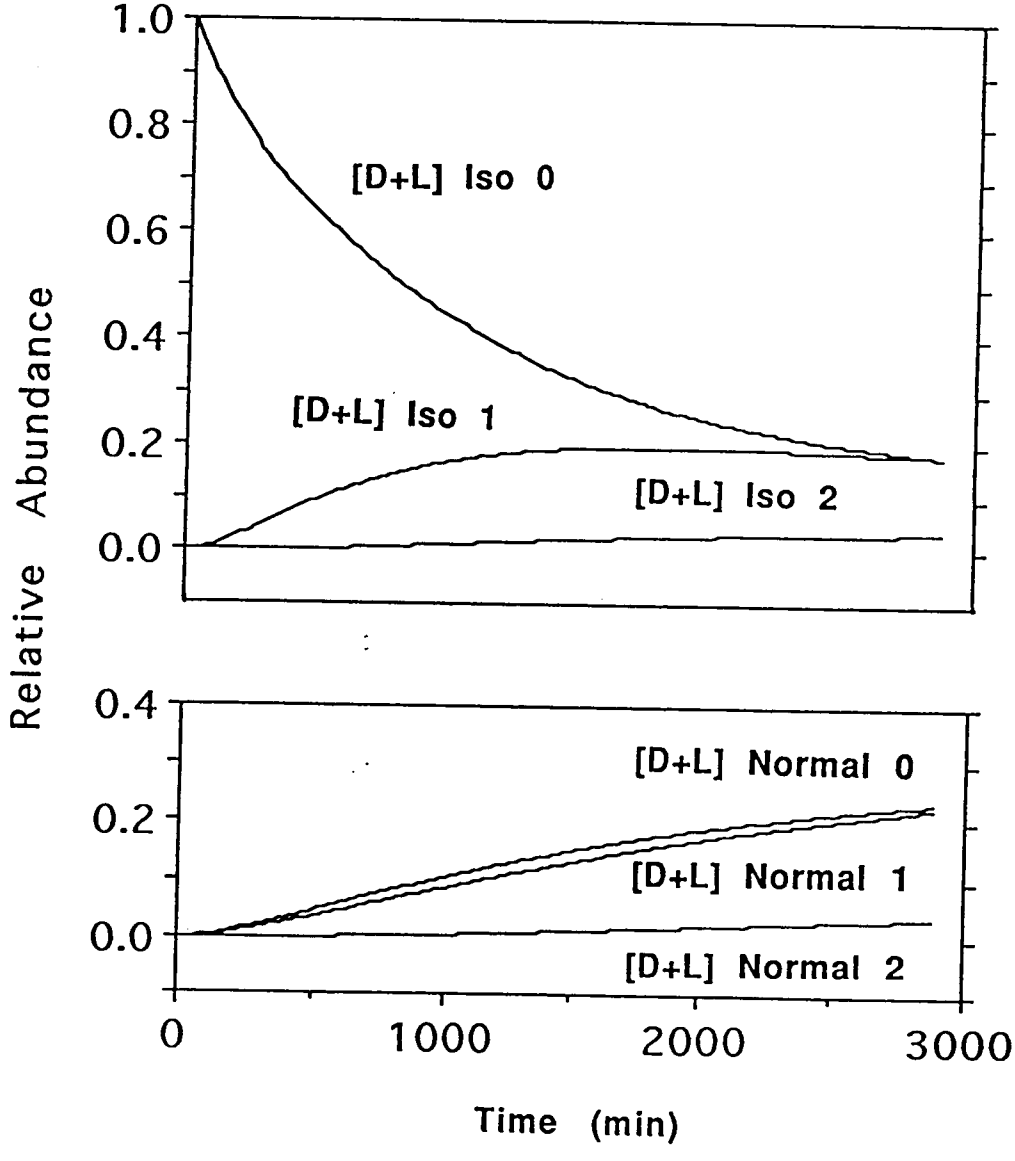
1.0
0.8
0.6
0.4
0.2
0.0
Relative Abundance
[D+L] Iso 0
[D+L] Iso 1
[D+L] Iso 2
0.4
0.2
0.0
[D+L] Normal 0
[D+L] Normal 1
[D+L] Normal 2
0
1000
2000
3000
Time (min)

FIGURE 6.6

kinetic considerations dictate that an L-isoaspartyl site would undergo an average of five conversions to a succinimide to allow stoichiometric repair: Given that L-Normal is formed by hydrolysis of L-Succinimide, then the time-dependence of the repair reaction is given by

$$d[\text{L-Normal}]/dt = 0.2*[\text{L-Succinimide}].$$

Integration to infinite time, by which time the [L-Normal] approaches 1.0, gives the just-mentioned value of 5 for the total amount of L-Succinimide formed during the time course. If the epimerization reaction is included in these considerations then the formation of unmethylatable D-isoAsp and slowly methylated D-Asp decrease the opportunity for succinimides to be formed by methylation and demethylation reactions. However, since epimerization is relatively slow, it remains a strong prediction that many cycles of succinimide hydrolysis will occur per mole of starting L-Iso. For example, the theoretical model that we have evaluated (Figure 6.5) includes the potential for epimerization of L-Succinimide to D-Succinimide, and numerical integration predicts that by infinite time about 4.2 [D+L]Succinimides would have formed per initial mole of L-Iso. Of course, a hypothetical enzyme-like activity that partitions a hydrolyzed succinimide toward L-Normal could improve the efficiency of methylation-driven repair, but no such activity is known.

Our ^{18}O repair reactions did not go to completion, as seen by HPLC traces that showed L-Iso remaining in the reactions, and as indicated by kinetic modeling that suggested that the repair reaction had halted to yield the set of products expected in the vicinity of 1600 minutes of reaction time. There are several possible reasons for why the

^{18}O repair reactions halted, including loss of activity of PCM due to its spontaneous denaturation, or a buildup of S-adenosylhomocysteine that acted as an end-product inhibitor. Even so, about 1.9 succinimide hydrolyses had occurred per mole of L-isotetragstrin that was initially present. Thus, while the theoretical limit of 4-to 5-succinimide hydrolyses was not reached, abortive cycles of succinimide hydrolysis had clearly taken place.

In heavy atom labeling experiments it is important to consider the possibility of chemical isotope effects on reactions rates. While water is a reactant in the repair mechanism, very little isotope effect on reaction rate is expected since introduction of the heavy atom results in a mass change of only about 11% in the water molecule, and only about 0.3% in the peptide molecule. We therefore assumed in our kinetic analysis that that there is no isotope effect on any reaction rate, and we made use of kinetic constants derived previously for the repair of L-isotetragastrin by bovine erythrocyte PCM (McFadden and Clarke, 1987).

Although the weight of evidence points to a repair function for PCM in vivo, the metabolic utlization of succinimide hydrolysis with its many abortive steps seems curiously inefficient. As estimated by Atkinson, the methyl donating AdoMet molecule is formed at the highest metabolic cost on a per carbon basis of any cellular compound (Atkinson, 1977). The use of the methyl group to drive an abortive reaction thus seems doubly wasteful. Though it is not necessary for individual steps in metabolic pathways to be efficient so long as the overall pathway serves a physiological need, the evidence acquired so far for the mechanism of in vitro peptide repair leaves open the interesting

possibility that other players in the physiological repair pathway may yet be found that improve its efficiency.

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CHAPTER 7

NON-METHYLATABLE SITES IN BOVINE ERYTHROCYTE PROTEIN (D-ASPARTYL/L-ISOASPARTYL) CARBOXYL METHYLTRANSFERASE

Jonathan A. Lindquist and Philip N. McFadden

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RATIONALE

During the repair of protein (D-aspartyl/ L-isoaspartyl) carboxyl methyltransferase (PCM) a methodology was used which involved the methylation of proteolytic fragments derived from PCM. The purpose of this chapter is to investigate the occurrence of methylatable sites that had not been identified during previous automethylation reactions.

NON-METHYLATABLE SITES IN BOVINE ERYTHROCYTE PROTEIN (D-ASPARTYL/L-ISOASPARTYL) CARBOXYL METHYLTRANSFERASE.

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The identification of unrepairable damage within in protein (D-aspartyl/L-isoaspartyl) carboxyl methyltransferase (PCM) has led to the question, "To what extent does damage accumulate within the enzyme?" [^{3}H] Methylation of peptides derived from PCM revealed the existence of "cryptic" sites of damage within the PCM molecule. Sites of damage, which the enzyme seems unable to repair when in its native conformation, provide further support for the observation that PCM had become slightly more acidic with damage and therefore bound somewhat more tightly to the anion exchange resin. The existence of cryptic sites, in conjunction with the identification of two sites of automethylation, has led to the proposal that αPCM is not a homogeneous population of damaged PCM molecules, but rather a subpopulation of molecules with a hierarchy of damage sites (α_1, α_2, α_3, α_4, α_5,...). The potential for the accumulation of damage at several unrepairable sites adds a further level of complexity to the hierarchical nature of protein damage.

INTRODUCTION

Two protocols have been used to map the sites of altered aspartate in protein (D-aspartyl/L-isoaspartyl) carboxyl methyltransferase (PCM). The first allows PCM to automethylate with [^{3}H-*methyl*]AdoMet, followed by digestion (see Chapter 4). In the second protocol, PCM was proteolytically digested and then active PCM was used to detect sites of methylation in the tryptic fragments. In principle, the second method allows complete detection of ALL methylatable sites, while the first method allows detection of only those sites accessible in intact PCM. This chapter provides evidence for the existence of sites that are damaged and CANNOT be methylated in intact PCM. Non-methylatable sites are an important consideration, as they may represent unrepairable damage in a repair enzyme.

EXPERIMENTAL METHODS

[^{3}H] Methylation of tryptic PCM fragments (Method 1)

The PCM used in this study was anion exchange FPLC purified PCM (see Chapter 2). For a typical reaction, 50 pmol of enzyme (90 µl) taken from the acidic, late-eluting shoulder was added to 63 µl of 0.2 M sodium citrate pH 6.0. This mixture was then digested with TPCK-treated trypsin (1:1) [Sigma] for 40 minutes at 37°C. Trypsin was prepared as described below under "proteolytic digestion". The digest was quenched with the addition of 2 µl of 100 mM phenylmethanesulfonyl fluoride in isopropanol

(PMSF). Tryptic fragments were then [^{3}H]methylated upon addition of 0.1 μM PCM and 4 μM [^{3}H-*methyl*]AdoMet [NEN, 15 Ci/mmol] for 40 minutes at 30°C. Following the methylation reaction, the tryptic fragments were separated by reversed phase HPLC on a C_{18} column as described under "HPLC purification". An aliquot (500 μl) of each HPLC fraction collected was assayed for base volatile radioactivity by pipetting it directly into a glass inner vial [15 mm x 45 mm, Kimble] containing 100 μl of 1 M NaOH. The inner vials were placed into 20 ml scintillation vials containing 4 mls of xylene based liquid scintillation cocktail [Scintiverse E, Fisher] and capped. Vials were allowed to equilibrate before counting and the efficiency of capture of the base-volatilized radioactive methanol determined using [^{14}C]methanol standards prepared in parallel.

[^{3}H] Methylation of tryptic PCM fragments (Method 2)

Typically, an aliquot containing 600 pmols (90 μl) of the FPLC peak fraction was further purified by reversed phase HPLC, lyophilized into 20 μl glycerol, and resuspended in 200 μl of 0.2 M sodium citrate pH 6.0. Proteolytic digestion with trypsin was performed as described under "proteolytic digestion". Immediately following digestion, the reaction was placed on ice and the protease inhibited with the addition of an excess of phenylmethanesulfonyl fluoride (70 μg). Methylation was performed using 1 μM PCM and 100 μM [^{3}H-*methyl*] AdoMet (250 mCi/mmol) at 30°C for 40 minutes as recommended for stoichiometric methylation of substrate by Johnson and Aswad (1991).

The sample was then mixed with an equal aliquot of PCM that had been proteolytically digested following [^{14}C]automethylation. The combined mixture was then purified by reversed phase HPLC on a C_{18} column.

[^{14}C] Automethylation

Typically, an aliquot containing 600 pmol (90 µl)of anion exchange FPLC peak fraction was mixed with 63 µl of 0.2 M sodium citrate pH 6.0. The reaction was initiated upon addition of 26 µl of [^{14}C-*methyl*]AdoMet [NEN] (57.6 mCi/mmol) and incubated for 40 minutes at 37°C. Following automethylation, the reaction was purified on the HPLC to remove the unincorporated [^{14}C] label and lyophilized into glycerol. The lyophilized sample was resuspended in 50 µl 0.2 M sodium citrate pH 6 and digested with trypsin (as described under proteolytic digestion). The digested sample was then pooled with [^{3}H]methylated tryptic PCM fragments as described under "[^{3}H]methylation of tryptic fragments (method 2)".

HPLC purification

High performance liquid chromatography (HPLC) was performed using a water/ acetonitrile gradient system. Solvent A is dI H_2O/ 0.01%(v/v) TFA [Sigma]. Solvent B is 90%(v/v) acetonitrile [Burdick & Jackson, UV grade]/ 9.9%(v/v) dI H_2O /0.1%(v/v) TFA. Protein purification was performed using a Vydac C_4 column [particle size 5 µ, 4.6 mm x 250 mm] with component elution monitored at 280 nm (as described in Chapter 2).

Peptide purification was performed using a Dynamax C_{18} column [particle size 5 μ, 4.6 mm x 250 mm] with elution monitored at 214 nm. Peptides were eluted from the column using a linear gradient from 0-70% solvent B in 70 minutes. Fractions were collected at 1 minute intervals during the run.

Proteolytic digestion

HPLC purified PCM was lyophilized to dryness, resuspended in 50 μl of 0.2 M sodium citrate pH 6.0 and digested with TPCK-treated Trypsin (1:10) [Sigma] for 40 minutes at 37°C. Trypsin (1 mg/ml in 1 mM HCl) was prepared freshly before use from a 10 mg/ml stock solution (in 1 mM HCl/10 mM $CaCl_2$, stored at 4°C) to minimize autodigestion. Tryptic fragments were resolved using reversed phase HPLC.

RESULTS

To determine the total methylatable sites within PCM, an aliquot of PCM was proteolytically digested to completion with trypsin. The tryptic fragments of PCM were then [^{3}H]methylated and resolved using reversed-phase HPLC. Measurement of the base-volatile [^{3}H] radioactivity of the material collected from the HPLC yielded the complex mixture of peaks shown in Figure 7.1. The large peaks eluting at 4-6 minutes, 12 minutes, and 17 minutes are due to unincorporated isotope, methanol generated during the methylation reaction, and/or degradation products of [^{3}H-*methyl*] AdoMet, as they are insensitive to digestion with pronase and are also visible in the control reactions (data not

FIGURE 7.1:

Reversed phase HPLC of [^{3}H] methyl esterified peptides derived from tryptic digestion of anion exchange purified PCM. Following a 40 minute digestion, the protease was inhibited with PMSF and the resulting peptide mixture, 0.7 μM total peptide which is estimated to be less than 0.1 μM substrate, was methylated for 40 minutes at 30°C using 0.1 μM PCM and 4 μM [^{3}H-*methyl*] AdoMet [15 Ci/mmol]. Following purification by reversed phase HPLC on a C_{18} column, 50% of each fraction was assayed for base volatile radioactivity.

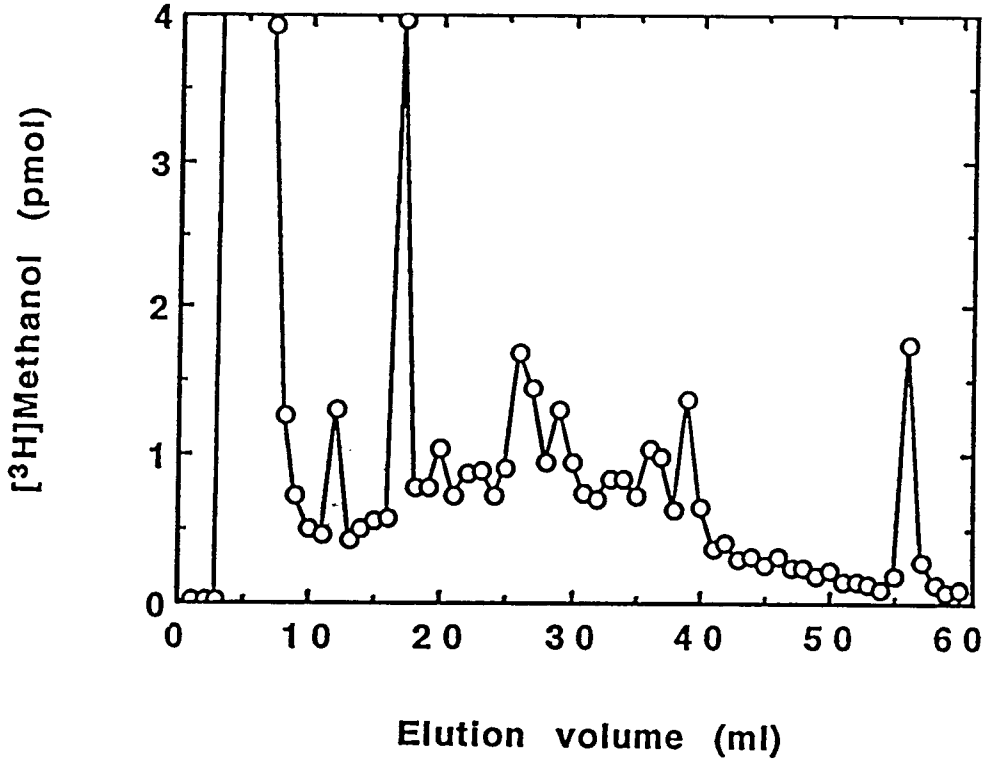

FIGURE 7.1

shown). The large peak eluting at 56 minutes is due to automethylation of the PCM fraction used to methylate the proteolytically derived fragments. The peaks eluting at 37 minutes and 39 minutes are the previously determined sites of automethylation, T-1 and T-2 (see chapter 4). The peaks eluting at 20 minutes, 22-23 minutes, 26-27 minutes, 29 minutes, and 33-34 minutes are novel peaks and represent sites of methylation that had previously not been seen during automethylation reactions.

To distinguish between repairable and unrepairable sites, two distinct isotopic methyl groups were utilized. By varying the period of methylation for each isotope to either before ([^{14}C]automethylation) or after ([^{3}H]methylation) proteolytic digestion, sites of methylation that were inaccessible to the enzyme while in its native conformation were identified (see Figure 7.2). New peaks were identified at 27 minutes, 42 minutes, and 48 minutes. The large background is due to unincorporated tritium label that could not be removed before the analysis and may have caused additional peaks to escape detection. The isotopically labeled samples were pooled prior to injection as co-elution would ensure positive identification of the automethylated peaks.

In a previous set of experiments (see Chapter 5), the stoichiometry of methylation of the cryptic peaks was also seen to increase with a 24 hr incubation at 37°C. The increase in the stoichiometry of methylation for the peak eluting at 27 minutes is shown in Figure 7.3. Condition 1 establishes the basal level of damage existing in the purified enzyme. Incubation in the absence of AdoMet (condition 2) shows an increase in number of methyl groups incorporated into this fragment upon [^{3}H]methylation, while

FIGURE 7.2:

Reversed phase liquid chromatography of methyl esterified peptides derived from either digestion of intact [^{14}C] automethylated PCM (filled circles) or by [^{3}H] enzymatic methylation of pre-digested PCM (open circles). One aliquot of purified PCM was digested with trypsin following [^{14}C] automethylation. A second aliquot was first digested with trypsin and then [^{3}H] methylated. The methylation reaction was performed by first inhibiting the protease with PMSF and then adding active PCM (2 μM) and [^{3}H-*methyl*] AdoMet (100 μM) to the tryptic peptides. Peptides were methylated for 40 minutes at 30°C and the reactions stopped by freezing in liquid N_2. The two samples were then thawed on ice, pooled, and purified by reversed phase HPLC on a C_{18} column. Fractions were collected and an aliquot assayed for both [^{3}H] and [^{14}C] radioactivity by direct counting. The arrow indicates the elution position of the intact protein.

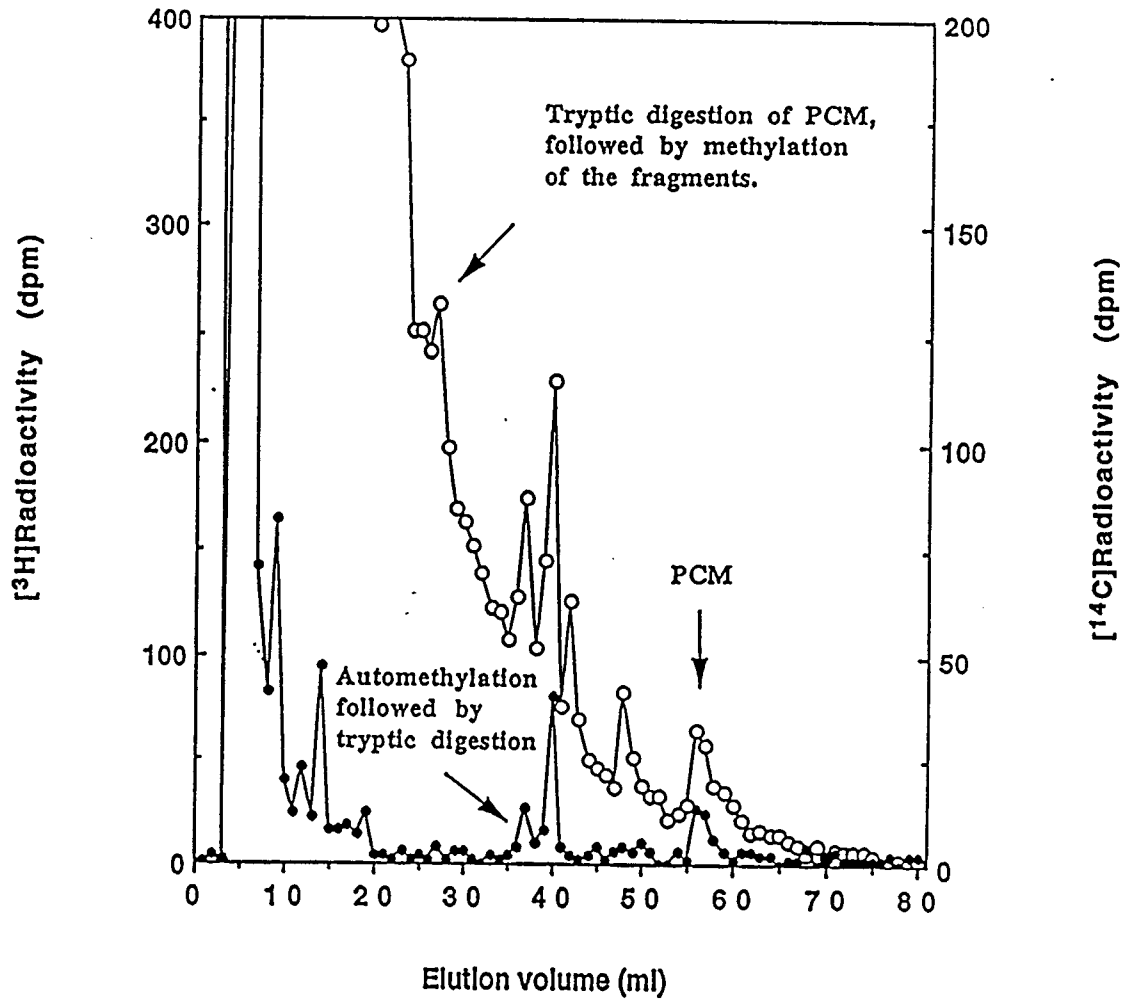
400
300
200
100
0
200
150
100
50
0
0 10 20 30 40 50 60 70 80
[3H]Radioactivity (dpm)
[14C]Radioactivity (dpm)
Elution volume (ml)
Tryptic digestion of PCM, followed by methylation of the fragments.
PCM
Automethylation followed by tryptic digestion

FIGURE 7.2

FIGURE 7.3:

A histogram showing an increase in the stoichiometry of methylation at a cryptic site that elutes at 27 minutes from reversed phase HPLC purification of tryptic digested PCM. Experimental conditions are described in the preceding chapter on self-repair. Condition 1, PCM with no incubation. Condition 2, PCM incubated in the absence of AdoMet. Condition 3, PCM incubated with AdoMet. Condition 4, PCM incubated with [^{3}H]AdoMet.

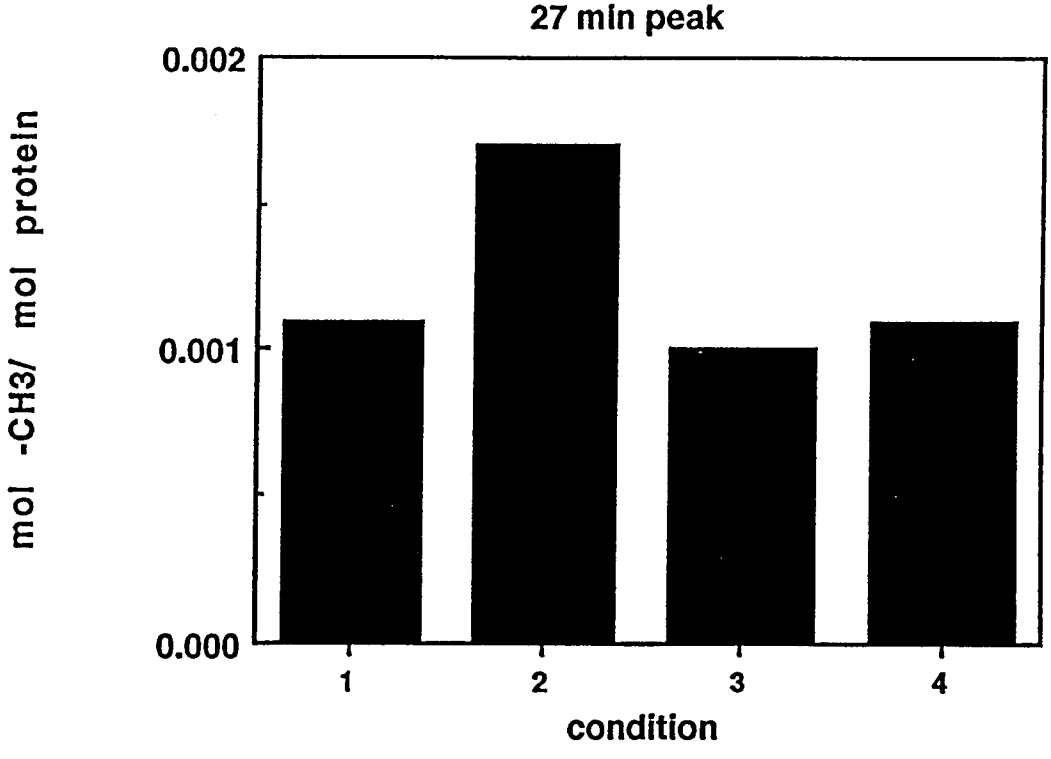
27 min peak
0.002
0.001
0.000
mol -CH3/ mol protein
1
2
3
4
condition

FIGURE 7.3

enzyme incubated in the presence of [^{3}H]AdoMet (condition 4) shows no increase in the number of methyl groups incorporated.

DISCUSSION

By incorporating a second radiolabel and reversing the order of treatment (Figure 7.2), sites of automethylation and sites of methylation were distinguished. The methylated sites appear to be inaccessible to the automethylation reaction when the enzyme is in its native conformation and only become accessible to repair when the higher order structure of the enzyme is lost (Figure 7.1). It is the presence of hidden or "cryptic" sites which leads to the hypothesis that unrepairable damage accumulates within the enzyme as it ages, as the level of damage in these sites is seen to increase with incubation at 37°C (Figure 7.3). The presence of cryptic sites may also contribute to the diversity of αPCM molecules and may contribute to the increased acidity of the later eluting anion exchange fractions.

Cryptic sites do not appear to be an artifact of the trypsinization procedure, as they are sub-stoichiometrically methylated and do not appear to increase with extended digestion times. The revealing of methylatable sites using trypsinization has also been previously identified in studies of the *in vitro* aging of calmodulin (Potter, et al. 1993). Another example of higher-order structure preventing repair was demonstrated in the enzyme serine hydroxymethyltransferase, where a second site of methylation was discovered upon denaturation of the enzyme (Schirch, 1995).

Altered aspartyl residues have been previously shown to accumulate within proteins and peptides with age. In several proteins, the effect of altered aspartyl formation has been a loss of biological activity (Teshima, et al. 1995). The accumulation of damaged sites within PCM, which the enzyme appears unable to repair, has led to the hypothesis that unrepaired damage could lead to a decrease in or possibly the loss of enzyme activity. It is not difficult to imagine how the formation of altered aspartyl residues could result in a loss of activity, as proteins are dependent upon a precise three-dimensional structure to function and altered aspartyl formation is known to result in a perturbation of that structure (Clarke, et al.1992).

A decrease in the efficiency of the enzyme, due to the accumulation of unrepaired damage and/or the apparent preoccupation with its automethylation reaction, could be responsible for a decrease in the rate of repair, and hence an increase in the rate of formation of altered aspartyl residues within other proteins. As this type of damage has been shown to cause a loss of biological activity, the net result would be an indiscriminant loss of activity throughout the cell resulting in an error cascade reaction that would ultimately culminate in cell death.

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CHAPTER 8

THE FUNCTIONAL STATE OF SPONTANEOUSLY DAMAGED PROTEIN (D-ASPARTYL/L-ISOASPARTYL) CARBOXYL METHYLTRANSFERASE

Jonathan A. Lindquist and Philip N. McFadden

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RATIONALE

The existence of damage within protein (D-aspartyl/ L-isoaspartyl) carboxyl methyltransferase (PCM) has been established in the preceding chapters. The presence of altered aspartyl residues has been shown to have serious effects on the activities of other proteins. The purpose of this chapter is to investigate the effects of altered aspartate upon the activity of PCM.

THE FUNCTIONAL STATE OF SPONTANEOUSLY DAMAGED D-ASPARTYL/L-ISOASPARTYL PROTEIN CARBOXYL METHYLTRANSFERASE

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The enzyme D-aspartyl/L-isoaspartyl protein carboxyl methyltransferase, PCM, is known to catalyze the transfer of a methyl group from S-adenosyl-L-methionine to the free carboxyl group of altered aspartyl residues that arise through a spontaneous aging reaction in proteins and peptides. Recently, the existence of a damaged subpopulation of PCM molecules has been identified (Lindquist and McFadden 1994a). The damaged molecules that are capable of incorporating a [^{3}H]methyl group have been termed the αPCM fraction. Reports of losses in biological activity resulting from the formation of altered aspartyl residues in other proteins raised the question, "To what extent do these residues affect PCM?" To address this question, *in vitro* [^{3}H]automethylated PCM was applied to an S-adenosyl-L-homocysteine-ω-aminohexyl-agarose affinity column. Upon addition of an elution buffer containing 50 μM S-adenosyl-L-homocysteine, a large portion of the [^{3}H]automethylated molecules was observed eluting from the column. This observation leads to the conclusion that αPCM molecules possess a functional S-adenosyl-L-methionine binding site and raises the possibility that these molecules may still maintain some catalytic activity.

INTRODUCTION

The formation of spontaneous protein damage at aspartyl and asparaginyl residues is currently believed to account for much of the heterogeneity observed in proteins, as it may lead to as many as seven possible products (L-Asn, L-Asp, D-Asp, L-Isoasp, D-Isoasp, L-Imide, and D-Imide) at any given site of damage (Aswad 1995a). As the understanding of this reaction has grown, so has the awareness of its presence and the list of proteins identified with sites of isoaspartyl damage continues to grow. So far, the reported effects of this damage upon the activity of these proteins cover a broad range, from no affect to the complete loss of biological activity (Teshima, et al.1995). The enzyme D-aspartyl/L-isoaspartyl protein carboxyl methyltransferase (PCM) has been proposed as a means by which cells can cope with this damage. In studies with model peptide substrates, PCM has been shown to be capable of repairing the L-isoaspartyl residues to L-aspartate (McFadden and Clarke 1987, Johnson, et al. 1987b). In studies with damage inactivated proteins, repair of these damaged sites by PCM has been shown to restore function as well (Johnson, et al. 1987a, Brennan, et al. 1994).

Recently, the existence of altered aspartyl residues has been identified within the repair enzymes themselves (Lindquist and McFadden 1994a). Reports of proteins losing biological activity as a result of the formation of altered aspartyl residues led to the question, "What effect do these residues have upon the activity of PCM?"

PCM is known to catalyze the transfer of a methyl group from S-adenosyl-L-methionine (AdoMet) to the free carboxyl group of altered aspartyl residues in proteins and peptides. To do this, PCM must be capable of binding both AdoMet and the

damaged substrate. Once bound, these molecules must be positioned in such a way as to stabilize the transition state so that methyl transfer can occur. And once done, PCM must then release S-adenosyl-L-homocysteine (AdoHcy) and the methyl esterified substrate into solution. As the crystal structure and catalytic mechanism of PCM are still unknown, the presence of multiple damaged sites in PCM could affect any combination of these steps.

Since the binding of PCM to immobilized AdoHcy had been used as a means of purification of the enzyme, it was determined that one of these questions had already been answered. To have bound to the affinity column during the initial purification, αPCM must have possessed a functional AdoMet binding site. However, it was impossible to prove that the damage observed in the enzyme had not occurred after this step in the purification. Therefore, it was decided to address a closely related question, "Could [^{3}H]automethylated PCM bind to the affinity column?" By allowing PCM to [^{3}H]automethylate prior to its application to the affinity column, the population of damaged molecules is already established, making any question of damage to the molecules after elution irrelevant.

EXPERIMENTAL METHODS

Automethylation reactions

The PCM used in this study was anion exchange FPLC purified PCM from the acidic, late-eluting shoulder (see Chapter 2). For a typical automethylation reaction, 35

pmol of enzyme (90 µl) was mixed together with 63 µl of 0.2 M sodium citrate pH 6.0, 3 µl of a protease inhibitor cocktail [containing 10 mM EDTA, Pepstatin A (10 mg/ml), Aprotinin (10 mg/ml), and Leupeptin (10 mg/ml)], and 4 µl of [^{3}H-*methyl*]AdoMet [NEN, 15 Ci/mmol]. The reaction was incubated at 37°C for 2 hrs, during which time methyl groups were expected to become incorporated into the αPCM molecules (see Chapter 2).

Affinity gel synthesis

S-adenosyl-L-homocysteine-ω-aminohexyl-agarose was synthesized according to the protocol of Paik and Kim (1980). 1.0 mmole of bromoacetic acid [Aldrich] and 1.2 mmole of N-hydroxysuccinimide [Aldrich] were dissolved in 8 mls of 1,4-dioxane [Aldrich] and mixed with 1.1 mmole of dicyclohexylcarbodiimide [Aldrich] in a glass test-tube (16 mm x 150 mm). This mixture was allowed to react at room temperature (22°C) for 70 minutes. The reaction was filtered through a sintered glass funnel to remove the dicyclohexylurea produced during the reaction. The filtrate, containing O-bromoacetyl-N-hydroxysuccinimide, was then added to 10 mls of ω-aminohexyl-agarose [Sigma], which had been equilibrated in 0.1 M sodium phosphate pH 7.5 at 4°C, and allowed to sit on ice for 30 minutes. The gel was then washed with 2 liters of 0.1 M NaCl (4°C) followed by a second wash with 200 mls of 0.1 M $NaHCO_3$ pH 9.0. The packed gel was then mixed with an equal volume of buffer containing 50 mg of S-adenosyl-L-homocysteine [Sigma] and gently mixed at room temperature for 3 days. The gel was then washed with 2 liters of 0.2 M NaCl and resuspended for 2 hrs in 40 mls 0.1

M $NaHCO_3$ pH 9.0 containing 0.2 M 2-mercaptoethanol [Sigma] to block unreacted bromoacetyl groups. The gel was then washed with 2 liters of 0.2 M NaCl and stored at 4°C in 0.2 M NaCl with 0.02% sodium azide until needed. The coupling efficiency of AdoHcy to the gel was measured to be 53%, as determined by absorption at 260 nm. [$\varepsilon_{260} = 16{,}000\ M^{-1}\ cm^{-1}$]

Affinity chromatography

The tip of a 1 cc syringe was packed with glass wool and a 2-way valve attached to regulate the flow of buffer during the pouring of the column. The column was filled with buffer Z [10 mM NaH_2PO_4 / 0.1 M NaCl / 10% (v/v) glycerol pH 6.6] (Gilbert et al. 1988 Biochemistry) at 4°C and any air bubbles were removed. 1.2 mls of a 25% suspension of gel were slowly added to the buffer in the column. The gel was allowed to settle and then washed with 10 volumes of buffer Z before use.

The automethylation reaction was diluted with 400 µl of buffer Z and applied to the gel. The column was then washed three times with 600 µl aliquots of buffer Z. Elution was performed using 6 x 400 µl aliquots of buffer Z containing 50 µM S-adenosyl-L-homocysteine (AdoHcy).

High performance liquid chromatography

Affinity column fractions were purified by reversed phase high performance liquid chromatography (HPLC) using a Vydac C4 column [particle size 5 µ, 4.6 mm x

250 mm]. Proteins were eluted with a gradient of 0.1%(v/v) TFA/ dI H_2O: 90%(v/v) acetonitrile/ 9.9%(v/v) dI H_2O/ 0.1%(v/v) TFA, as previously described (Lindquist and McFadden 1994a) with component elution monitored at 280 nm. Fractions were collected at 1 minute intervals during the run.

Electrophoresis: Proteins were electrophoretically separated under acidic conditions (pH 2.4) on a 12% polyacrylamide gel following the method of Fairbanks and Avruch (1974). After separation, the gels were fixed in 12% trichloroacetic acid for 45 minutes and the proteins visualized using a colloidal coomassie stain according to the method of Neuhoff, et al. (1988).

Measurement of radioactivity

Affinity fractions

An aliquot (100 µl) of each fraction was assayed in duplicate for base volatile radioactivity by pipetting the sample directly into a 1 dram glass inner vial [15 mm x 45 mm, Kimble] containing 100 µl of 1 M NaOH. The inner vials were placed inside 20 ml scintillation vials containing 4 mls of xylene-based liquid scintillation cocktail (LSC) [Scintiverse E, Fisher] and capped. The efficiency of capture of volatile radioactive methanol in the LSC was determined using [^{14}C]methanol standards assembled in parallel.

HPLC fractions

An aliquot (100 µl) of each reversed phase HPLC fraction was assayed for total radioactivity by pipetting directly into 7 ml scintillation vials containing 2 mls of LSC.

Gel slices

Acid-gel slices (1 cm x 1 cm) were placed into 1 dram glass inner vials into which 0.5 mls of 8 M NaOH were added to hydrolyze the ester bonds. The inner vials were placed inside 20 ml scintillation vials containing 4 mls of LSC and capped. The efficiency of vaporization was determined using [^{14}C]methanol standards.

Immunodetection

Immunodetection of PCM was performed using the protocol of Harlow and Lane (1988). Briefly, aliquots of the affinity column fractions were electrophoretically separated on 15% SDS-polyacrylamide gels, according to the method of Laemlli (1970), and then electroblotted onto nitrocellulose membranes using semi-dry transfer. Proteins were visualized with Ponceau S stain and the membranes were then blocked in 10%(w/v) milk in 50 mM Tris/ 150 mM NaCl pH 7.4 (TBS). Primary detection was made using a rabbit anti-PCM antibody diluted 1:200 in 2%(w/v) milk-TBS. Secondary detection was made using a goat anti-rabbit alkaline phosphatase conjugated antibody [Sigma] diluted 1:3000 in 2% milk-TBS. The color reaction, using nitro blue tetrazolium and 5-bromo-4-

chloro-3-indolyl phosphate, was developed by eye and quenched with 25 mM ethylenediaminetetraacetic acid in TBS.

RESULTS AND DISCUSSION

To test if [^{3}H]automethylated PCM could bind to the S-adenosyl-L-homocysteine affinity column, an aliquot of PCM (35 pmol) was first allowed to [^{3}H]automethylate for 2 hrs and then applied to the affinity column. The elution profile of this sample from the affinity column is shown in Figure 8.1. A large peak of radioactivity can be seen eluting during the load and initial washes, which accounts for 56% of the counts applied to the column. Addition of the elution buffer containing 50 μM AdoHcy yielded a second peak, shown in the inset box of Figure 8.1, which accounted for 4% of the counts applied to the column. The difference in the total counts recovered may be due to loss during the handling of the sample, as the estimate of total counts applied is based upon an identically prepared reaction that was frozen at -20°C upon completion of the incubation. Analysis of these fractions using immunodetection revealed PCM to be present in both the flow through and the eluent.

To ensure that the material eluting from the affinity column was truly PCM, the affinity fractions were pooled and further purified using HPLC. Resolution of the affinity fractions revealed a small radioactive peak eluting at 26-27 minutes, which corresponds to the elution time previously determined for bovine PCM (Figure 8.2). A control injection of [^{3}H]automethylated PCM identically prepared, which was not passed over the affinity column, revealed the stoichiometry of methylation for this material to be

FIGURE 8.1:

Profile of base volatile [^{3}H] radioactivity eluting from an S-adenosyl-L-homocysteine-ω-aminohexyl-agarose affinity gel after the application of [^{3}H]automethylated PCM (35 pmol). The inset box is an enlargement of the samples W3 to E2. Wash fractions are represented by 'W' and elution fractions by 'E'.

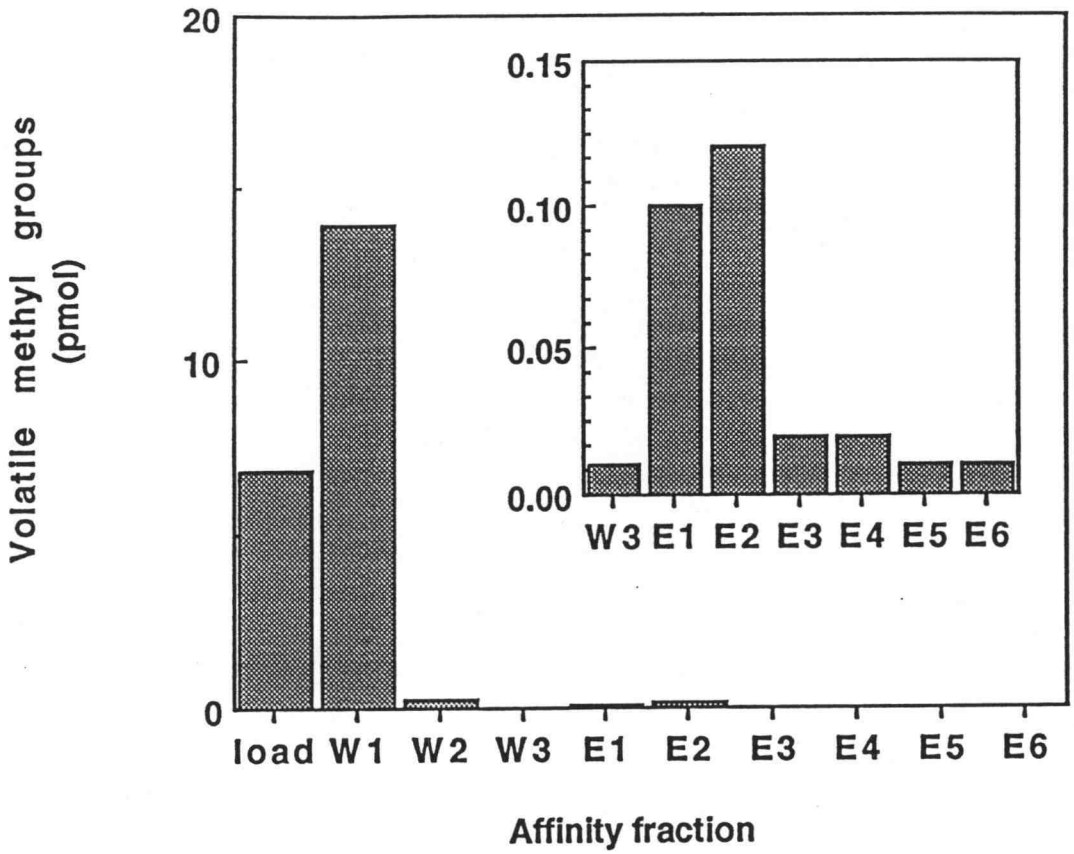
Volatile methyl groups (pmol)
20
10
0
0.15
0.10
0.05
0.00
W3 E1 E2 E3 E4 E5 E6
load W1 W2 W3 E1 E2 E3 E4 E5 E6
Affinity fraction

FIGURE 8.1

FIGURE 8.2:

[^{3}H] Radioactivity profile for affinity-purified samples resolved by reversed phase HPLC. The bottom profile is from an aliquot of an automethylation reaction prepared identically to the samples applied to the affinity column. The large peaks eluting at 4 to 6 minutes and 9 minutes are either due to methanol generated during the automethylation reaction, unincorporated [^{3}H] radiolabel, and/or its degradation products. The arrow indicates the elution position of PCM as previously determined by activity assay and immunodetection (Lindquist and McFadden 1994a). The inset box shows the radioactivity profile of [^{3}H] automethylated PCM obtained from elution off of the affinity gel.

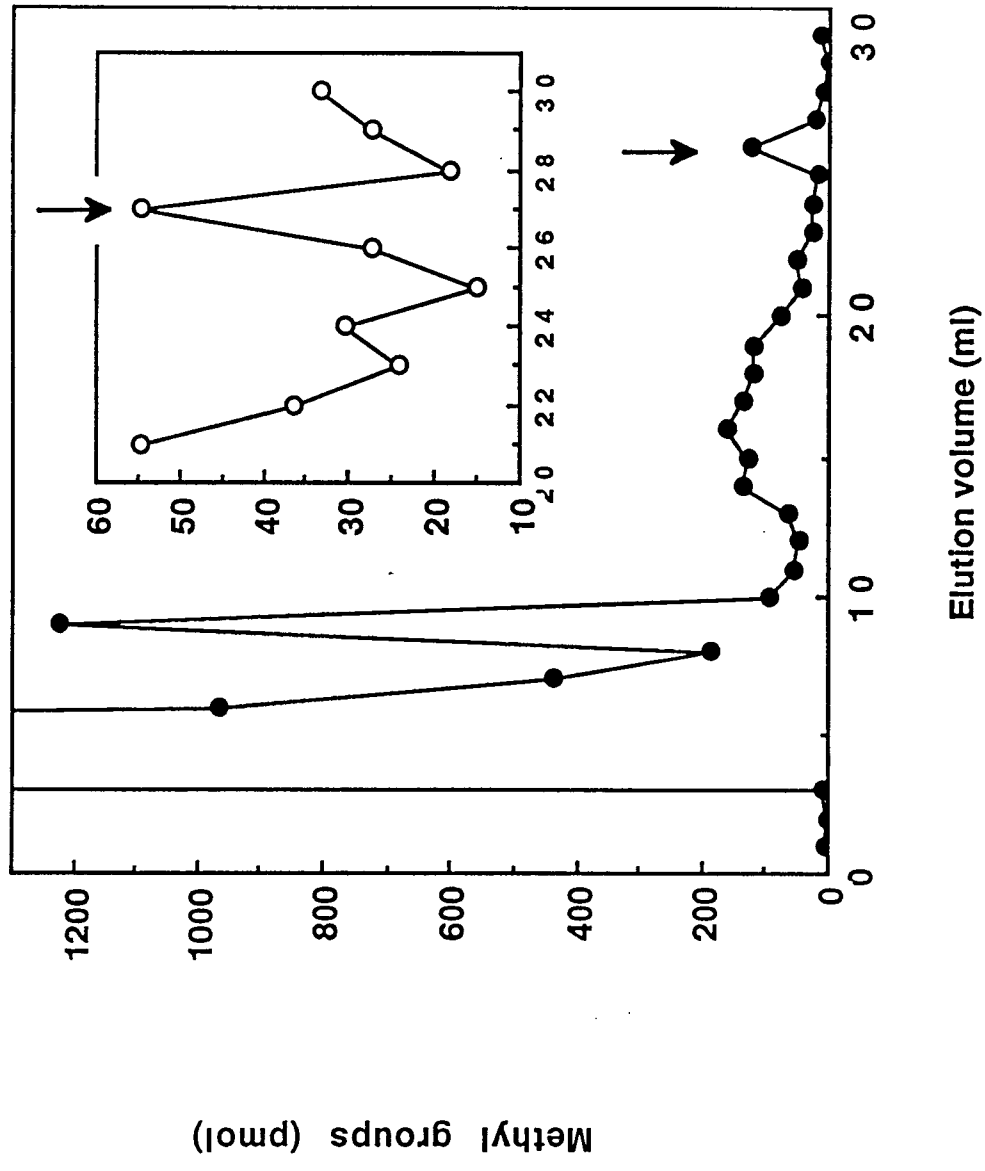
Methyl groups (pmol)
Elution volume (ml)
0
200
400
600
800
1000
1200
10
20
30
40
50
60
0
10
20
30
20
22
24
26
28
30

FIGURE 8.2

0.006 mol of methyl groups per mol of protein. Analysis of the UV absorbing material, from HPLC purification of the fractions eluting from the affinity column, showed there to be almost complete recovery of the PCM.

As an independent measure of the recovery of [^{3}H]automethylated PCM from the affinity column, aliquots of the affinity fractions were electrophoretically separated under acidic conditions. Electrophoretic analysis of the material eluting from the affinity column (Figure 8.3) showed recovery of 79% of the radioactive counts incorporated into PCM and that 46% of the methylated material, αPCM, bound to the column and 33% was contained in the flow through. Analysis of an aliquot from the control reaction, for the purpose of determining loss of sample due to handling, showed the stoichiometry of methylation to be 0.005 mol of methyl groups per mol of protein, which is in agreement with the value determined using the HPLC. Since analysis of the UV absorbing material showed nearly complete recovery of PCM, the stoichiometry of the material recovered from the affinity column can be estimated to be 0.002 mol of methyl groups per mol of protein.

Purification of the enzyme showed that a similarly prepared affinity gel was capable of binding 700-1500 Units of PCM per ml of gel (1 Unit = 4 pmols, pure PCM = 10,000 U/mg). Based upon this estimate, a sufficient number of sites for complete binding of the material applied to the column had been provided. The observation that not all of the αPCM bound to the affinity gel can be explained in two ways. First, a single application of the sample may not have provided an opportunity for all of the enzyme to bind to the column. The second possibility is that αPCM is a heterogeneous

FIGURE 8.3:

A histogram showing the profile of base volatile [^{3}H] radioactivity obtained from acidic gel slices after electrophoretic separation of samples obtained from the affinity column. The molecular weight range for proteins contained in the slice is indicated at the bottom. Molecular weight ranges were determined using three protein standards: bovine serum albumin (MW 66 kDa), carbonic anhydrase (MW 30 kDa), trypsin inhibitor (MW 21 kDa). As explained in Figure 8.1, 'W' represents a wash fraction and 'E' an elution fraction.

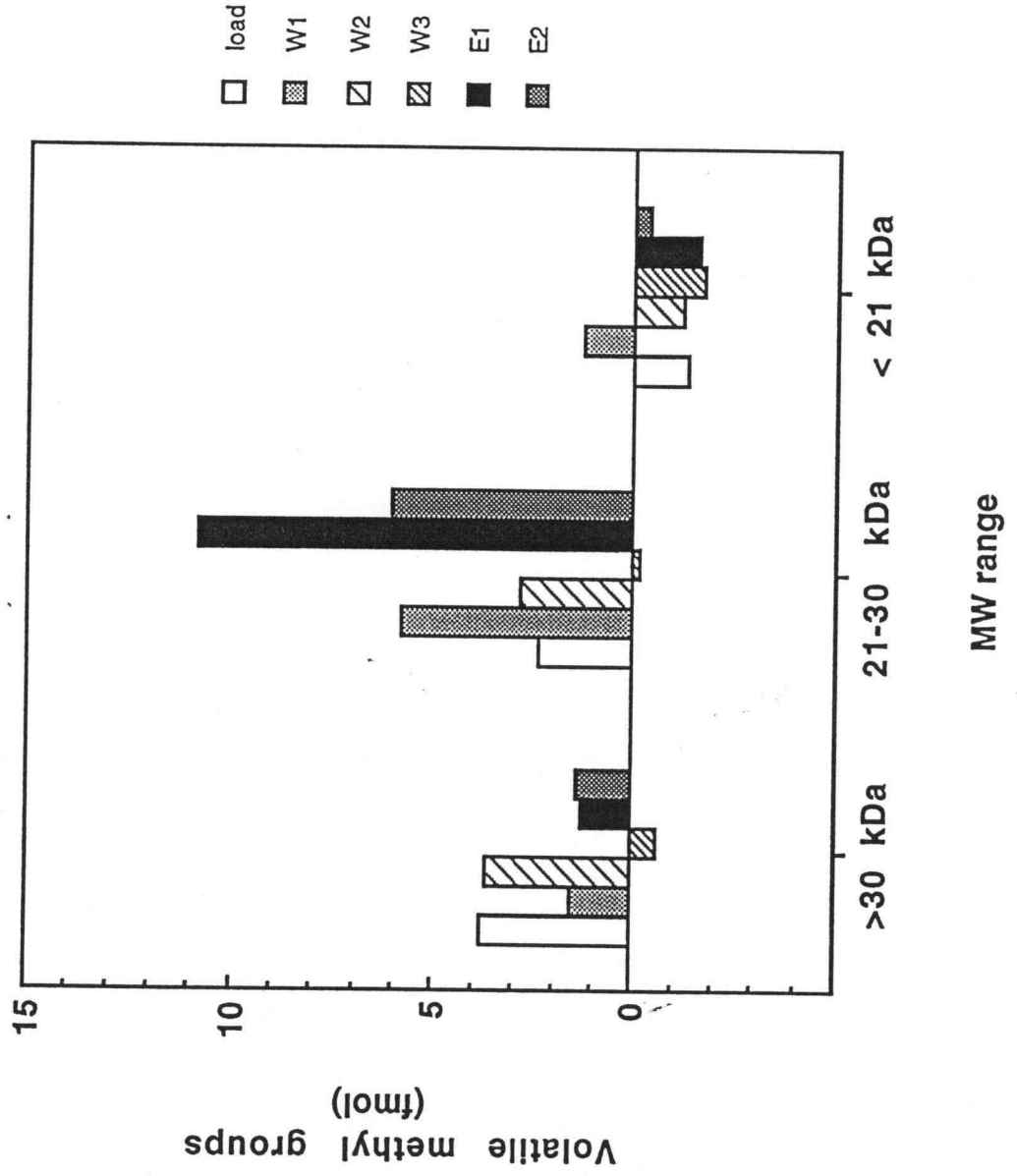
load
W1
W2
W3
E1
E2
Volatile methyl groups (fmol)
15
10
5
0
>30 kDa
21-30 kDa
< 21 kDa
MW range

FIGURE 8.3

population of damaged molecules, as previous results suggest, and that some of these molecules lose their ability to bind AdoHcy when they become damaged in a structurally "sensitive" region. The ability to recover the majority of the UV absorbing material using the affinity gel suggests that a sufficient opportunity for complete binding of the enzyme had been provided. However, in a similar experiment analyzing the flow through of crude cytosol applied to the gel, it was found that a portion of this material did bind to the gel when reapplied, arguing in favor of the first alternative, that the lack of binding was due to insufficient opportunity and not a functional defect in the enzyme.

The binding of the majority of the [^{3}H]methylated αPCM to the affinity gel shows that these molecules possess a functional AdoMet binding site. The ability of the [^{3}H]methylated molecules to bind to the affinity column also supports the argument that αPCM must have bound to the affinity column during its initial purification.

Since there are several steps involved in catalyzing the transfer of a methyl group from AdoMet to the damaged substrate, the binding of AdoHcy by αPCM does not guarantee that the rest of the enzyme is functioning properly. However, the ability to bind and release AdoHcy demonstrates a functional AdoMet binding site within αPCM and raises the possibility that these molecules may still be catalytically active.

Although the exact effects that altered aspartyl residues have upon the catalytic activity of PCM remains to be determined, the presence of altered aspartyl residues is known to result in a perturbation of the structure in other proteins. As the three-dimensional structure of a protein is important to its function, any disruption in this structure is likely to affect the function of the protein. In the case of PCM, the most

logical effect would be a loss of activity. A decrease in the efficiency of repair is most likely to result in a gradual accumulation of unrepaired damage in all of the cellular proteins, including PCM. The effect of the accumulation of damage in these proteins will be a perturbation of structure that may lead to a loss of activity or perhaps only make these proteins more susceptible to other forms of damage. In any case, the net result of the accumulation of damage throughout the cell will be the same; a loss of the cell's ability to maintain itself. And a cell that has lost this ability will senesce.

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CHAPTER 9

INTRACELLULAR AUTOMETHYLATION OF PROTEIN (D-ASPARTYL/L-ISOASPARTYL) CARBOXYL METHYLTRANSFERASE IN RABBIT ERYTHROCYTES

Jonathan A. Lindquist and Philip N. McFadden

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RATIONALE

Having shown that protein (D-aspartyl/ L-isoaspartyl) carboxyl methyltransferase (PCM) is damaged *in vitro*, the purpose of this chapter is to see if PCM automethylates *in vivo*.

INTRACELLULAR AUTOMETHYLATION OF PROTEIN (D-ASPARTYL/L-ISOASPARTYL) CARBOXYL METHYLTRANSFERASE IN RABBIT ERYTHROCYTES.

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Sequential purification under acidic conditions, which are known to stabilize methyl ester linkages, was used to isolate protein (D-aspartyl/L-isoaspartyl) carboxyl methyltransferase (PCM) from erythrocytes that had been previously incubated with [^{3}H-*methyl*]methionine. Base-volatile [^{3}H] radioactivity that copurifies with the immunodetectable peak of PCM was identified. Copurification provides the first evidence for the existence of intracellular automethylation and establishes the presence of damage in a repair system within a living cell.

INTRODUCTION

The presence of altered aspartyl residues has been reported to result in the loss of biological activity within several proteins (Teshima, et al. 1995). In studies with model peptides containing L-isoaspartyl residues, the enzyme protein (D-aspartyl/L-isoaspartyl) carboxyl methyltransferase (PCM) has been shown to repair the majority of these damaged sites to L-aspartate. PCM has also been shown to restore activity to damage-

inactivated proteins. Recently, the existence of altered aspartyl residues was identified within PCM (Lindquist and McFadden 1994a). In investigating the effect of altered aspartyl residues upon the activity of PCM, a methodology was developed that allowed the sequential purification of PCM under acidic conditions. The advantages of this methodology are two-fold. Firstly, PCM is neither an abundant protein in the erythrocyte nor the only substrate for enzymatic methylation, making the analysis of crude cytosolic extracts impractical for identifying this phenomenon. Secondly, normal purification techniques are performed at physiological pH where esters are relatively unstable. Given the length of a typical enzyme purification, it is likely that all of the esters would have hydrolyzed before purification was achieved. The existence of this methodology provided an opportunity to address the question, "Does PCM automethylate *in vivo*?"

Erythrocytes were selected as the model system to investigate this phenomenon *in vivo* for several reasons. First, erythrocytes are easy to isolate. Second, PCM is present in erythrocytes and has been purified from the erythrocytes of several species. Third, much of the work done on the methylation of damaged aspartyl residues in proteins has been done using erythrocytes making it a reasonably well characterized system (Freitag 1981, McFadden 1982, O'Connor 1984, Barber 1984, Barber 1986). Further, mature erythrocytes lack a nucleus and therefore have no capacity for protein synthesis. It is the lack of protein synthesis that makes erythrocytes an ideal system for the study of the mechanisms of cellular maintenance and self-repair. Also, the lack of protein synthesis eliminates any background problems due to incorporation of the radiolabeled amino acid into the protein of interest. Finally, erythrocytes are physiologically important and

possess a life-span of 60 days (rabbit) making protein aging an important problem in these cells.

Computer modeling of protein aging within a human erythrocyte, using experimentally determined rate constants, showed that a significant amount of altered aspartyl residues could form during its 120 day life-span in the absence of a repair system (Lowenson, J.D. and Clarke, S. (1991) Gerontology 37, 128-151). Hemoglobin, which accounts for 97% of the protein in an erythrocyte, is a rather stable protein and does not appreciably turn over within the 120 day life-span. During this time, the damage accumulated within hemoglobin, although sub-stoichiometric, would represent seven times the amount of damage accumulated with all other non-hemoglobin proteins combined. Non-hemoglobin proteins, which represent the major structural and metabolic components of an erythrocyte, are predicted to accumulate seven isoaspartyl residues per polypeptide during the 120 day life-span of an erythrocyte in the absence of repair. Recall that damage to as few as one or two sites has been shown to have significant effects on the activity of many proteins, often resulting in the loss of biological activity. So, with this level of damage, it is likely that these proteins would have long since ceased to function. While this model considers only one of several forms of protein damage that can occur, it exemplifies the potential problem that protein aging could pose to a cell in the absence of a repair system.

In the absence of protein synthesis, as in erythrocytes, or under conditions when protein synthesis would be regulated, such as starvation, self-repair would become a necessity to keep the repair systems active. However, automethylation has never been

shown to occur within a living cell. To measure intracellular automethylation, the cellular pool of S-adenosyl-L-methionine would need to become radiolabeled. This is easily accomplished, as methionine is an essential amino acid and is readily taken up by cells. The metabolism of methionine in erythrocytes is simplified by the lack of protein synthesis in these cells. Methionine is coupled to ATP by the enzyme methionine adenosyltransferase to produce AdoMet, which may serve as a substrate for the cellular transmethylation reactions (Finkelstein, J.D. and Martin, J.J. (1984) J.Biol.Chem. 259, 9508-9513). Utilizing [^{3}H-*methyl*]methionine and glucose, as a source of ATP, erythrocytes will produce [^{3}H-*methyl*]AdoMet. Therefore, the detection of intracellular automethylation becomes a matter of isolating [^{3}H-*methyl*]esterified PCM.

EXPERIMENTAL METHODS

Erythrocyte isolation

Rabbit blood (5 mls) was obtained by cardiac puncture using a heparinized syringe. The blood was first centrifuged at 1200 x g in a clinical centrifuge [IEC] for 10 minutes at room temperature. Following removal of the blood plasma, the erythrocytes were washed three times with ice cold isotonic, glucose-containing, phosphate buffer (IP 7.4) with the following formulation: 118 mM NaH_2PO_4/ 18 mM glucose to pH 7.4 w/ 5 M NaOH. The cells were centrifuged for 3 minutes at 3000 x g to pack the erythrocytes. Supernatants were carefully removed by aspiration to remove the buffy coat of lymphocytes. The packed cells were then resuspended with an equal volume of IP 7.4.

Erythrocye incubation

Following the method of Barber (1986), one nmol of non-radioactive methionine was added to an aliquot (50-100 µl) of [^{3}H-*methyl*]methionine [70-80 Ci/mmol NEN] and the mixture was lyophilized just to the point of dryness. To minimize oxidation of the sample, the methionine was immediately resuspended in 100 µl of IP 7.4 and placed on ice. Following the addition of 3 µl of penicillin-streptomycin stock solution [10,000 U/ml Gibco-BRL], 200 µl of a 1:1 cell suspension was added. The samples were incubated for 5 hrs in a 37°C water bath with shaking to allow the cellular pool of methyl groups to become labeled.

Preparation of cytosol

Following the five hour incubation, the samples were centrifuged at 3000 x g for one minute and the reaction supernatant removed. A small amount of hemolysis was observed in the reaction supernatant. The packed cells (100 µl) were washed three times with ice cold IP 7.4 and then lysed in 6 volumes of buffer Z [10 mM NaH_2PO_4/ 0.1 M NaCl/ 10% glycerol (v/v) pH 6.6] by vortexing and freezing in liquid nitrogen. The frozen cells were thawed in an ice/water bath and then centrifuged at 15,000 x g for 20 minutes at 4°C. The cytosol was removed to prevent contamination by the membrane pellet.

Affinity Chromatography

The cytosol (~ 600 µl) was diluted to 3 mls with buffer Z and the sample loaded onto an affinity column containing 1 ml of S-adenosyl-L-homocysteine-ω-aminohexyl-agarose. The sample was passed over the column several times to optimize binding. The column was then washed with buffer Z that had been adjusted to 0.2 M NaCl to minimize nonspecific binding of protein to the column. Following washing of the column with 10-20 column volumes of buffer, the bound protein was eluted with 5-10 mls of buffer Z containing 50 µM AdoHcy. Fractions (0.5 ml) were collected, acidified with the addition of 1/10th volume 1 M H_3PO_4 to promote stability of the esters, and stored at -20°C. Fractions were assayed for protein methyl esters as base-volatile radioactivity using an inner vial assay described under "radioactivity assays". The elution position of PCM was determined by immunodetection on Western blots.

High performance liquid chromatography

Affinity fractions that contained immunodetectable PCM, were further resolved using high performance liquid chromatography (HPLC) on a water/acetonitrile gradient system (see chapter 8). Samples were purified using a Vydac C_4 column [particle size 5 µ, 4.6 mm x 250 mm] with component elution monitored at 280 nm. The elution position of rabbit PCM was confirmed by immunodetection.

Electrophoresis

HPLC fractions from each affinity fraction were lyophilized to dryness and resuspended with 50 µl 0.1% TFA. The corresponding fractions from each HPLC run were pooled and the pooled fractions were lyophilized to dryness. The pooled fractions were then resuspended in 25 µl of 1x sample buffer pH 2.4 and electrophoretically separated on a 12% acid gel according to the method of Fairbanks and Avruch (1972). Following electrophoresis, the gels were fixed in 12% trichloroacetic acid (TCA) and the proteins visualized using a colloidal Coomassie stain [Neuhoff, et al. (1988)]. The gels were then sliced into 1 cm x 1 cm bands and assayed for base-volatile [^{3}H]radioactivity.

Immunodetection

Western blots were performed according to the methods described by Harlow & Lane (Antibodies, A Laboratory Manual 1988 Cold Spring Harbor Press). Briefly, proteins were electrophoretically separated on SDS-polyacrylamide gels (Laemlli, U.K. (1970) Nature 277, 680) and transferred to nitrocellulose membranes [Protran, Schleicher & Schull] using semi-dry transfer. Proteins were visualized with Ponceau S stain and then the membranes were blocked with 10% milk (w/v) in 50 mM Tris/ 150 mM NaCl pH 7.4 (TBS). Primary detection was performed using a 1:200 dilution (in 2% milk-TBS) of a rabbit polyclonal antibody raised against a denatured PCM-Trp E fusion protein and previously found to be specific for PCM (unpublished data). Secondary detection was performed using a goat anti-rabbit alkaline phosphatase conjugated

antibody [Sigma A-8025] diluted 1:3000 in 2% milk-TBS. The color reaction, using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate, was developed and quenched with 25 mM ethylenediaminetetraacetic acid (EDTA) in TBS.

Radioactivity assays

Affinity fractions

An aliquot (50 µl) of each affinity fraction was pipetted into a 1 dram glass inner vial [15 mm x 45 mm, Kimble] containing 100 µl of 1 M NaOH. This vial was then placed into a 20 ml scintillation vial containing 4 mls of liquid scintillation cocktail [Scintiverse E, Fisher] and capped. Vials were allowed to equilibrate before counting and the degree of equilibration monitored using [^{14}C]methanol standards.

HPLC fractions

An aliquot (500 µl) of each fraction was pipetted into a 1 dram glass vial containing 100 µl of 1M NaOH. These vials were then processed as described above under Affinity fractions.

Gel slices

Acid gel slices (1 cm x 1 cm) were placed into 1 dram glass inner vials, to which 0.5 ml of 8 M NaOH was added. Following the addition of base, the inner vials were placed into 20 ml scintillation vials as described above.

RESULTS

After incubating erythrocytes with [^{3}H-*methyl*]methionine for five hours, a time previously determined to yield maximal protein methyl ester radiolabeling by both TCA precipitable and base-volatile assays (data not shown), the cells were washed and lysed by freeze/thaw using liquid N_2. Membranes were removed by centrifugation and the cytosol loaded onto an S-adenosyl-L-homocysteine-ω-aminohexyl-agarose column. The elution profile of base-volatile [^{3}H] radioactivity from this column is shown in figure 9.1. The large radioactive peak eluting in the load and first wash fractions contains [^{3}H] methanol that was formed by the repair reaction, unincorporated [^{3}H]methionine, unincorporated [^{3}H]AdoMet, and [^{3}H]methylated proteins that have no affinity for the immobilized ligand. The smaller radioactive peak, shown in the inset box, corresponds to the elution of [^{3}H]methyl esterified protein from the affinity column upon addition of buffer Z containing 50 μM S-adenosyl-L-homocysteine. This radioactive peak corresponded with the elution of PCM from the column as determined by immunodetection. The ability of [^{3}H]automethylated PCM to bind to the affinity resin was established using *in vitro* automethylated bovine PCM (see chapter 8).

FIGURE 9.1:

Elution profile of base-volatile [^{3}H] radioactivity from S-adenosyl-L-homocysteine-affinity chromatographic separation of erythrocyte cytosol following a 5 hour incubation with [^{3}H-*methyl*]methionine. Column fractions were electrophoretically separated, electroblotted, and probed with an anti-PCM polyclonal antibody (as described under immunodetection). The position of immunoreactive PCM eluting from the column is indicated (*).

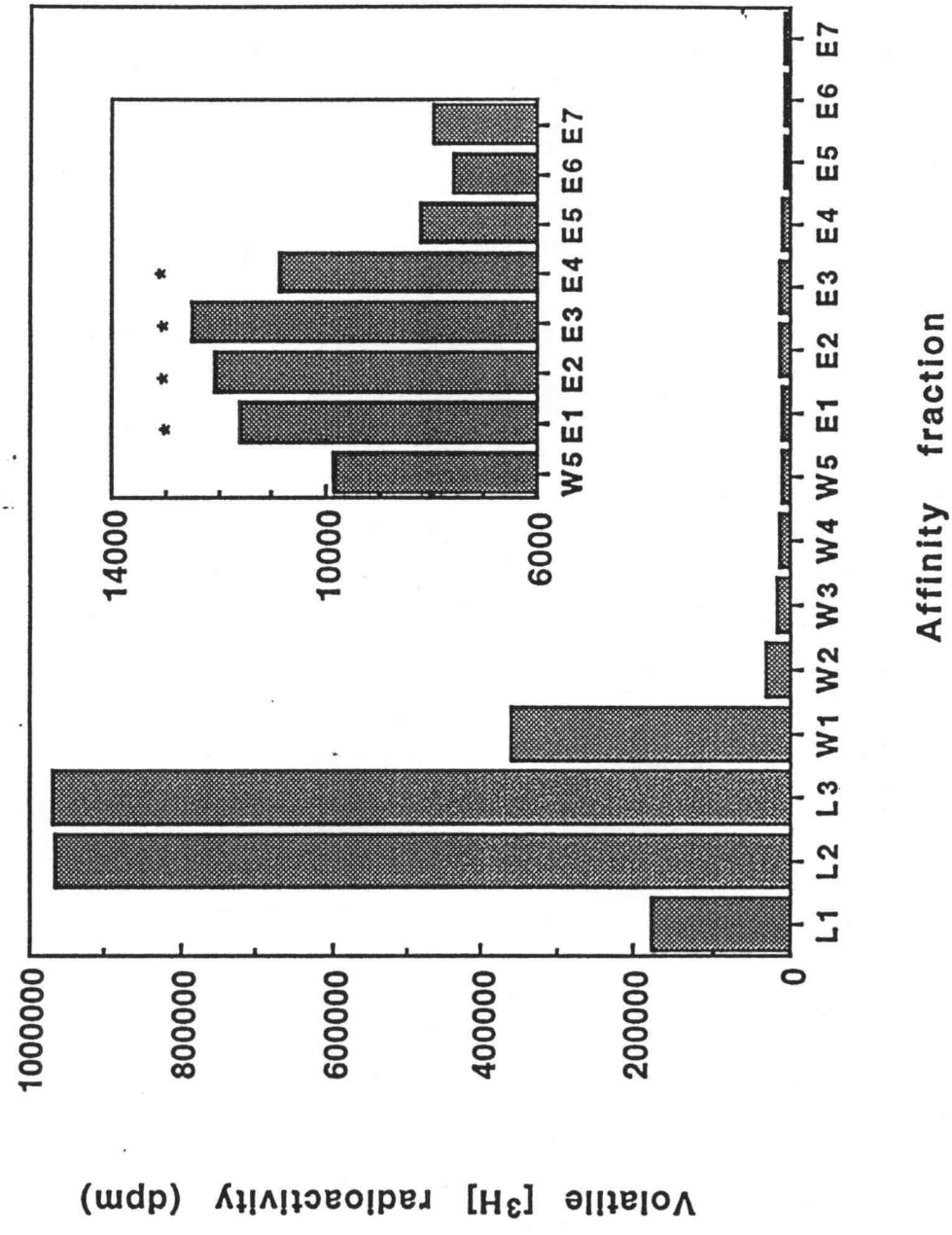
Volatile [3H] radioactivity (dpm)
1000000
800000
600000
400000
200000
0
L1 L2 L3 W1 W2 W3 W4 W5 E1 E2 E3 E4 E5 E6 E7
Affinity fraction
14000
10000
6000
W5 E1 E2 E3 E4 E5 E6 E7
* * * *

FIGURE 9.1

The affinity fractions containing immunodetectable PCM were then further purified by reversed phase HPLC using a C_4 column. The elution of base-volatile [^{3}H] radioactivity from the HPLC column corresponds with the elution of UV absorbing material and also coincides with the elution position of PCM (Figure 9.2) The elution position of rabbit PCM is very similar to that of bovine PCM (Lindquist and McFadden 1994a). The similarity in the elution of the two enzymes from the HPLC column is not surprising, because the sequence of PCM among the mammalian forms of this enzyme is highly conserved (Gilbert, et al. 1988, Sato, et al. 1989, Ingrosso, et al. 1989, Fu, et al. 1991).

Electrophoretic separation of the HPLC purified material under acidic conditions yielded insufficient quantities of protein for detection with a colloidal Coomassie stain. However, as shown in Figure 9.3, there is base-volatile radioactivity detected in the gel slices corresponding to the known electrophoretic migration of PCM, and radioactivity in this electrophoretic range was observed only in the HPLC fractions that also were PCM-positive by Western blot..

To verify this result by a second method, high resolution separation was employed using HPLC. Fractions (100 µl) were collected in a three-minute window, from 23.0 to 26.0 minutes, which had previously been determined for the elution of rabbit PCM in Figure 9.2. Analysis of the base-volatile [^{3}H]radioactivity showed a peak that corresponds to the elution of PCM (Figure 9.4). The elution position of PCM was confirmed by immunodetection.

FIGURE 9.2:

Reversed phase high performance liquid chromatographic separation of a PCM-positive affinity fraction and its corresponding base-volatile [^{3}H] radioactivity. Component elution was monitored at 280 nm. The elution position of rabbit PCM is indicated by the arrow and was determined by immunodetection as previously described.

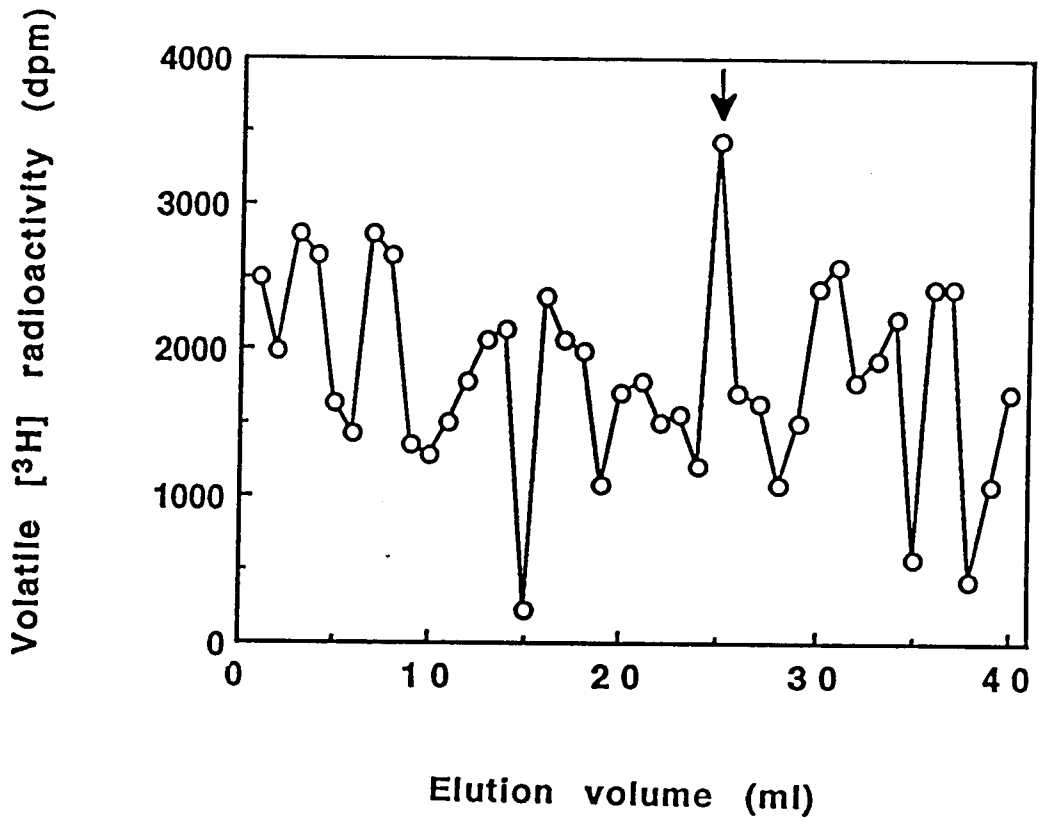
Volatile [3H] radioactivity (dpm)
4000
3000
2000
1000
0
0
10
20
30
40
Elution volume (ml)

FIGURE 9.2

FIGURE 9.3:

A histogram showing the base-volatile [^{3}H] radioactivity corresponding to gel slices (1 cm x 1 cm) of HPLC fractions that had been electrophoretically separated under acidic conditions and visualized with a colloidal Coomassie stain. The arrow indicates the elution position of PCM following reversed phase HPLC purification.

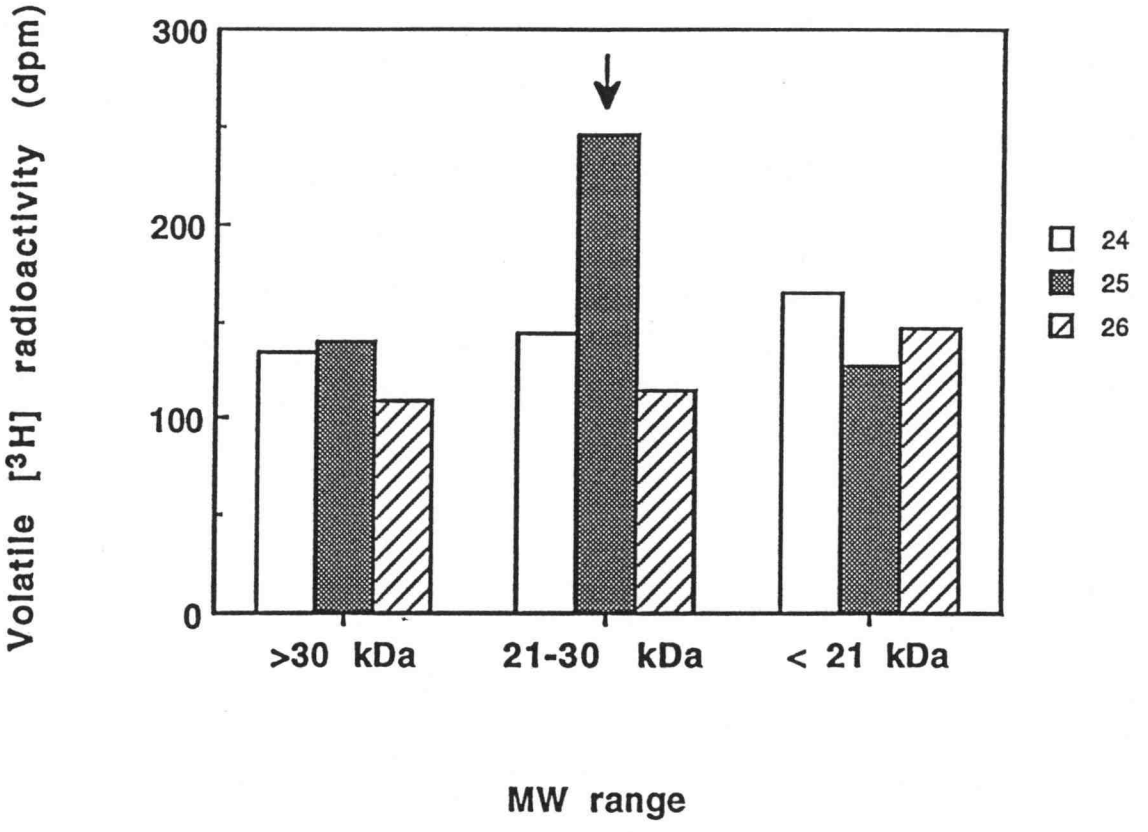
Volatile [3H] radioactivity (dpm)
300
200
100
0
>30 kDa
21-30 kDa
< 21 kDa
MW range
24
25
26

FIGURE 9.3

FIGURE 9.4:

High resolution reversed phase liquid chromatographic separation of a PCM-positive affinity fraction and the corresponding base-volatile [^{3}H] radioactivity. Fractions (100 µl) were collected in a three-minute window centered on the previously determined elution position of PCM (Figure 9.2). An aliquot of each fraction (50 µl) was assayed for base-volatile [^{3}H] radioactivity. The arrow indicates the elution position of PCM as determined by immunodetection.

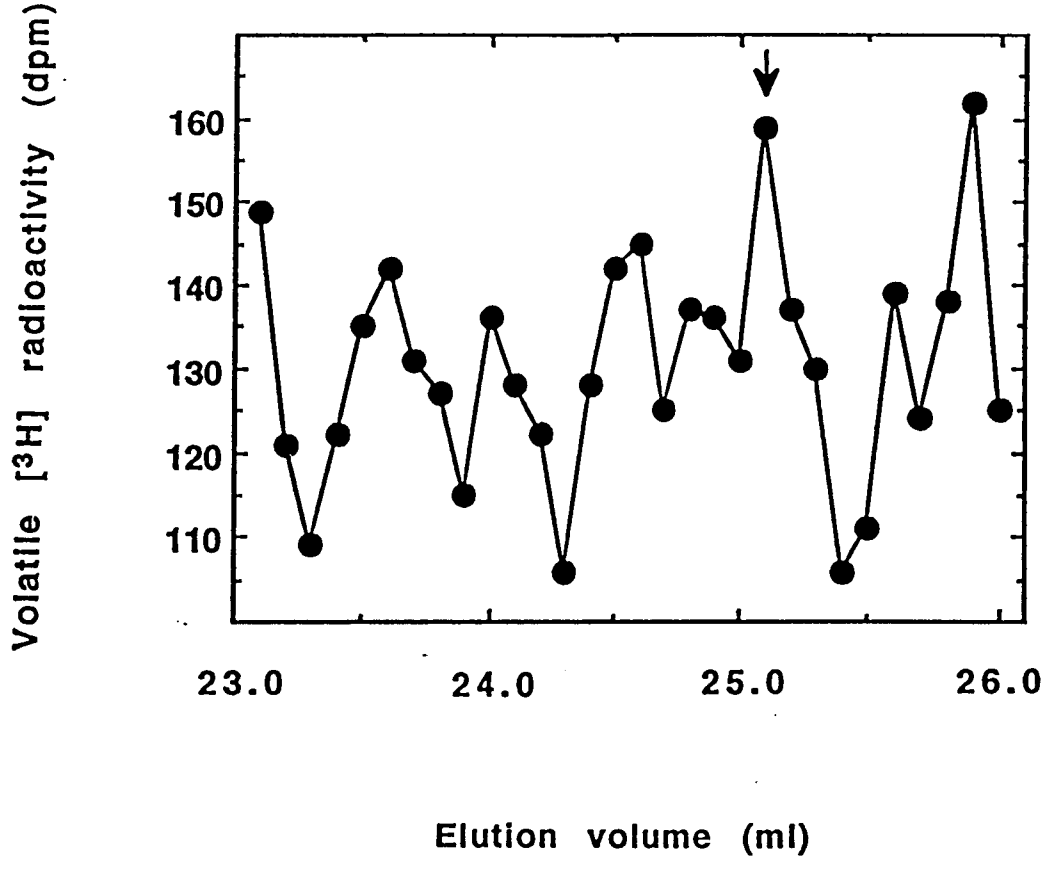
Volatile [3H] radioactivity (dpm)
160
150
140
130
120
110
23.0
24.0
25.0
26.0
Elution volume (ml)

FIGURE 9.4

Comparison of the high resolution HPLC fractions, corresponding to the elution position of PCM for each of the immunoreactive affinity fractions, yields the histogram shown in Figure 9.5. The peak of base-volatile [^{3}H] radioactivity corresponds with the peak of elution of PCM from the affinity column.

DISCUSSION

Using sequential separation of *in vivo* [^{3}H-*methyl*] labeled samples, a peak of base-volatile radioactivity characteristic of methyl esterified protein was found, which comigrates with rabbit PCM. The inability to separate [^{3}H-*methyl*] esterified material from PCM under acidic conditions lead to the conclusion that it is the PCM molecules themselves that were labeled and that PCM is capable of automethylating *in vivo*. The validity of this methodology, to sequentially purify [^{3}H] automethylated PCM, was verified using *in vitro* automethylated bovine PCM (data not shown).

The ability of PCM to automethylate *in vivo* provides evidence for the existence of damage in the repair enzymes themselves and supports previously published work characterizing a damaged subpopulation of PCM molecules, termed αPCM. The effect of this damage upon the activity of PCM is still unknown, although recent work on this topic suggests that αPCM molecules possess a functional AdoMet binding site (see chapter 8). However, a functional AdoMet binding sites does not prove these molecules to be catalyticly active; although it does raise the possibility.

There are several possible alternatives as to the affect damage could have on the activity of PCM: First, damage may result in a loss of activity. Second, damage may

FIGURE 9.5:

Base-volatile [^{3}H] radioactivity profile for PCM-positive/high resolution HPLC fractions. Each Western-positive affinity fraction was purified using a high resolution technique (Figure 9.4). The radioactivity of the PCM peaks from the different fractions is compared here. The material used in this experiment was similarly prepared to the material used in Figure 9.1. However, due to an increase in the volume of gel used in the affinity column and a decrease in the volume of the elution fractions, the location of the peak of Western-positive material is shifted. This shift is responsible for the difference in the sample notation observed on the X-axis.

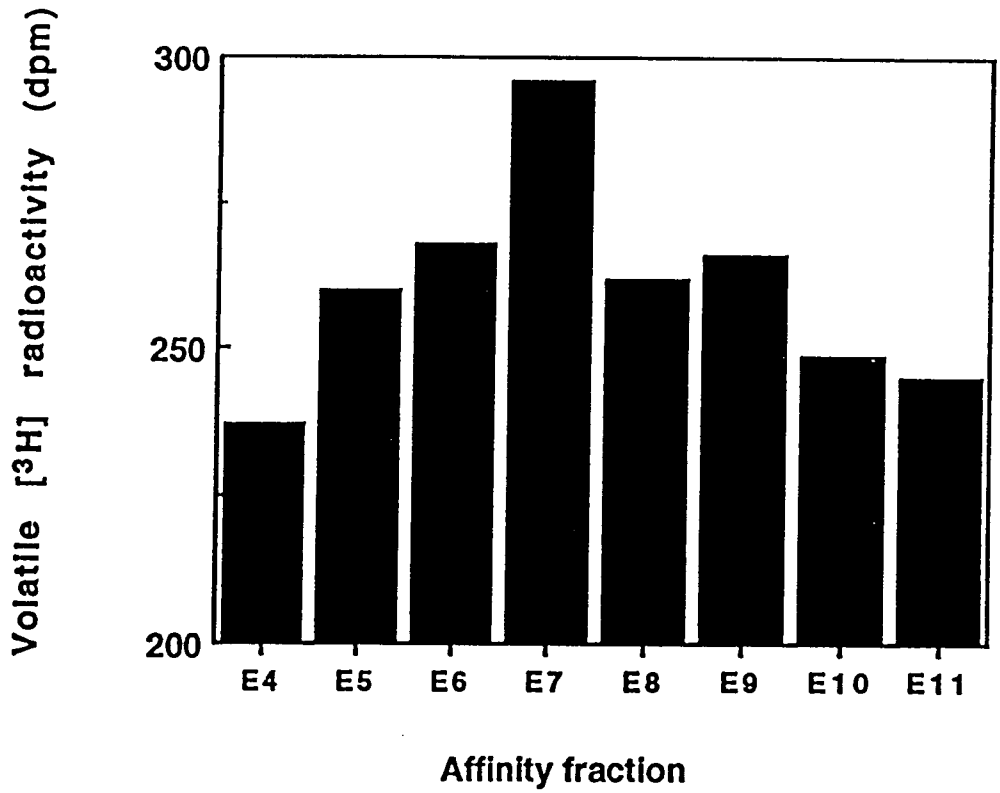
Volatile [3H] radioactivity (dpm)
300
250
200
E4
E5
E6
E7
E8
E9
E10
E11
Affinity fraction

FIGURE 9.5

have no affect on activity. Third, damage may increase activity. And fourth, damage may alter the activity.

A loss in activity has been demonstrated in other damaged proteins and is perhaps the most reasonable of the hypotheses. Since damage results in a perturbation of structure, either through deamidation, isomerization, and/or epimerization, it is not difficult to imagine how this damage could result in a loss of activity, as three-dimensional structure is very important to a protein's ability to function. A loss of activity within a repair system could lower the effectiveness of the system by reducing the efficiency of repair. Inefficient repair could lead to a steady increase in unrepaired damage not only within the repair enzymes, but within all of the cellular proteins; the result of which would be an error cascade reaction within the cell. A steady loss of a cell's ability to function with age would be compatible with current observations in senescence.

It is also not difficult to imagine how perturbation of a protein's structure could have no affect on its activity, providing that damage occurred in flexible regions that were less important; such observations have been made for a handful of proteins. If this was the case with PCM, damage might still manifest itself through time wasted on ineffective repair reactions that may also result in the accumulation of unrepaired damage in other proteins. These other proteins might not be as forgiving to damage and so an error cascade reaction could also result.

Increased activity as a result of damage may be a bit more difficult to imagine. If damage served as a switch to activate the repair system, then this would make for a truly efficient repair system. However, no such "efficient" systems have been described.

Lastly, an abnormal activity arising as the result of damage may also be difficult to imagine. However, as the effect of substituting a key residue is unknown, such damage might result in an altered substrate affinity or a change in the positioning of the molecules in the active site allowing a previously undescribed reaction to occur.

Of the hypotheses that have been proposed, the more reasonable ones suggest that damage to the repair system results in a steady decrease in repair. The effect of this decrease would be a steady increase in the accumulation of unrepaired damage throughout the cell. The immediate effect of this damage may be a loss of activity or merely a perturbation of structure, which makes the damaged protein susceptible to other forms of damage. However, regardless of the type of damage, the net result will be the same. The accumulation of damage will lead to a loss in the ability of a cell to maintain itself and a cell that loses this ability will senesce.

Acknowledgments

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CHAPTER 10

CONCLUSION

CONCLUSION

The broad goal of this dissertation was to gain a better understanding of the effects of damage upon the damage-control enzymes. Several approaches have been used, all of which were related to this central question. Fundamental to several of the approaches was the purification of PCM and tests of its *in vitro* activities.

AUTOMETHYLATION

The purification of PCM from bovine erythrocytes involved several chromatographic steps: affinity, gel filtration, and anion exchange FPLC. During each of these steps, PCM peak fractions were incubated with [^{3}H-*methyl*]AdoMet and the reaction mixture separated using gel electrophoresis under acidic conditions that are known to promote the stability of the methyl ester linkages. In each of these separations, [^{3}H]methylated protein migrating with PCM was detected. The inability to separate this material from PCM, even after the addition of a fourth chromatographic step, reversed phase HPLC, led to the conclusion that it was the PCM molecules themselves that were methylated. Using two separate measures, Coomassie dye binding and absorption at 280 nm, to quantitate the PCM concentration, the stoichiometry of methylation for PCM was determined to be 1-2% (Chapter 2). This value is typical for the sub-stoichiometric occurrence of altered aspartyl residues within proteins. Analysis of the ability of PCM molecules to self-methylate determined that methylation is limited to a subpopulation of PCM molecules, since the methylation reaction plateaued after approximately 24 hrs.

The realization that damaged PCM molecules are a subpopulation and directly proportional to the total enzyme concentration led us to term these damaged molecules "αPCM". Analysis of the "α" content in the anion exchange FPLC purified material yielded values of 0.1%, 1%, and 10% for the three fractions respectively. Apparently, the later eluting fractions had been enriched in their content of αPCM. This suggested that these damage molecules had become slightly more acidic and therefore bound a little more tightly to the anion exchange column. The analysis of the stability of these [^{3}H-*methyl*] linkages, showed them to be rapidly hydrolyzed in base and volatile upon acidification; both characteristics of a methyl ester linkage. In fact, this is what one would expect, given that the majority of damage events result from the deamidation of asparagine [Clarke, S. (1987) Int. J. Peptide Prot. Res. 30, 808-821. Lura, R. and Schirch, V. (1988) Biochemistry 27, 7671-7677. Aswad (1995)] and the exchange of an amide for a carboxylate would reduce the pK_a of the deamidated protein.

Since the catalytic mechanism of PCM is still undetermined, it was necessary to show that the methylated molecules observed were not methylated-enzyme intermediates. To test this, PCM was allowed to self-methylate and then an excess of non-radioactive AdoMet and L-isoaspartyl substrate, either peptide (isotetragastrin; Trp-Met-isoAsp-Phe-NH_2) or protein (ovalbumin), were added to see if the methylated species could be chased away (Chapter 2). In each of these tests, methylation of the isoaspartyl substrate was observed, but there was no noticeable decrease in the level of methylated PCM. This result led to the conclusion that the methyl group incorporated into PCM was not an enzyme intermediate.

The next question to be addressed was how active PCM incorporates methyl groups into the subpopulation of αPCM. In one set of experiments, by adding an excess of isoaspartyl substrate prior to the addition of [^{3}H-*methyl*]AdoMet, methylation of the isoaspartyl substrate was observed with no decrease in the methylation of αPCM. This result suggests that the reaction was intramolecular and that αPCM molecules might be methylating themselves. However, this is probably incorrect since in other experiments, a series of enzyme dilutions was performed prior to the addition of [^{3}H-*methyl*]AdoMet. If the reaction was truly intramolecular, then dilution of the enzyme would have had no effect upon the reaction rate. However, if the reaction was intermolecular, occurring between two PCM molecules, then there would be a concentration dependence upon the rate of the reaction. Although the data did not fit the theoretical curve for a bimolecular reaction, it was quite obvious that the reaction was not intramolecular, as changes in the concentration did affect the reaction rate. From this result, it was concluded that the order of the automethylation reaction was bimolecular. Thus, the dilution experiments appeared to contradict the conclusion that was made based upon competition between automethylation and isoaspartyl substrate methylation.

The term "automethylation" does not distinguish how the pool of PCM molecules self-methylate. It was chosen, as it was analogous to the term "autophosphorylation". The autophosphorylation reaction of CheA, a protein involved in bacterial chemotaxis, has been shown to be an intermolecular reaction (Stock, J.B., Surette, M.G., McCleary, W.R., and Stock, A.M. (1992) J.Biol.Chem. 267, 19753-19756), while the autophosphorylation reaction of protein kinase C has been shown to be intramolecular

(McFadden, P.N., Mandpe, A., and Koshland, D.E.Jr. (1989) J.Biol.Chem. 264, 12765-12771).

KINETICS

A parsimonious explanation was ultimately obtained by close analysis of the data from the dilution experiment. Here, the dissociation constant for the automethylation reaction, related to K_m, was determined [$K_{s\ app}$ = 0.5 μM](Chapter 3). The high affinity of PCM for αPCM, in comparison to other damaged substrates [K_m <1 μM to K_m > 1 mM](Lowenson and Clarke 1990 and 1991, Aswad 1995), explains how the methylation of αPCM can take precedence over the repair of other damaged substrates. Also obtained from the data was the $k_{p\ app}$ [0.0095 min^{-1}], related to the V_{max}. This is a very slow constant, signifying that a relatively stable complex between active PCM and αPCM exists.

Using an estimate of 5 μM for the intracellular PCM concentration and the previously determined rate constants, the combination of high affinity and low turnover leads to the estimate that greater than 90 in 100 αPCM molecules exist as a stable complex with PCM. However, the slow turnover of αPCM molecules, one per hundred minutes, suggests that PCM becomes preoccupied with its repair reaction, perhaps at the expense of other proteins. Such high-affinity, slow turn over behavior is typical for dead-end inhibitor complexes and therefore it is possible that the stability of this complex serves some undetermined regulatory function.

SITE MAPPING

Having established that PCM was damaged and capable of incorporating methyl groups into its sequence in a manner consistent with the presence of altered aspartyl residues, a further question was which are the sites of aspartyl damage in PCM. Using a combination of chemical and proteolytic mapping methods, the sites of automethylated damage in αPCM were isolated to two amino acids within the protein, Asn_{188} and Asp_{217} (Chapter 4). Thus, αPCM that is automethylated is a heterogeneous species, involving at least two subpopulations. From the quantitative analysis, approximately one-half of αPCM is damaged at each site. It is not clear whether the unusual kinetics of automethylation (high affinity, slow turnover) are attributes of both subspecies of αPCM. It is unclear if these are the only possible sites, as the Asn-Gly is predicted by theoretical arguments to be a potential site which is prone to damage.

SELF-REPAIR

Next, the ability of PCM to repair Asn_{188} and Asp_{217} was investigated. Until now, the ability of PCM to recognize these damaged sites had been established under conditions which promoted ester stability, so no assessment of the enzyme's ability for self-repair could be made. To enable quantitation of self-repair back to normal amino acids, a methodology that allowed assessment of the total methylatable sites within the enzyme was developed. This methodology involved the proteolytic digestion of PCM followed by [^{3}H]methylation of the proteolytic PCM fragments with active PCM. To test

if PCM was capable of self-repair, the enzyme was incubated at physiological pH in the presence of AdoMet for 24 hrs, during which time methylation and demethylation were expected. Subsequent digestion and [^{3}H]methylation revealed a decrease in the quantity of the two damaged sites, Asn_{188} and Asp_{217}. Note that Asn_{188} and Asp_{217} are associated with trypsin fragments T-2 and T-1, respectively. To test if this reduced availability of the sites was truly due to repair, the enzyme was incubated with [^{3}H-*methyl*]AdoMet. Digestion of this material revealed that the decreased quantity of sites in T-1 and T-2 to be due to the occupation of these sites with non-radioactive methyl groups.

The above finding indicated that repair had not lowered the amount of damage that was initially present in PCM. However, the [^{3}H] incubated enzyme also showed the same stoichiometry of methylation as a control incubation of enzyme without AdoMet. Both samples showed a two-fold increase in the methylation of these sites over enzyme that had no prior incubation. These results suggest that PCM is indeed capable of recognizing and methylating damage at T-1 and T-2, but due to the stability of these esters at physiological pH, no noticeable level of repair has occurred at either site. These results also showed that damage occurs quite rapidly at these sites and that PCM is capable of recognizing and methylating this damage almost as fast as it occurs. As methylation is the first step in repair, these sites are likely to be repaired upon demethylation. However, the rather slow rate of this demethylation leads to the conclusion that the rate of repair is not sufficient to keep up with the rate of damage under these conditions. The resulting imbalance would lead to the accumulation of

damage at these sites. At best then, under *in vitro* conditions the self-repair activity of PCM can only slow and not stop the accumulation of spontaneous amino acid damage.

To further assess this situation, the stability of the methyl ester linkages was measured (Chapter 5). PCM was allowed to [^{3}H]automethylate for 24 hrs, after which an excess of non-radioactive AdoMet was added and a series of time points taken. Analysis of the time points for total ester content revealed a curve with two apparent phases. The slopes of these lines were calculated and yielded the decay constants ($-k_d$) of -0.2050 hr^{-1} and -0.0177 hr^{-1} that were then converted into half-lives of 3.4 hrs and 39.2 hrs.

To determine which site was represented by each half-life, the time points were proteolytically digested and the fragments T-1 and T-2 analyzed for their methyl ester content. Surprisingly, both of these sites appeared to maintain the same quantity of methyl esters in each digestion. This result suggests that both of these sites fall into the slow category with a half-life of 39.2 hrs. This, however, does not explain which sites demethylate with the more rapid half-life, which coincidentally appears to be fast enough to allow self-repair in a 24 hr time frame. Analysis of the time course shows that these sites may account for up to one-half of the methyl groups incorporated into PCM.

The initial explanation for this observation is that upon proteolytic digestion, the half-life of the fast sites becomes even more rapid, such that these sites completely demethylate during the time of the digestion. Why then are these sites not observed using chemical digestion, which is performed under more acidic conditions that should stabilize these esters? One possible explanation is that the methyl group has shifted the migration of this fragment away from its parent peak, such that it co-migrates beneath

another radioactive peak, perhaps making this peak appear broader. As this material is sub-stoichiometrically methylated, there would be insufficient material to detect its presence. Such a broad peak has been observed eluting at 35 minutes from a cyanogen bromide digest. A second explanation is that the fast sites are lost during the lengthy time of chemical digestion, 18 hrs.

18-O INCORPORATION

To investigate the efficiency of the repair reaction and explore an alternative method for identifying damaged sites, an isoaspartyl peptide, isotetragastrin [Trp-Met-isoAsp-Phe-NH_2] was incubated with PCM and an excess of AdoMet at physiological pH in a buffer made with $H_2[^{18}O]$ (Chapter 6). Analysis of the purified reaction products, D-/L-tetragastrin and D-/L-isotetragastrin, using fast atom bombardment (FAB) mass spectrometry detected peaks containing one and two ^{18}O atoms, which had been incorporated into the peptide during the repair reaction. This confirmed that hydrolysis of the succinimide occurs as a step in the repair reaction. This repair reaction was modeled using previously determined kinetic constants for the repair of isotetragastrin [McFadden and Clarke 1987]. The theoretical values for the incorporation of ^{18}O agree well with the experimental observations. Using the model, an average value of five cycles of methylation and demethylation was determined for complete repair of a damaged site. Although this is an excellent method for detection of repaired sites, as the oxygen atom incorporated during repair is not readily exchanged with solvent, there are still some limitations to the application of this technique. The most significant limitation

of this method is the sub-stoichiometric nature of altered aspartate in proteins. However, if one could obtain a higher atomic percentage of ^{18}O, this limitation might be overcome.

NON-METHYLATABLE SITES

Having established that PCM is damaged and that the repair of this damage is inefficient under the experimental conditions tested, the extent to which damage accumulates within PCM was next examined. Methylation of proteolytic fragments derived from tryptic digestion of PCM identified multiple peaks that had not been previously detected during automethylation reactions (Chapter 7). Analysis of the control reactions supported observations made by others in the field that these non-automethylated sites of damage were not a result of the trypsinization procedure. This led to the conclusion that the revealed sites were sites of damage that had previously existed within PCM and were inaccessible to methylation when the enzyme was in its native conformation. During the observation that incubation of the enzyme increased the stoichiometry of methylation at the T-1 and T-2 sites, an increase in the methylation of the hidden or "cryptic" sites was also observed. These results, when taken together, suggest that not only does damage accumulate within PCM as a result of inefficient repair, but unrepairable damage accumulates within the enzyme as well.

FUNCTIONAL STATE

Since the presence of damage has been reported to cause a loss of biological activity in other proteins, the possible effects of this damage upon PCM were investigated. Recall that the reaction catalyzed by PCM involves the transfer of a methyl group from AdoMet to the free carboxyl group of the damaged aspartyl residue. Therefore, to perform this reaction PCM must be capable of binding both the damaged substrate and AdoMet. Also, these molecules, once bound, must be positioned in such a way that the transition state is stabilized and catalysis may occur. Once the reaction is catalyzed, both AdoHcy and the methyl esterified substrate must be released from the active site. As the structure and catalytic mechanism of PCM are still unknown, damage to PCM may affect any or all of these steps.

In considering these possibilities, it was realized that the purification of PCM had, in fact, already answered the first of these questions. Recall that purification involved the use of an affinity column, S-adenosyl-L-homocysteine-Sepharose 4B. To have been purified with PCM, αPCM must have bound to the affinity ligand and therefore it must possess a functional AdoMet binding site. However, the possibility that this damage had occurred to the enzyme after affinity purification could not be ruled out.

Since there is no practical way to rigorously rule out that damage had not occurred after the enzyme was eluted from the affinity resin, a closely related question was chosen. Could [^{3}H]automethylated PCM bind to the affinity column? In this way, the population of damaged molecules was established prior to beginning the affinity-binding experiment. Analysis of the material eluting from the affinity column showed

that [^{3}H]automethylated PCM had indeed bound and was eluted upon addition of AdoHcy (Chapter 8). This provided an indisputable answer to the first of these questions, that damaged PCM possesses a functional AdoMet binding site. This result also opens the possibility that these molecules may in fact possess catalytic activity, although this remains to be tested as the binding of AdoMet is only one of many steps involved in catalysis.

The presence of a functional AdoMet binding site in damaged PCM molecules is not difficult to rationalize. Based upon "consensus sequences" among other known AdoMet binding proteins, Ingrosso et al. (1989) proposed that three regions within PCM were involved in the binding of AdoMet. Region I consists of residues 81-97 and contains the sequence Gly-Ser-Gly-Ser-Gly which is believed to be a nucleotide binding motif involved in hydrogen bonding with the sugar-ring (Aitken 1990). The other regions of conservation were residues 151-157 and residues 171-180, although no specific interactions for these residues have been proposed. Photo-affinity labeling of PCM using 8-azido-S-adenosyl-L-methionine (Syed et al. 1993) identified a peptide containing residues 113-121 that contains two adjacent aspartyl residues. These aspartyl residues were hypothesized to be involved in charge negation of the sulfonium ion in AdoMet. Since neither of the residues identified as damaged sites within PCM, Asn_{188} and Asp_{217}, is located in one of the regions that are proposed to interact with AdoMet, this result is understandable. Flexibility is an important contributing factor to the probability that damage will occur (Clarke 1987, Wright 1991). If the regions containing Asn_{188} and Asp_{217} possessed some structural flexibility that allowed damage to occur, then this

flexibility might also make these regions more "forgiving" to the perturbation that damage would invoke.

INTRACELLULAR

The ability to purify [^{3}H]automethylated PCM under acidic conditions provided an opportunity to address perhaps the most relevant question to cellular biochemistry. "Can PCM automethylate *in vivo*?" To address the existence of this reaction *in vivo*, erythrocytes were incubated with [^{3}H-*methyl*]methionine. The lack of protein synthesis in erythrocytes makes them an ideal system for the study of the mechanisms of cellular maintenance and self-repair. To see if PCM was indeed [^{3}H]automethylated *in vivo*, sequential steps of purification were performed under acidic conditions to stabilize the esters. At each step of purification, base-volatile [^{3}H]radioactivity was identified that co-eluted with immunodetectable levels of PCM (Chapter 9). It is the co-purification of this material through multiple sequential steps that led us to conclude that PCM is indeed automethylated *in vivo* and establishes the existence of damage within the cellular repair systems.

It is very common for aspartyl alterations to affect the function and activities of proteins. Thus, damage in PCM and a loss in activity would be consistent with the observations made in the majority of damaged proteins identified so far (Aswad 1995). A loss of activity in PCM upon damage would result in a decrease in the concentration of active PCM molecules within the cell, unless PCM was capable of efficient self-repair, of which it appears not to be. Also, due to the high affinity between PCM and αPCM, a

second active PCM molecule would become preoccupied with attempting to repair its damaged counter-part, resulting in a loss of essentially two PCM molecules per damage event.

Even if damage had no effect upon the catalytic ability of PCM, the high affinity of PCM for αPCM seems to suggest that for every damaged molecule, an active molecule would be preoccupied with attempting repair. The result of this preoccupation would be a loss of one PCM molecule per damage event.

In either case, damage to PCM results in a decrease in the number of PCM molecules capable of repairing damage in other proteins. A decrease in the number of effective molecules would result in a decrease in the net rate of repair and a decrease in the rate of repair would result in an apparent increase in the rate of protein damage.

This scenario would be consistent with observations made regarding changes that occur in aging cells (Stadtman 1988, Wright 1991, Shakespeare and Buchanan 1976). These observations include, a decrease in the activity of enzymes without a corresponding decrease in protein concentration, an increase in the levels of modified proteins, and an increase in the level of methyl acceptors. A contributing factor to this phenomenon may include reports of decreased proteolysis with age, especially towards abnormal proteins. The cause of the decrease in proteolytic activity is unknown, but without the continual resynthesis of proteins, spontaneous unrepaired protein damage may become an ever-increasing problem in aging organisms.

An increase in the level of altered aspartyl residues may also increase the levels of other forms of protein damage. The perturbation of structure that results from the

formation of altered aspartyl residues may expose other residues to oxidation, deamidation, glycosylation, isomerization, or may result in a loosening or loss of structure. A loss of structure that results in the exposure of hydrophobic surfaces may cause proteins to aggregate and may lead to their deposition in insoluble structures.

An alternative means by which damage may accumulate is by occurring below the surface in proteins (Aswad 1993 Prot. Sci., Aswad 1995). As long as a protein maintains its folded conformation, only the surface that is exposed to solvent is accessible to repair. Therefore, any damage that occurs beneath the surface would be unrepairable damage. However, the possibilities for this type of damage are more restricted, as the folded structure of the protein is itself somewhat restrictive. The effects of solvent and damaging agents in the solvent, such as oxidants, would also be more limited.

One obvious means by which protein damage can affect cellular function is through the inactivation of enzymes. A loss in metabolic function would have serious effects within the cell. At some point a threshold would be reached in which the cell would lose a crucial level of activity, and at that point the cell would die.

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APPENDIX

APPENDIX

MEASUREMENT OF ASP/ASN DAMAGE IN AGING PROTEINS, CHEMICAL INTERCONVERSION OF ASPARTYL ISOMERS, ^{18}O TAGGING OF ENZYMATICALLY REPAIRED ASPARTYL SITES, AND ENZYME AUTOMETHYLATION AT SITES OF ASP/ASN DAMAGE

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INTRODUCTION

Asp/Asn damage in aging proteins, resulting from the propensity of L-Asn and L-Asp residues to spontaneously convert to a mixture of α-epimerized and β-isomerized aspartyl products via succinimide intermediates (Figure A.1), is a practical problem from the standpoint of researchers seeking to isolate and study pure proteins. In particular, the advent of protein overexpression systems and the convenience of working with large quantities of protein has made it increasingly common for investigators with no prior intention of studying spontaneous protein damage to find that a protein of interest has undergone a transformation that is ultimately found to be due to Asp/Asn damage. The first indication of this problem is generally the detection of isoforms of a polypeptide with altered chromatographic or electrophoretic properties, often as a function of a heat-step involved in the purification of the protein. Other times the formation of these

FIGURE A.1:

Asp/Asn damage in proteins. L-Aspartic acid and L-asparagine 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-aspartic acid, D-aspartric acid, L-isoaspartic acid, and D-isoaspartic acid.

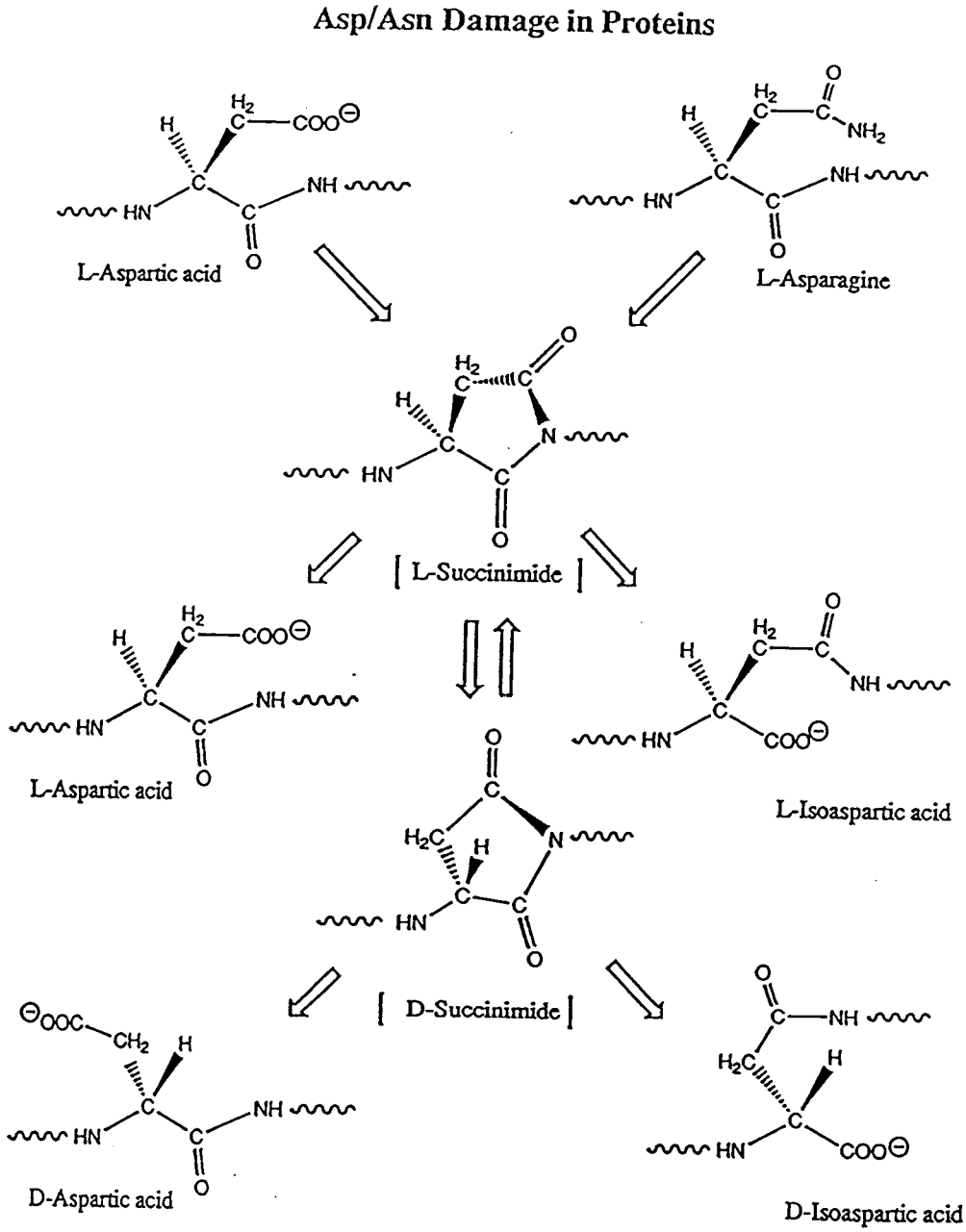
Asp/Asn Damage in Proteins
L-Aspartic acid
L-Asparagine
L-Succinimide
L-Aspartic acid
L-Isoaspartic acid
D-Succinimide
D-Aspartic acid
D-Isoaspartic acid

FIGURE A.1

spontaneously formed isoforms has been traced to a prolonged fermentor run or a lengthy storage period during the production of the protein. Though the isoforms of the protein may make up only a few percent of the total material, their presence is troubling since the purity of the protein is compromised.

Successful efforts have been made in the chemical and physical characterization of such spontaneously altered proteins, with recent examples pertaining to characterization of Asp/Asn damage in overexpressed forms of hirudin, deoxyribonuclease I, calbindin, interleukin-1β, epidermal growth factor, human growth hormone, anti-p185^{HER2}, tissue plasminogen activator, phosphocarrier protein, CD4, somatotropin, and interleukin-1α (Bischoff, et al., 1993, Cacia, et al., 1993, Chazin, et al., 1989, Daumy, et al., 1991, George-Nascimento, et al., 1990, Johnson, et al., 1989, Kwong and Harris, 1994, Paranandi, et al., 1994, Sharma, et al., 1993, Teshima, et al., 1991, Violand, et al., 1990, Wingfield, et al., 1987). Such efforts have made use of a varied combination of methods to pinpoint sites of Asp/Asn damage in the respective proteins, the most general of which have included a) peptide mapping and detection of chromatographically altered protein fragments obtained through enzymatic and/or chemical cleavage reactions, b) determination of asparagine deamidation by mass spectral analysis, c) the failure of the Edman cleavage reaction at β-isomerized aspartyl residues, and d) diagnostic enzymatic methylation of peptide fragments by protein (D-aspartyl/L-isoaspartyl) carboxyl methyltransferase (PCM). The widespread occurrence of protein Asp/Asn damage in many sequence contexts and in many different classes of

proteins indicate that this type of protein damage will continue to plague researchers and companies who are interested in producing large amounts of pure protein.

Asp/Asn damage of aging proteins is also a physiological problem for organisms dependent on the integrity of their proteins. Many tissue and cell proteins have been found to contain such forms of damage, including, for example, a large proportion of the β-amyloid protein associated with Alzheimer's dementia (Roher, et al., 1993). Perhaps an even stronger indication of the physiological relevance of Asp/Asn damage is the presence in most cells of the above-mentioned enzyme, PCM, whose function is the methylation and processing of D-aspartyl and L-isoaspartyl residues that form as the result of intracellular Asp/Asn damage (Figure A.2). The methylation of these sites and their rapid demethylation by a spontaneous mechanism at physiological pH has been shown in model studies to convert these abnormal aspartyl isomers to normal L-aspartyl residues (Figure A.3). Hence, the function of this enzyme evidently relates to the repair of sites of Asp/Asn damage, which could either restore a protein's function or could allow complete proteolytic degradation of a protein that might otherwise resist degradation because of the presence of abnormal amino acid isomers.

This chapter covers four aspects related to Asp/Asn damage and the D-Asp/L-Isoasp enzymatic methylation pathway. First, some suggestions are made for improvements in the use of chemical reduction assays for the products of Asp/Asn damage (Carter and McFadden, 1994a, Carter and McFadden, 1994b). Second, a technique is described that can chemically interconvert normal aspartyl residues and isoaspartyl residues for purposes of synthesizing model damaged peptides, and

FIGURE A.2:

Enzymatic methylation of damaged protein. Protein (D-aspartyl/L-isoaspartyl) carboxyl methyltransferase (PCM) incorporates the methyl group from S-adenosylmethionine into ester linkage with the β-carboxyl of D-aspartyl residues and the α-carboxyl of L-isoaspartyl residues. The same enzyme active site is capable of methylating both forms of Asp/Asn damage, possibly because, as shown in the box, free rotation about the N-C bond can yield similar configurations of D-Asp and L-Isoasp in which the esterified carboxyl group is in approximately the same position in space relative to the α-carbon and the rest of the protein backbone.

Enzymatic Methylation of Damaged Protein

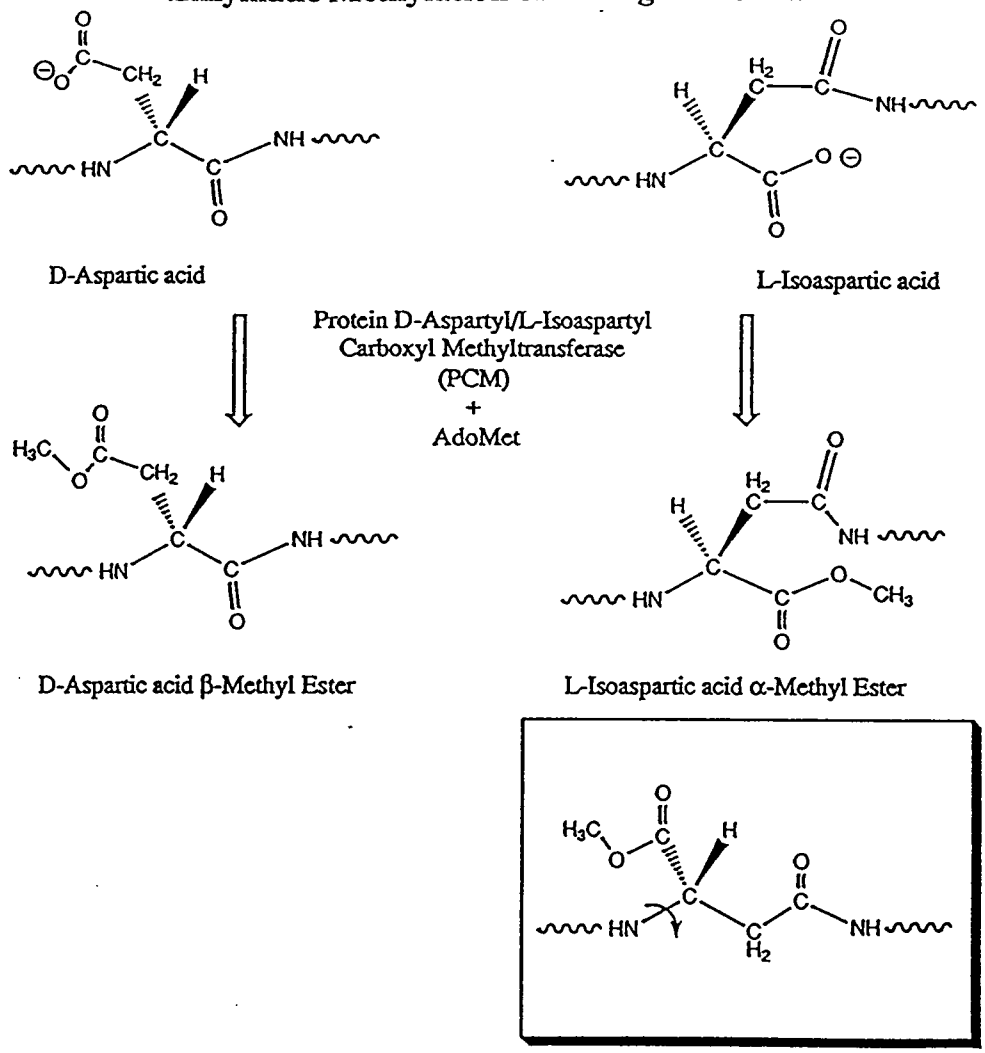

FIGURE A.2

FIGURE A.3:

Methylation-dependent protein repair. Enzymatically formed methyl esters of D-aspartyl and L-isoaspartyl residues are subject to rapid nonenzymatic displacement by nucleophilic attack by the amide nitrogen of the adjacent amino acid residue toward the C-terminus. This results in formation of succinimides, which are in turn subject to spontaneous epimerization and hydrolysis to yield four products (L-Asp, D-Asp, L-Isoasp and D-Isoasp). Two of these (D-Asp, L-Isoasp) can be methylated again by PCM, affording another opportunity for demethylation, succinimide formation, and succinimide hydrolysis. Eventually, the products of the process are highly enriched in L-Asp and D-Isoasp. L-Asp, being a normal α-amino acid, may restore function to the damaged protein, or may at least permit the aging protein to be fully destroyed by proteolytic mechanisms. While D-Isoasp is not a normal α-amino acid, it is possible that D-Isoasp may be a functional equivalent for L-Asp since L-Asp and D-Isoasp might adopt similar configurations as might D-Asp and L-Isoasp as shown in Figure A.2.

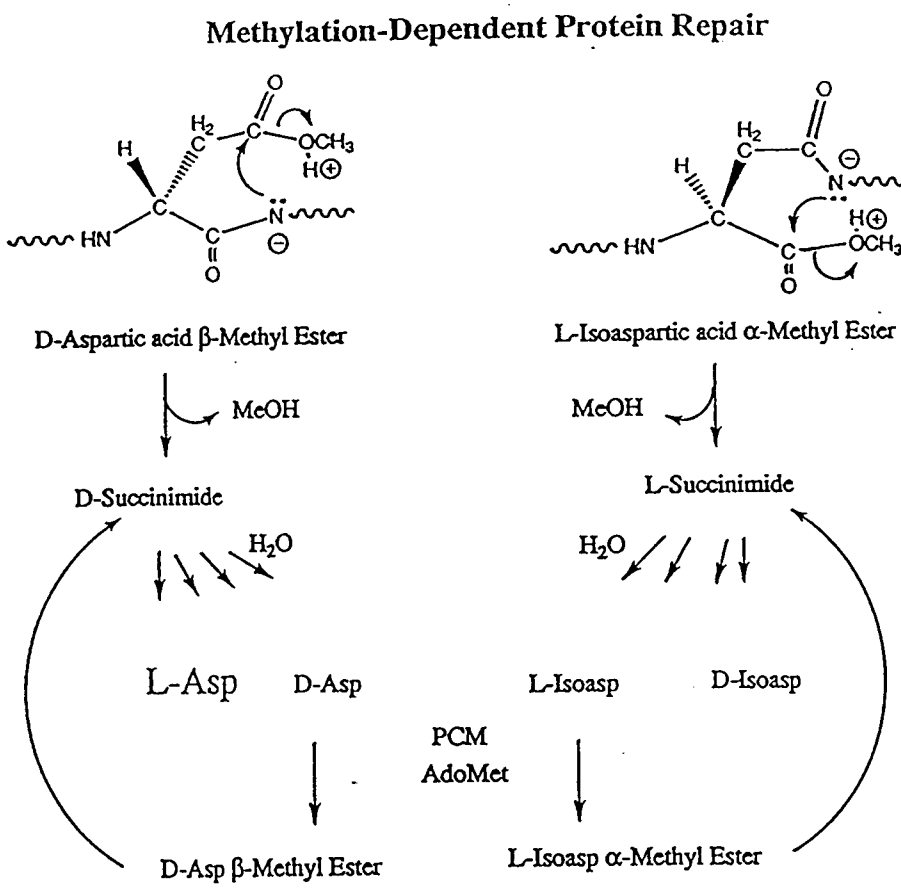
Methylation-Dependent Protein Repair
D-Aspartic acid β-Methyl Ester
L-Isoaspartic acid α-Methyl Ester
MeOH
MeOH
D-Succinimide
L-Succinimide
H2O
H2O
L-Asp
D-Asp
L-Isoasp
D-Isoasp
PCM
AdoMet
D-Asp β-Methyl Ester
L-Isoasp α-Methyl Ester

FIGURE A.3

conversely, for enabling the Edman sequencing through an isoaspartyl linkage. Third, recent isotope-labeling evidence in support of methylation-dependent repair of Asp/Asn damage is reviewed. Fourth, automethylation involving an Asp/Asn-damaged subpopulation of PCM (termed the αPCM fraction) is described (Lindquist and McFadden, 1994a), and a model is presented for the unusual kinetics of PCM automethylation.

MEASUREMENT OF ISOASPARTIC ACID AND PROTEIN SUCCINIMIDES BY CHEMICAL REDUCTION

The hydrolysis of a protein to free amino acids results in the loss of any information as to how an aspartyl residue was linked within the protein, and so it has not been possible to measure succinimidyl and isoaspartyl residues in conjunction with conventional amino acid analysis. Recently we investigated whether two related approaches of chemical reduction might show promise in converting succinimides and isoaspartic acid to derivatives that are stable to protein hydrolysis. The first method involves reductive ring-opening of protein succinimides by sodium borohydride to yield homoserine and isohomoserine upon protein hydrolysis (Figure A.4). This technique was validated for model compounds containing known quantities of succinimides (Carter, et al., 1994a). The second method uses borane (BH_3) reduction to convert the free α-carboxyl group of isoaspartyl residues to an alcohol, resulting in isohomoserine that can

FIGURE A.4:

Trapping succinimides by chemical reduction. The reductive ring-opening of succinimides (Kondo and Witkop, 1968) as applied to protein succinimides, results in formation of a mixture of homoserine and isohomoserine residues. These are stable derivatives that can be analyzed following protein hydrolysis.

Trapping Succinimides by Chemical Reduction

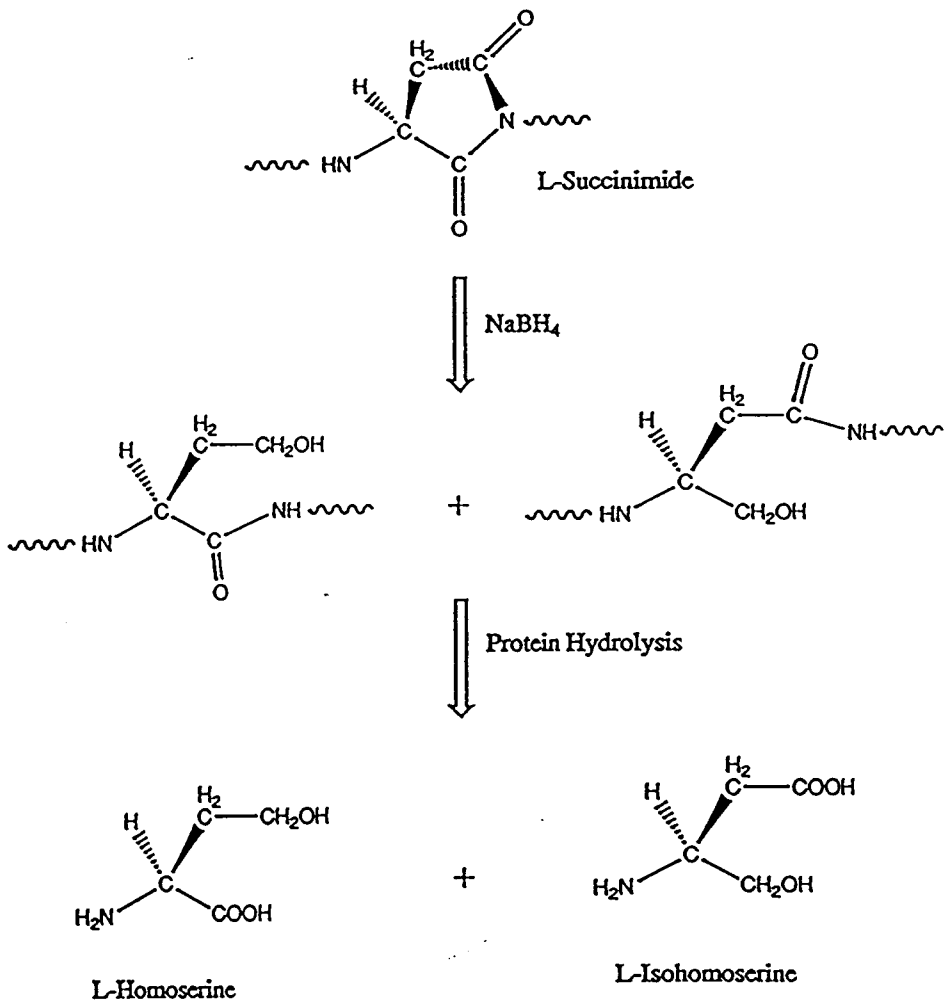

FIGURE A.4

be detected by protein hydrolysis and amino acid analysis (Figure A.5). This method has also been validated with model polypeptides (Carter, et al., 1994b).

An improvement in borane reduction of polypeptides can be made by using as a reducing agent the commercially available borane dimethylsulfide complex in place of borane tetrahydrofurn complex that was the reagent used previously (Carter, et al., 1994b) This change is to be recommended partly because borane dimethylsulfide is a more stable and less hazardous reagent than borane tetrahydrofuran, and because borane dimethylsulfide excelled in a direct comparison of the effectiveness of the two reagents in the reduction of N-carbobenzoxy-L-aspartic acid-β-benzyl ester to N-carbobenzoxy-L-isohomoserine-β-benzyl ester.

A general difficulty in applying reduction methods to the assay of Asp/Asn damage in large proteins is in detection of the substoichiometric content of isohomoserine that typically is expected to result from borohydride or borane reduction. For example, less than one percent of the total aspartic acid and asparagine present may be present as succinimide and/or isoaspartic acid, and so only a small amount of isohomoserine could possibly be expected. A useful adjunct in such cases, then, is to degrade most of the α-amino acids in an amino acid hydrolyzate by snake venom L-amino acid oxidase, leaving the β-amino acid isohomoserine as a stronger signal above the background. However, a further difficulty in the analysis of small amounts of isohomoserine is the low ninhydrin color constant exhibited by this amino acid. The reddish color intensity following ninhydrin spraying of thin-layer separated isohomoserine is less than 1/10th that of equivalent amounts of aspartic acid, and ion-

FIGURE A.5:

Detection of isoaspartic acid as the corresponding alcohol. The reduction of protein carboxyl groups by borane treatment (Atassi and Rosenthal, 1969, Rosenthal and Atassi, 1967) as applied to protein isoaspartyl groups results in formation of isohomoserine.

Detection of IsoAsp as the Corresponding Alcohol

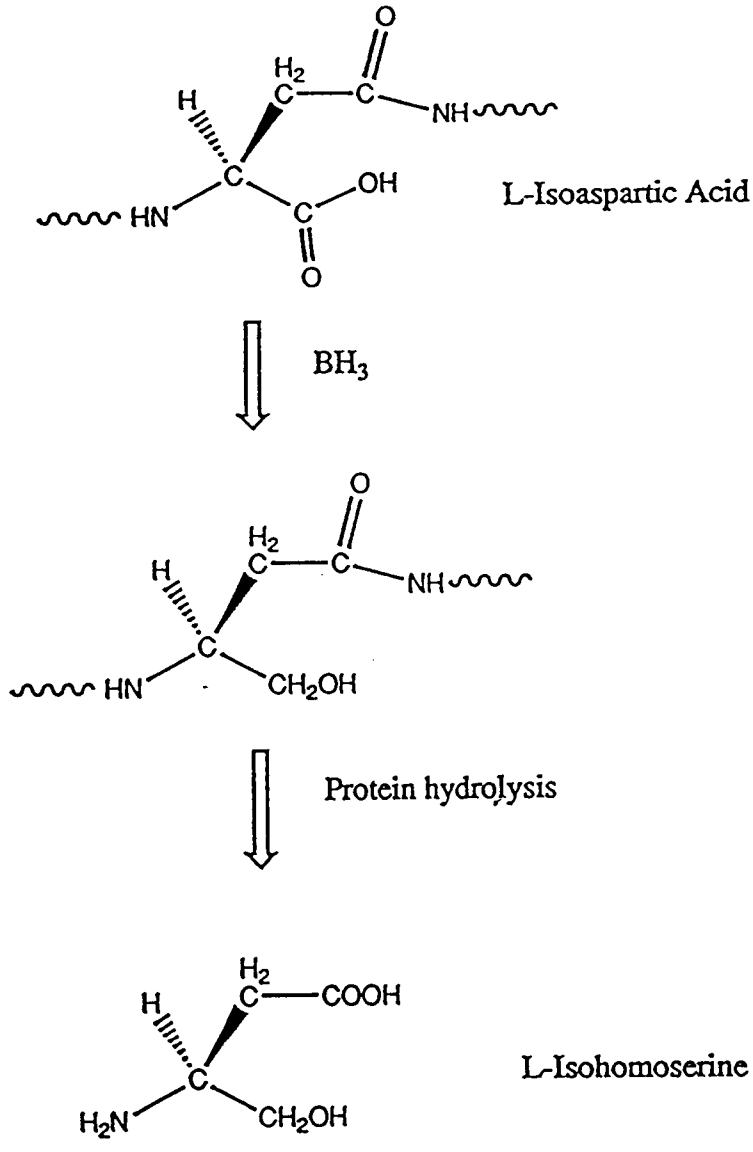

FIGURE A.5

exchange separation of isohomoserine with post-column ninhydrin detection indicates a ninhydrin color yield (570 nm+460nm) that is as little as 1/50th that of equivalent amounts of aspartic acid. Depending on the amount of starting material, then, a means other than ninhydrin may be necessary for detection of isohomoserine. Derivatization of isohomoserine with either phenylisothiocynate or dabsyl chloride, and chromatographic separation of the conjugates by reversed phase HPLC are promising routes to isohomoserine analysis since the isohomoserine derivatives absorb in the ultraviolet (PTC-isohomoserine) and at 460 nm (dabsyl-Ihser) approximately as well on a molar basis as derivatives of α-amino acids.

INTERCONVERSION OF ASP AND ISOASP RESIDUES BY CHEMICAL ESTERIFICATION AND DE-ESTERIFICATION.

There is a convenient procedure for converting normal L-Asp peptides to peptide mixtures containing the several aspartyl isomers (McFadden and Clarke, 1986). Here the methyl ester of the L-aspartyl side chain is first formed by acidic methanol tretament. The ester is then displaced via a succinimide intermediate upon mild alkaline treatment. Hydrolysis of the succinimide finally gives rise to the mixture. The main product in the mixture is the L-isoaspartyl derivative, which can be purified to serve as a stoichiometric substrate in various studies of protein (D-aspartyl/ L-isoaspartyl) carboxyl methyltransferase. For example, L-Trp-L-Met-L-Isoasp-L-Phe-NH2 was prepared in this manner for studies described below, and recently an esterified/de-esterified preparation

of bovine serum albumin has been prepared and found in enzymatic assays to be extensively methylated by PCM. Similar chemistry could be used to convert isoaspartyl linkages to normal aspartyl linkages, which could permit Edman sequencing beyond an otherwise sequence-terminating isoaspartyl site.

The major limitation of this procedure is that glutamic acid and C-terminal carboxyls are also methyl esterified and are not extensively de-esterified under mild alkaline conditions. This results in additional complexity of the mixture with the varied presence of these other methylated carboxyl groups. We have recently explored a general solution to this problem by chemically forming the benzyl esters of peptide carboxyl groups. Here, the mild alkaline treatment of the peptide ester is again expected to facilitate the formation of succinimide rings that are then hydrolyzable to mixtures of aspartyl isomers. The remaining benzyl esters of glutamic acid residues and of the C-termini can then be removed by catalytic reduction, most conveniently by palladium catalyst with a hydrogen donor such as formate. This approach has met limited success thus far in our hands because the initial formation of the benzyl ester is not as facile as the formation of the methyl ester. Even so, it will be worthwhile to develop improved esterification conditions since this approach can enable the interconversion of aspartyl isomers without modifying the other carboxyls.

METHYLATION-DEPENDENT PROTEIN REPAIR VIA REPEATED PASSAGE THROUGH A SUCCINIMIDE INTERMEDIATE.

A rather inefficient aspect of the pathway for methylation-dependent repair of L-isoaspartyl sites in peptides is the requirement for repeated formation and hydrolysis of the succinimide intermediate. While kinetic evidence suggested that indeed on the order of 5 cycles of methylation, demethylation, and succinimide hydrolysis are necessary to effect the complete repair of a peptide, this assumption had not been directly tested. Recently, the expectation that ^{18}O from [^{18}O]water would be incorporated into the peptide upon succinimide hydrolysis was used to test for multiple passages through the succinimide intermediate during peptide repair (Figure A.6). Here, the model peptide used was L-Trp-L-Met-L-Isoasp-L-Phe-NH2, which itself had been prepared by chemical esterification/de-esterification as described above. [^{18}O]Water (43%) was included in the reaction medium, which, in addition to the isopeptide, consisted of bovine erythrocyte protein carboxyl methyltransferase, S-adenosylmethionine (AdoMet), and pH 7.8 phosphate buffer. Following a 48 hour reaction, the peptide products were purified by reversed phase HPLC, and peptide masses were measured by fast-atom bombardment mass spectrometry. The identification and quantification of doubly-^{18}O labeled normal aspartyl peptide as a repaired product fit closely with quantitative predictions of the extent of methylation/ demethylation/ succinimide hydrolysis that would occur in a period of about 27 hours, rather than the actual 48 hours of the reaction (Lindquist and McFadden, 1994b) . Thus, the theoretical kinetics of repair closely matched the experimental work, verifying that multiple succinimide hydrolyses take place, but with

FIGURE A.6:

L-Succinimide (L-imide) postulated as the central intermediate during peptide repair, showing the sites (asterisks) of potential incorporation of ^{18}O during hydrolytic ring opening in the presence of [^{18}O]water.

Potential sites of ^{18}O incorporation during peptide repair

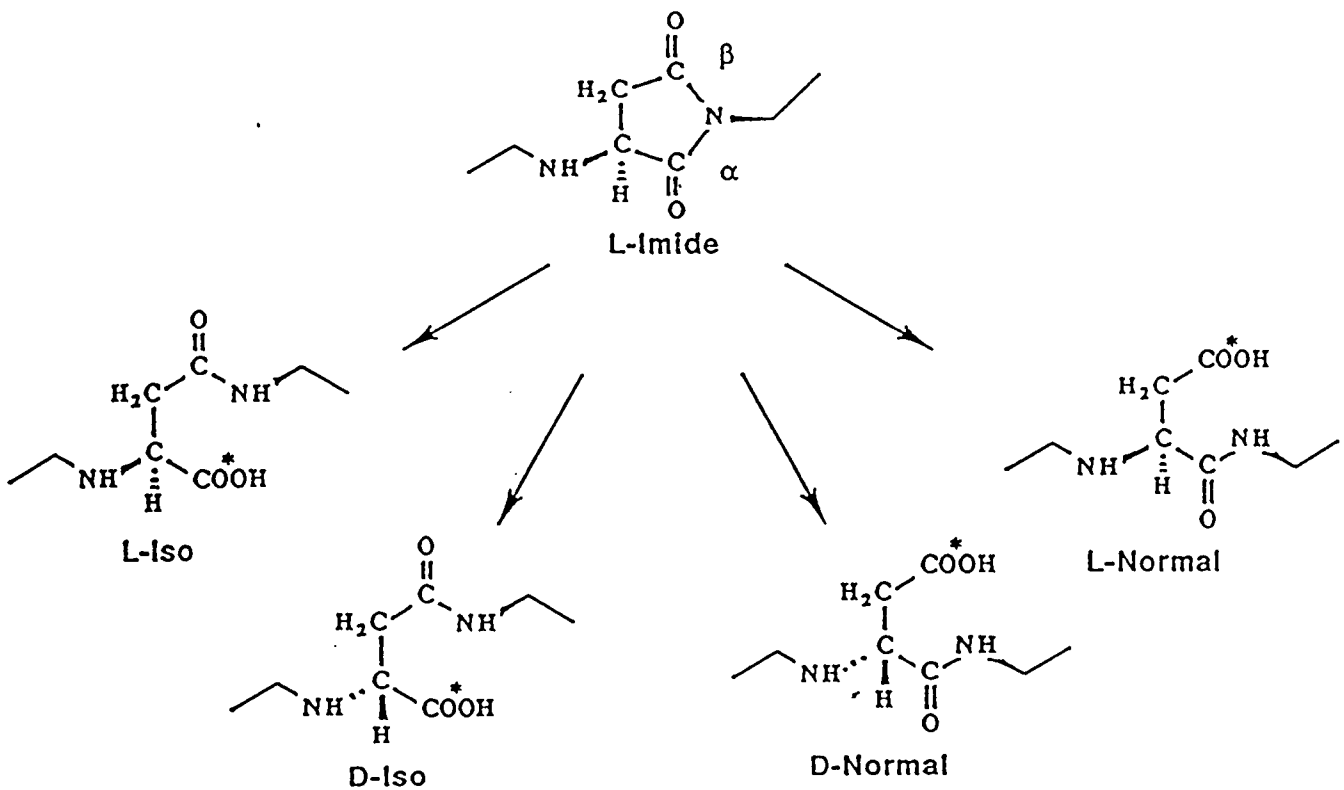

FIGURE A.6

the caveat that during the final ~21hours of the reaction little peptide repair took place. We originally ascribed the failure of the repair reaction to go to completion to either enzyme denaturation during the lengthy incubation, or to the buildup of S-adenosylhomocysteine (AdoHcy), the end-product inhibitor of methyltransferases. Recently, however, we have found an additional factor that may block the completion of the repair reaction in that S-methylthioadenosine (MTA) has been detected in repair reaction mixtures as a substantial spontaneous breakdown product of AdoMet. Since MTA may be equal to or more potent than AdoHcy as a methyltransferase inhibitor, this spontaneous side-reaction may be a major cause of the slowing of the rate of peptide repair.

A DAMAGED SUBPOPULATION OF PROTEIN (D-ASPARTYL/ L-ISOASPARTYL) CARBOXYL METHYLTRANSFERASE IS METHYLATED BY A HIGH AFFINITY, LOW-TURNOVER REACTION.

Interesting possibilities for feedback can be predicted to occur if enzymes that detect damage in other aging proteins are themselves damaged with age. As an example, bovine erythrocyte PCM was recently found to methylate itself on a subpopulation of enzyme molecules (Lindquist, et al., 1994a). The subpupulation of presumably damaged L-Isoasp- and D-Asp-containing enzyme molecules has been termed the αPCM fraction. From the known specific activity of [^{3}H-*methyl*]S-adenosylmethionine used in the radioactive automethylation assay it is calculated that αPCM molecules make up

approximately 1% of the total PCM population in the cell. Such a low stoichiometry of methylation is expected, given that over the lifetime of the cell there is only a partial spontaneous conversion of amino acid sites to D-aspartyl and L-isoaspartyl residues, and given the hypothesis that part of this spontaneous damage is repaired by the enzymatic methylation pathway. αPCM can be partly enriched by anion-exchange chromatography and then quantified by HPLC (Figure A.7), probably on the basis that deamidation of an Asn yields the slightly more negatively charged αPCM. Preparations with up to about 10% αPCM have been obtained in this manner.

To investigate the mechanism of PCM automethylation, assays were performed at several different enzyme dilutions. It was found that the specific rate of αPCM methylation increases with PCM concentration. This shows that the automethylation reaction involves more than a single PCM molecule since an intrapeptide methylation reaction would not have its rate affected by enzyme concentration. Most likely, enzyme automethylation involves the incorporation of a methyl group into an αPCM molecule by the activity of a second PCM molecule.

The specific rate of αPCM plateaus at high concentrations of total PCM (Figure A.8), indicative of a saturating reaction. The assumption of a rapid equilibrium in the interaction between αPCM and active PCM has allowed the derivation of a rate equation that lends a good theoretical fit to our dilution experiments (McFadden and Lindquist, 1994). In this equation,

$$v' = v/[PCM]_{tot} = \alpha k_p [PCM]_{tot} / (K_s + [PCM]_{tot}),$$

FIGURE A.7:

High-pressure liquid chromatography of [^{3}H]automethylated PCM, showing that the acidic shoulder of PCM as purified by anion exchange chromatography is enriched in automethylatable αPCM. A) HPLC elution profile of UV-absorbing species after [^{3}H]automethylation of PCM. Left chromatogram: An aliquot (60 µl) from an anion-exchange fraction containing the peak of PCM activity was reacted with [^{3}H-*methyl*] S-adenosylmethionine for 18 hours and then injected onto a reversed phase (C_4) HPLC column. Elution by acetonitrile gradient yielded the chromatogram shown here of reaction components absorbing at 280 nm. The arrow marks the position of the sharp peak attributable to the PCM polypeptide, the other peaks being evident as background species in similarly analyzed reaction mixtures in which PCM was not present (data not shown). The left inset shows the units of PCM activity surviving the denaturing HPLC conditions in fractions 24-28 in a parallel HPLC analysis of nonradiolabeled PCM. The right inset shows Western blot detection of the PCM polypeptide (horizontal arrow; M_r=27,000) in HPLC fractions 25-27 by specific PCM antiserum and colorimetrically detectable alkaline-phosphatase-linked secondary antibody. A small amount of dimerized PCM (M_r=54,000) was also immunodetectable, with control experiments showing that it was formed after the enzyme incubation, probably during sample lyophilation. Right chromatogram: An aliquot (60 µl) from an anion-exchange fraction immediately following the peak of PCM activity, and hence enriched in the slightly more acidic (and thus presumably deamidated) αPCM, was similarly reacted with [^{3}H-*methyl*]S-adenosylmethionine. Only a ten-fraction window of the right-hand chromatogram is shown, as the rest of the chromatogram was essentially identical to the 60-fraction chromatogram on the left. B) HPLC elution of [^{3}H]radioactivity. HPLC fractions from the two runs in "A" were assayed for their content of [^{3}H] volatile radioactivity in a vapor-phase assay for protein methyl esters (Chelsky, et al., 1984) (●). From reference 16.

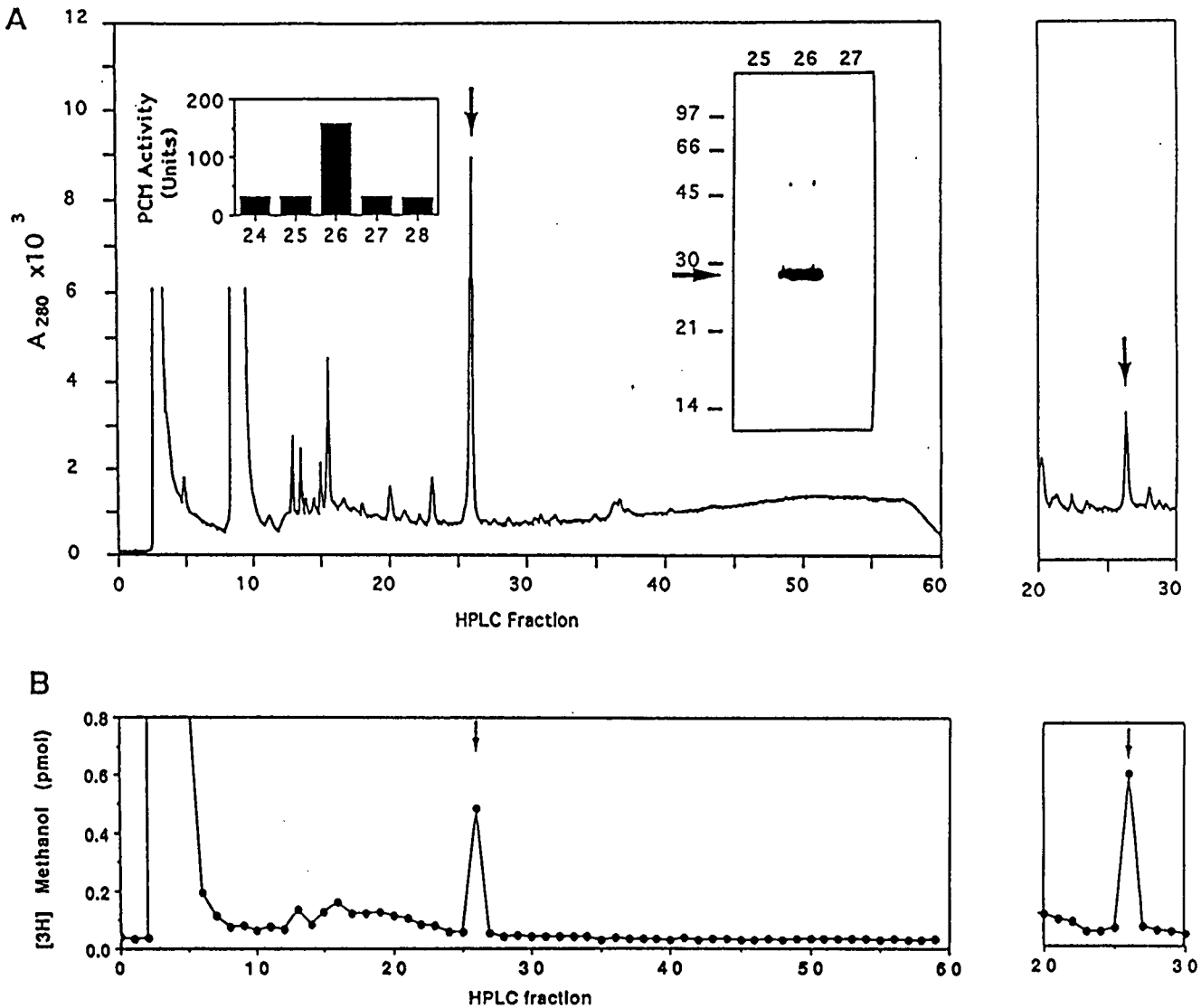
A
12
10
8
6
4
2
0
A_{280} x10^3
PCM Activity (Units)
200
100
0
24 25 26 27 28
25 26 27
97
66
45
30
21
14
0 10 20 30 40 50 60
HPLC Fraction
20 30
B
[3H] Methanol (pmol)
0.8
0.6
0.4
0.2
0.0
0 10 20 30 40 50 60
HPLC fraction
20 30

FIGURE A.7

FIGURE A.8:

The effect of dilution on the rate of PCM automethylation. The initial rate of PCM automethylation (percent of the total PCM methylated per minute) was measured as a function of enzyme dilution. Since the reactions contained differing volumes of purified PCM, the additional volumes were replaced with buffer containing identical salts as in the purified PCM solution. The experiment was repeated using different samples of anion-exchange fractionated PCM, containing estimated percentages of αPCM as follows: ❍ , 8-10% content of αPCM; ● , 2% αPCM; ■ , 1.5% αPCM. These estimates of αPCM content were made by measuring the final level of automethylation in extended time courses, and taking into account a small competing rate of ester hydrolysis that occurs in such time courses. Methyl ester formation in the PCM polypeptide chain was quantified following either HPLC separation (●) or following acidic gel electrophoresis (❍, ■). The theoretical saturation curves were generated by the equation in the text, using $k_p = 0.0095$ min^{-1}, $K_s = 0.50$ μM PCM_{tot}, and the values for α shown in this figure.

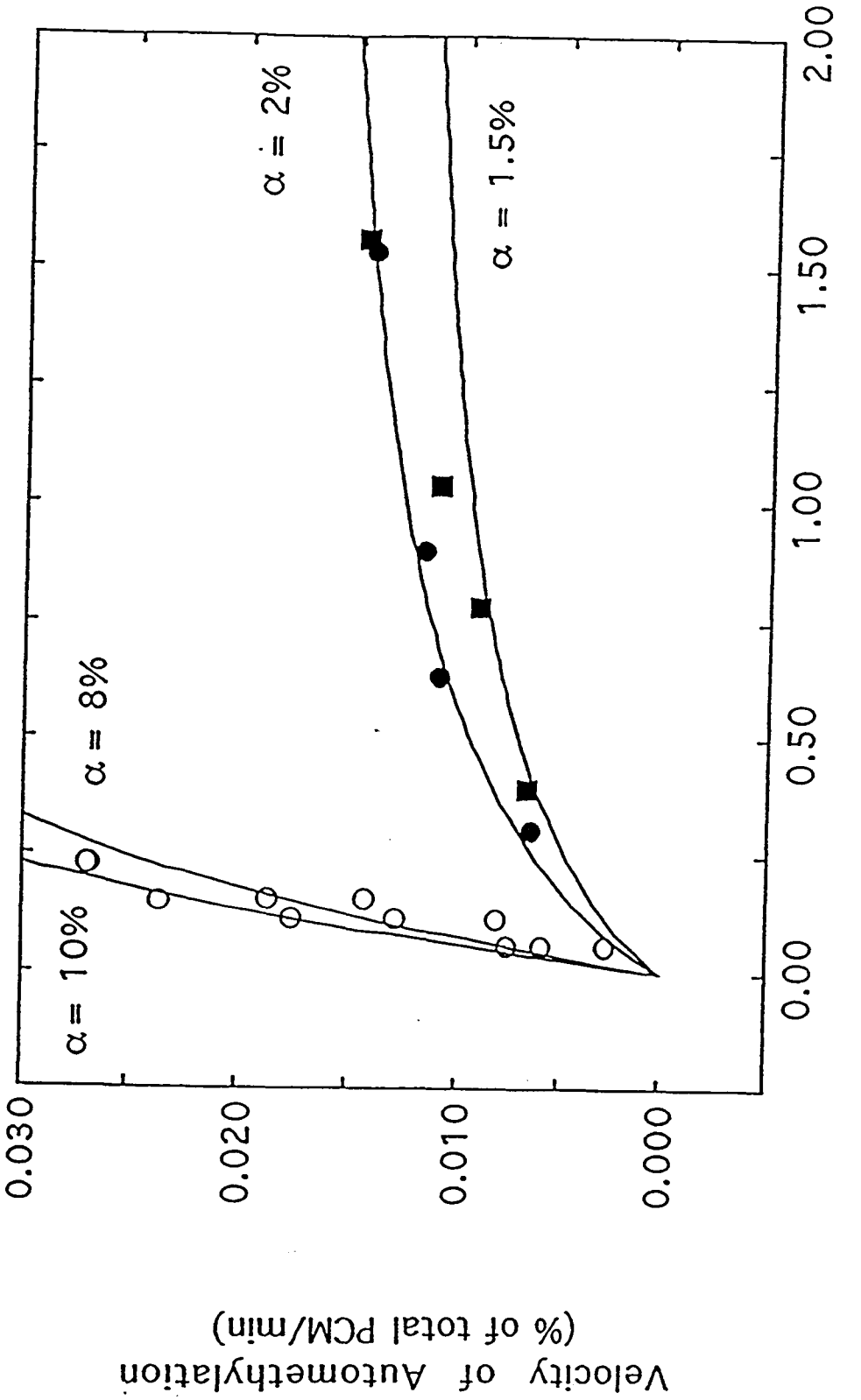
α = 10%
α = 8%
α = 2%
α = 1.5%
0.030
0.020
0.010
0.000
0.00
0.50
1.00
1.50
2.00
PCM (μM)
Velocity of Automethylation
(% of total PCM/min)

FIGURE A.8

the specific rate, v', of αPCM methylation is given as a function of the total PCM concentration, $[PCM]_{tot}$; α is the fractional population of αPCM (e.g.2%); k_p is the turnover number for the αPCM methylation reaction; and K_s is the dissociation constant between PCM and αPCM. By applying the above equation to the experimental dilution studies, values for the kinetic constants were calculated to be k_p, $0.0095min^{-1}$, and K_s, 0.5 μM. These values were constant in experiments with different PCM preparations, including those containing different percentages of αPCM (Figure A.8). These values for K_s and k_p are interesting and somewhat surprising. The turnover number for αPCM methylation, K_s, is lower than for the methylation of most other polypeptides by PCM (Lowenson and Clarke, 1991), which could indicate that PCM has a high affinity for its damaged "brethren". While this could be considered a logical adaptation to preserve the integrity of the repair system, the turnover number for the automethylation reaction, k_p, is so low as to suggest that the enzyme-substrate complex, PCM*αPCM, is nearly a dead-end since the complex decays to methylated product at a rate of less than once every hundred minutes. This combination of high affinity and low turnover suggests that as more αPCM is formed by spontaneous aging, the enzyme could conceivably become self-occupied by its slow self-methylation reaction, interfering with the methylation and further metabolic processing of other age-damaged proteins.

CONCLUSIONS

Asp/Asn damage affects numerous if not all proteins both in vitro and in vivo. Combined approaches, including the chemical reduction methods described here, can be used to measure Asp/Asn damage in a given protein. While protein engineering can enable the elimination of particularly troublesome sites that are prone to Asp/Asn damage, this may not be a complete answer to the practical problem of Asp/Asn damage since it is not uncommon for proteins to develop multiple sites of Asp/Asn damage, and in the long run, essentially every Asp or Asn residue could develop some degree of damage. Nature has evidently taken an active approach to solving the problem of Asp/Asn damage by selecting for the presence in most living cells of a PCM activity that specifically methylates and metabolizes Asp/Asn protein damage. PCM is being exploited increasingly as an in vitro tool to diagnose sites of Asp/Asn damage, and given the present good understanding of methylation-dependent repair of Asp/Asn damage it is now conceivable that a similarly active approach can be used to repair damaged proteins of pharmaceutical or industrial importance and to help ensure the preservation of activity of proteins during in vitro reactions of various kinds. Untangling the full complexity of the metabolism of intracellular Asp/Asn damage is still in the future, though, and factors such as Asp/Asn damage in PCM itself and the resulting automethylation of PCM may have considerable importance in determining the effectiveness of the metabolic systems that process damaged protein.

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