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Protein L-Isoaspartyl, D-Aspartyl *O*-Methyltransferases: Catalysts for Protein Repair

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ABSTRACT

Protein L-isoaspartyl, D-aspartyl *O*-methyltransferases (PIMTs) are ancient enzymes that are distributed through all phylogenetic domains. PIMTs catalyze the methylation of L-isoaspartyl, and to a lesser extent D-aspartyl, residues arising from the spontaneous deamidation and isomerization of protein asparaginyl and aspartyl residues. PIMTs catalyze the methylation of isoaspartyl residues in a large number of primary sequence configurations, which accounts for the broad specificity of the enzyme for protein substrates both *in vitro* and *in vivo*. PIMT-catalyzed methylation of isoaspartyl substrates initiates the repair of the polypeptide backbone in its damaged substrates by a spontaneous mechanism that involves a succinimidyl intermediate. The repair process catalyzed by PIMTs is not completely efficient, however, leaving open the possibility that unidentified enzymatic activities cooperate with PIMT in the repair process. Structurally, PIMTs are members of the Class I family of AdoMet-dependent methyltransferases. PIMTs have a unique topological arrangement of strands in the central β-sheet that provides a signature for this class of enzymes. The regulation and physiological significance of PIMT have been studied in several model organisms. PIMTs are constitutively synthesized by cells, but they can be upregulated in response to conditions that are potentially damaging to protein structures or when proteins are stored for prolonged periods of time. Disruption of PIMT genes in bacteria and simple eukaryotes produces subtle phenotypes that are apparent only under stress. Loss of PIMT function in transgenic mice leads to fatal epilepsy, suggesting that PIMT function is particularly important to neurons in mammals.

CONTENTS

I. INTRODUCTION

Due to the inherent chemical reactivities of amino acid side chains, proteins are subject to a variety of spontaneous modifications that can negatively affect the functionality of a protein. These modifications include oxidative damage [1,2], deamidation [3] and racemization [4] reactions. If not metabolized, the abnormal products of these reactions can accumulate over time in long-lived proteins. Therefore, cells use both proteolytic systems and repair enzymes to reduce the burden of structurally damaged proteins. Much of the damage incurred by proteins is irreparable, and the destruction of damaged proteins by proteolytic systems is well-established. By contrast, only a very small number of protein repair enzymes have been identified. The focus of this chapter is one of these repair enzymes, the protein L-isoaspartyl/D-aspartyl methyltransferase (PIMT[1]: E.C.2.1.1.77) that catalyzes the S-adenosylmethionine (AdoMet)-dependent methylation of L-isoaspartyl (L-isoAsp) and D-aspartyl (D-Asp) residues in age-damaged proteins. The goal of this chapter is to review the biochemistry of PIMT-catalyzed methylation, structural studies of PIMT, the distribution and regulation of PIMT activities in living organisms, and insights into the biological significance of protein isoAsp methylation gained from transgenic models. In addition to articles that focus on PIMT, the review includes information obtained from high-throughput genomic studies. Because of the large number of published manuscripts, it has not been possible to include all of the studies whose results have contributed to our current understanding of PIMT function. Many questions remain unanswered with respect to PIMT function, and this chapter attempts to point out questions in need of further investigation.

A. Historical overview

PIMT was serendipitously discovered in 1965 by Axelrod and Daly [5] while analyzing the metabolism of [^{14}C-methyl]AdoMet in extracts prepared from bovine pituitaries. The specificity of the reaction was not recognized at the time. Instead, the activity was described as a "methanol-forming enzyme," based on the presence of radioactive methanol in the extracts, which we now know was derived from the spontaneous hydrolysis of protein carboxyl [^{14}C-methyl] esters formed by PIMT. A few years later, the enzyme was correctly identified as a protein carboxylmethyltransferase [6,7] and purified to homogeneity from calf thymus [8]. It was another 10 years before D-Asp [9,10] and L-isoAsp [11,12] residues were identified as the substrate residues modified by PIMT.

The complete amino acid sequences of the human erythrocyte and bovine brain PIMTs were described in 1989 [13,14], paving the way for the first molecular cloning of the gene [15], molecular analyses of PIMT expression [16,17] and the development of transgenic models. The first three-dimensional structure of PIMT was solved for the *Thermatoga* enzyme [18] and followed by additional crystallographic studies exploring the catalytic mechanism of PIMT [19,20]. Physiological roles for PIMT continue to be explored in model organisms ranging widely in their biological complexity [21-24]. Results from these studies, discussed in more detail below, indicate that PIMT is an ancient enzyme that has been strongly conserved during evolution. PIMT activity is expressed constitutively in most cells, but its function is not essential under many conditions. Evidence suggests, however, that PIMT plays an important role in the maintenance of functional protein structures under stress conditions and during long-term storage.

II. BIOCHEMISTRY OF PIMT-CATALYZED REACTIONS

A. Physical properties of PIMTs

PIMT activities were first characterized and purified from mammalian sources [8], but have subsequently been shown to be widely distributed throughout all domains of life (Section IV). In mammals, PIMT activity can be detected in all tissues, although the measured specific activities vary considerably between tissues [25-27]. The enzyme has been purified to homogeneity from several tissues, including spleen [8], brain [14] and erythrocytes [13,28]. With few exceptions [29], PIMT fractionates as a cytosolic enzyme with a calculated molecular weight of 24,000 – 27,000. The chromatographic behavior of PIMT on gel filtration columns is consistent with that of a globular, monomeric protein [28,30]. Two major isozymes of PIMT have been purified from bovine brain [31] and human erythrocytes [30,32] which differ only in their C-terminal sequences [33,34]. Analysis of cDNA clones indicates that the isozymes arise by differential splicing [33,35]. Substitution of an –RDEL for an –RWK terminus changes the surface charge of the native protein sufficiently that the two isozymes can be resolved by anion exchange chromatography. The significance of the different C-termini is unknown. Although the –RDEL terminus of the more acidic isozyme is similar to that of an endoplasmic retention signal, both isozymes are found in the cytosolic fraction and neither form is associated with microsomes [34]. Biochemically, there are no significant differences in the substrate preferences or the kinetic parameters of the reactions catalyzed by the isozymes [30-32,36], suggesting that they play similar roles in cells. Supporting this conclusion, both isozymes also function similarly in transgenic models [37].

PIMTs are ubiquitously distributed in plants as well (Section VIIB), but the enzymes have been less thoroughly studied. A PIMT activity has been purified to homogeneity from wheat germ [27] which displays chromatographic properties consistent with a slightly acidic, monomeric enzyme with a molecular weight of 25,000. Measured K_ms for the wheat enzyme with isoAsp substrates are almost two orders of magnitude higher than those of human PIMT with the same isoAsp substrates, suggesting that the active sites of mammalian and plant PIMTs have undergone considerable divergence. From genomic information, it is clear that higher plants have duplicated *PIMT* genes and it is likely that two closely related PIMTs are expressed (Section VIIB). In this respect, two different PIMTs have been overexpressed from *Arabidopsis* cDNAs, but the biochemical properties of the second PIMT have not been investigated in detail [38,39].

Most of the biochemical information on PIMTs from lower eukaryotes and microbes has been obtained using recombinant proteins. The physical properties of these recombinant enzymes are similar to those previously isolated from mammalian and plant tissues. With the exception of two PIMTs from hyperthermophiles (Section IIIC). the enzymes are approximately the same size as those from higher eukaryotes , and they catalyze the methylation of both protein and peptide substrates. Table I lists PIMTs which have been studied in some detail, together with additional sources of information about the enzymes, including relevant accession numbers for online databases.

B. PIMT catalyzes the formation of protein carboxyl methyl esters

PIMT catalyzes the transfer of a methyl group from AdoMet to either the α- carboxyl group of isoAsp residues or the β-carboxyl group of a D-Asp residues in peptide and protein substrates (Fig. 1). The evidence supporting the unusual nature of these substrate residues and

their spontaneous generation during aging is discussed in Section IIC. Stereochemically, D-Asp and L-isoAsp residues are surprisingly similar in the distribution of functional groups [11,40], accounting for PIMT's ability to recognize both classes of substrates. The products of the reaction are a protein carboxyl methyl ester and a molecule of S-adenosylhomocysteine (AdoHcy), which acts as a potent end-product inhibitor of the reaction [41,42].

Kinetics studies have suggested that PIMT works by a rapid-equilibrium random sequential bi-bi mechanism [41,42], but structural studies are more consistent with an ordered sequential mechanism (Section IIIB) in which AdoMet binding precedes peptide binding and the release of methylated peptide precedes AdoHcy release [20,43]. The turnover number of PIMT calculated from kinetic data is always very low, on the order of 0.1-1.0 mol min^{-1}, although V_{max} varies with different isoAsp substrates [20,44,45]. In general, the turnover number with peptide substrates is significantly higher than that with protein substrates, suggesting that the greater conformational flexibility of peptide substrates facilitates product formation.

The protein carboxyl methyl esters formed by PIMT are unstable, turning over with half-times ranging from minutes to hours at physiological pH [46,47]. The processing of protein carboxyl methyl esters appears to be completely nonenzymatic. At the present time, there is no compelling evidence for protein demethylases or any other enzymatic activities that might participate in processing esters [48]. Studies with synthetic peptide substrates indicate that the immediate product of demethylation is a succinimide structure (Fig. 1), which forms spontaneously as methanol is released [11,49]. The chemical mechanism is postulated to involve the nucleophilic attack of the carboxyl peptide nitrogen on the ester carbonyl group. The actual rate of succinimide formation is significantly greater than the rate of ring hydrolysis and shows a strong dependence on the nature of the carboxyl amino acid. Studies with synthetic peptides indicate that small side chains facilitate succinimide formation, in the relative order of glycine, serine and alanine [50]. Protein methyl esters formed in intact cells also show wide variations in the rate of methanol release, reflecting the different primary sequence configurations surrounding physiological methylation sites [47].

C. Protein D-aspartyl and L-isoaspartyl residues are modified by PIMT

The unusual nature of the substrate sites for PIMT was first discovered by Steven Clarke's laboratory in 1982, due in part to the fortuitous congruence of chemical and biological investigations of protein carboxyl methylation reactions in the human erythrocyte [10]. Erythrocytes presented an ideal model for studying protein carboxylmethylation reactions because of their relatively simple biochemistry and because the incorporation of methyl groups from L-[methyl-^{3}H]methionine into protein methyl esters is not obscured by the incorporation of radioactivity into newly synthesized proteins or into methylated nucleic acids. Clarke's group showed that the protein methyl esters formed in either a lysed cell preparation [46] or in intact cells [51] were associated with a large number of membrane proteins and that these methyl esters showed a hydrolytic stability characteristic of Asp methyl esters. Aspartic acid β-[^{3}H]methyl esters could also be isolated from biosynthetically labeled membranes by digestion with carboxypeptidase Y [9], but yields were low, because most esters were hydrolyzed to [^{3}H]methanol during the digestion.

At the same time, the physiological experiments, discussed in more detail (Section V.A.) revealed features of the methylation reactions that were difficult to reconcile with a regulatory role for PIMT in erythrocytes. The experiments demonstrated that all of the methylation reactions involving membrane proteins were markedly substoichiometric [51]. In addition, the

number of methyl esters in an erythrocyte was positively correlated with the age of the cell [52], raising the possibility that methyl-accepting sites arose from age-related damage. Consistent with this hypothesis, the Asp β-methyl esters released from erythrocyte membranes by carboxypeptidase Y digestion were found to possess the D-stereoconfiguration. These Asp β-methyl esters were degraded by D-amino acid oxidase, but not L-amino oxidase, and they could be chemically derivatized to L-Leu-D-Asp β-methyl esters [10]. Protein D-Asp residues were hypothesized to originate post-translationally from the nucleophilic attack of the C-terminal peptide amino group on the carbonyl carbon in an Asp or Asn side chain, forming an unstable succinimide structure that is susceptible to racemization [10]. This succinimide structure is identical to the one that forms during the hydrolysis of protein methyl esters and deamidation reactions (Fig. 2).

Hydrolysis of a peptide succinimide generates a mixture of Asp- and isoAsp-containing peptides [50,53]. The first evidence that L-isoAsp residues were substrates for PIMT was provided by the Clarke and Aswad laboratories. Building on an earlier observation that adrenocorticopin hormone (ACTH) was methylated by PIMT *in vitro* [54], Murray and Clarke [11] tested all of the potential deamidation products of the ACTH-derived hexapeptide sequence VYPNGA for methyl-accepting activity. In this experiment, the L-isoAsp variant incorporated 0.8 mol/mol methyl groups during incubation with PIMT, while neither the L-Asp, D-Asp nor D-isoAsp variants possessed detectable methyl-accepting activity. In a complementary experiment, Aswad demonstrated that an isoAsp variant of deamidated full-length ACTH also acted as a high affinity substrate for PIMT [12]. The K_m of full-length isoaspartyl-ACTH was 1.9 μM, similar to the 6 μM value calculated for the hexapeptide [11].

Figure 2 shows the relative kinetic constants for the spontaneous deamidation, isomerization and racemization reactions involving the ACTH-derived model peptide VYP(X)GA, where X corresponds to an Asn, Asp, succinimide or Asp-*O*-methyl ester. The data were calculated from the combined data from two experimental studies [50,53]. Values for the kinetic constants have been normalized to that for Asn deamidation, which has been assigned a value of 1.0, since this is the rate-limiting reaction in cells. Generalizing from this model, L-isoAsp-containing peptides are predicted to comprise the major products of deamidation reactions. By contrast, D-Asp residues are the least frequent products of deamidation reactions, because the rate of succinimide hydrolysis is an order of magnitude faster than that of succinimide racemization. In addition, cleavage of succinimide rings is biased, producing about three times more isoAsp than Asp isomer. Thus, protein deamidation is likely to generate isoAsp-containing proteins with high frequency and proteins with racemized Asp residues with a much lower frequency.

The sequence requirements surrounding the L-isoAsp methyl-accepting site have been explored by using synthetic peptides as PIMT substrates. These experiments indicate that PIMT recognizes L-isoAsp residues in a wide range of primary sequences with limited commonality. In one comparative study [44], the affinity of human erythrocyte PIMT for 35 different L-isoAsp-containing peptides varied over several orders of magnitude. Measured K_ms for the most active substrates were in the 0.1-1. μM range, while PIMT recognized less active substrates with K_ms approaching and even exceeding 1 mM. High affinity substrates had at least one amino acid N-terminal and two amino acids C-terminal to the modified isoAsp residue, suggesting that neighboring residues were important in positioning substrates within the active site. The wide range of peptide substrates modified by PIMT is consistent with the broad specificity of the enzyme observed in intact cells (Section II.D.).

Considering that D-Asp β-[^{3}H]methyl esters have been isolated from intact erythrocytes incubated with L-[^{3}H-methyl]-L-methionine [10,55,56], it is surprising that peptides with D-Asp residues are generally not methylated or are poorly methylated by PIMT. Both human [56] and *Pyrococcus* [40] PIMTs modify D-Asp residues in synthetic peptides , but the measured K_ms are two to three orders of magnitude higher than the K_ms for the corresponding L-isoAsp peptides. The peptides may not provide good models for physiological methyl-accepting sites, since purified PIMT catalyzes the formation of D-Asp β-[^{3}H]methyl esters using erythrocyte membranes as a substrate *in vitro* [36]. Furthermore, the production of D-Asp β-methyl esters in the purified system was reduced in the presence of L-isoAsp peptides, suggesting that D-Asp and L-isoAsp substrates compete for the PIMT active site. Taken together, the biochemical data is best explained by a dual role for PIMT in recognizing both L-isoAsp and D-Asp substrates.

D. Methyl-accepting sites in protein substrates

From the very first identification of a protein carboxylmethyltransferase (then termed a carboxymethylase) activity in calf spleen [6], it has been clear that PIMT recognizes a broad range of protein substrates. Endogenous methyl-accepting proteins can be detected in extracts prepared from all mammalian tissues [25,57] and across phyla [58-60]. The identification of these endogenous methyl-accepting proteins in cellular extracts is complicated by the lability of protein methyl esters under mildly basic conditions [6,25,46], thus requiring the use of acidic gel systems for their resolution. Under the appropriate separation conditions, methyl-accepting activity is always found to be distributed among a heterogeneous group of proteins characteristic of a particular cell or tissue [46,61-63]. This broad specificity for protein substrates has proven useful for biochemical analyses of PIMT. A variety of commercially-available, inexpensive proteins, including most notably ovalbumin and gelatin, have been useful for monitoring the purification of PIMT activities and characterizing its enzymatic properties [6,31].

The broad specificity demonstrated by PIMT is readily explained by the unusual nature of protein D-Asp and L-isoAsp residues, which arise spontaneously as proteins age. Because all Asn and Asp residues in a protein are theoretically subject to the kinds of spontaneous damage that give rise to D-Asp and L-isoAsp residues (Fig. 2), cells would be expected to contain many different methyl-accepting proteins. Detailed studies with individual proteins indicate, however, that structural features of a protein strongly influence the actual rate of protein isomerization. In general, isoAsp residues arise at higher rates in flexible regions of polypeptides [64-66], most probably because of steric difficulties associated with succinimide formation in more structured regions of polypeptides [67]. In this respect, methyl-accepting sites have been identified near the N-termini of several proteins, including, serine hydroxymethyltransferase [68], α-globin [69] and protein kinase A [70]. Calmodulin (CaM) has provided a particularly useful model for exploring the effects of protein conformation on the generation of isoAsp residues, because of the profound conformational changes associated with Ca^{2+}binding. In the presence of Ca^{2+}, CaM is highly structured and demonstrates very low methyl-accepting activity at sites near the N-terminus and in the flexible central helix [71]. In the absence of Ca^{2+}, CaM is very flexible and isoAsp residues form rapidly under physiological conditions at multiple sites in the protein, but most significantly in the third and fourth EF hands responsible for high affinity Ca^{2+}binding [64,65,72]. Methyl-accepting sites in the EF-hands are derived from both Asn and Asp residues, altering CaM's Ca^{2+}binding properties [73] and contributing to a severe reduction in enzymatic activity [72].

Additional evidence for the formation of protein isoAsp residues on a physiologically significant time scale has come from the biotechnology industry. In a growing number of cases, the appearance of an isoAsp residue is reported to adversely affect the stability of a recombinant protein designed for therapeutic use. Examples of recombinant proteins negatively affected by isoAsp formation include human growth hormone [74], epidermal growth factor [75,76], interleukin-1β [77], the thrombin inhibitor hirudin [78], tissue plasminogen activator [79], stem cell factor [80], and recombinant antibodies [81] [82]. Sequence analyses indicate that isoAsp residues do not arise randomly in protein sequences. Instead, there are clearly hot spots for isoAsp generation in the sequences. Comparison of the primary sequences surrounding isoAsp residues reveals a strong tendency for a glycine residue to be situated at the *N+1* position, consistent with an increased propensity for succinimide formation at these sites [50,83,84].

Protein methyl-accepting activity has been widely used to assess the isoAsp content of proteins and has even been developed into a commercial kit, but the presence of an isoAsp residue is not sufficient to constitute a methyl-accepting site. This has been shown most clearly with a naturally occurring isoAsp variant of bovine ribonuclease that has been purified to homogeneity and studied in considerable detail. The isoAsp residue, which arises from deamidation of Asn-67 [85,86], is located in the crystal structure in a highly structured surface loop [87] where it is refractory to modification by PIMT [88]. Structural constraints in the intact protein are responsible for this poor methyl-accepting activity, because a tryptic fragment containing isoAsp-67 is readily methylated by PIMT [88]. This results provides a cautionary note to investigators using protein methyl-accepting activity to assess the isoAsp content of proteins [89], which is difficult to measure with chemical methods.

E. PIMT-catalyzed methylation initiates the repair of polypeptide backbones

The appearance of an isoAsp residue in a protein introduces an additional carbon into the polypeptide backbone, profoundly affecting local structure. Biochemical evidence indicates that PIMT-catalyzed methylation initiates the repair of the polypeptide backbone. The mechanism underlying protein repair has been studied extensively with peptide substrates [49,88,90,91]. As discussed in Section IIB, methyl esters formed by PIMT spontaneously hydrolyze to form an internal succinimide (Fig. 1), which hydrolyzes to a mixture of isoAsp and Asp peptides (Fig. 2). L-Asp products of the hydrolysis can be considered repaired, in that the normal polypeptide backbone configuration is restored, while L-isoAsp hydrolysis products can be methylated again by PIMT. Thus, by multiple cycles of methylation and demethylation, isoAsp residues in synthetic peptide substrates are converted to L-Asp residues in high yields [88,90,91]. A minor fraction of synthetic peptide substrates, representing 10-20% of the initial substrate, is not repaired following incubation by PIMT, because the succinimide has racemized. The D-isoAsp products generated by succinimide hydrolysis are considered irreparable, while D-Asp products are generally poor substrates for PIMT (Section II.B.).

Because of their greater structural complexity, it has been more difficult to demonstrate the structural repair of isoaspartyl-containing proteins by PIMT. Fortunately, several proteins can be converted to isomerized forms in sufficient quantity to provide useful models for potential repair, and PIMT has been shown to restore impressive amounts of enzymatic activity to isoAsp-containing forms of both CaM [72] and the bacterial HPr phosphocarrier protein [92]. Significantly, PIMT was unable to completely restore activity to either CaM or HPr even after exhaustive methylation, probably because irreparable isomers were generated during succinimide hydrolysis.

The inefficiency of the protein repair pathway outlined in Fig. 2 is striking in that it lacks the stereochemical specificity and efficiency characteristic of most cellular processes. In addition to the fact that the products of the processing pathway initiated by PIMT are heterogeneous, no mechanism has been identified for converting isoAsp residues to Asn residues, even though Asn are the likely origin of many isoAsp residues in proteins. It is an open, but important, question whether other proteins participate in and help to guide the repair process *in vivo*.

III. CRYSTALLOGRAPHIC STUDIES OF PIMTs

A. Class I methyltransferases with a signature topology

The first crystal structure of a PIMT was solved by Skinner *et al.* [18] for the enzyme from *Thermatoga maritima*, a hyperthermophilic eubacterium. The structure predicted from their model is a modified Rossman fold consisting of a central seven-stranded β-sheet flanked by an α-helix on either side. Similar αβα structures have been subsequently observed in the orthologous PIMTs from *Pyrococcus furiosus* [19], *Sulfolobus tokodaii* [93], *Drosophila melanogaster* [20], and humans [43,94], consistent with strong evolutionary conservation. Fig. 3 shows the topology diagram and three-dimensional structure of fly PIMT as an example. PIMTs consist of a PIMT-specific N-terminal subdomain (lightly shaded in Fig. 3) and a central methyltransferase domain (dark shading) that is also common to class I AdoMet-dependent methyltransferases with other substrate specificities [95,96]. The β-sheet contains seven strands, one of which is antiparallel to the other six strands. In PIMTs, the central sheet has the topological arrangement of 3↑2↑1↑4↑5↑6↓7↑, which is a defining characteristic of PIMTs. By contrast, the strands in the central β-sheets in other Class I AdoMet-dependent MTases are arranged in a 3↑2↑1↑4↑5↑7↓6↑ topology [95,96]. As might be expected, strongly conserved amino acid side chains in the conserved methyltransferase domain are involved in binding AdoMet and AdoHcy. Fig. 3 shows a molecule of AdoHcy bound to PIMT, with an asterisk indicating the position of the sulfur atom.

B. Substrate binding and catalysis

Two highly conserved regions that are unique to PIMTs form the sides of the substrate binding cleft and provide functional groups important in binding isoAsp substrates [18,19]. One side of the substrate binding cleft is contributed by helix α4 and a small β hairpin, consisting of strands βa and βb, located at the end of the N-terminal subdomain. The short β hairpin extends over the AdoHcy binding cleft in the crystal structure (open arrow in Fig. 3). The other side of the substrate binding cleft consists of a short hydrophobic coil near the C-terminus, extending from the end of strand β7 in the central sheet. From a co-crystal of *P. furiosus* PIMT, adenosine, and an isoAsp-containing hexapeptide substrate, VYP(iso)DHA, it is possible to identify interactions important in substrate binding [19]. The crystallographic model shows that peptides bind to the enzyme in an extended conformation suggestive of local denaturation. Many stabilizing interactions arise from hydrogen bonds between the substrate and the polypeptide backbone of PIMT. The carboxylate group of the bound isoaspartyl residue forms hydrogen bonds with residues on both sides of the substrate binding cleft. One hydrogen bond involves an invariant serine in the N-terminal subdomain. In fly PIMT, this Ser-60 is located in a short loop connecting the βb strand with the α4 helix (Fig. 3). Mutagenesis has confirmed the importance of Ser-60 in catalysis. Substitution of Ser-60 with either Thr or Gln progressively reduces

catalytic activity, and a S60A mutant is completely inactive [20]. The isoAsp carboxylate group also forms hydrogen bonds with the backbone nitrogen of Val-219 in the conserved C-terminal sequence on the other side of the substrate binding cleft.

The orientation of the C-terminus in fly PIMT is rotated approximately 90° relative to the C-termini in other PIMT crystals. Effectively, this displacement generates a more open conformation in the substrate binding cleft that allows greater solvent access to AdoHcy. In the homologous structures from *P. furiosus* and humans, the C-terminus folds back over the bound AdoHcy, limiting solvent access to 1.4% and 0.5% of the respective structures. By contrast, 9% of the AdoHcy in the *D. melanogaster* structure is accessible to solvent. The differences between PIMT structures suggests that PIMT alternates between open and closed conformations. These conformational changes at the C-terminus could be important in the exchange of substrates and products [97]. The open conformation would facilitate the exchange of AdoHcy and AdoMet, whereas the closed conformation would facilitate the binding of isoAsp-containing substrates, while dehydrating the active site. Considering all of the structural data together, including the relative placements of bound cofactors and peptide substrates, the data are consistent with an ordered sequential mechanism for PIMT-catalyzed reactions. The structural data predicts the AdoMet binding would precede peptide binding and that methylated peptide release would precede AdoHcy release (see also Section II.A.).

C. Additional C-terminal domains in PIMTs from some hyperthermophiles

The αβα domain described above is common to all PIMTs and represents the entire structures of PIMTs from humans, flies, and *P. furiosus*. Additional species-specific C-terminal domains are present, however, in *T. maritima* and *S. tokodaii* PIMTs. *T. maritima* PIMT contains an additional irregularly structured C-terminal domain consisting of 103 amino acids [18]. The structure of the domain is unlike other known folds and the sequence appears to be unique to *T. maritima*. Nonetheless, the domain is required for enzymatic activity, because a recombinant protein lacking the C-terminal domain displays much lower levels of activity that the full-length protein [98].

An entirely different C-terminal domain and domain structure has been identified in PIMT from the archaeon, *S. tokodaii*. The additional C-terminal domain in *S. tokodaii* PIMT consists of approximately 30 amino acids arranged in a single loop and a short α-helix [93]. The C-terminal domain in *S. tokodaii* PIMT is postulated to promote oligomerization. In the crystal structure, C-terminal domains interact with one another to form a coiled coil structure at the center of a hexamer. The hexamer is thought to be assembled from three homodimers held together by disulfide linkages involving Cys-149, a residue that is notably lacking in orthologous PIMT sequences. Cys-149 is located in a surface helix connecting strands of the central sheet and is required for dimerization. Mutagenesis indicates that the C-terminal domains are not required for hexamer formation, but they help to stabilize the hexamer, as shown by differential scanning calorimetry. It is not clear if oligomerization is required for PIMT activity, because the enzymatic properties of *S. tokodaii* PIMT have not been studied. Interestingly, the *S. tokodaii* genome contains a coding sequence for another PIMT without the additional C-terminal domain, so it is not clear if both forms are physiologically important.

IV. PHYLOGENETIC DISTRIBUTION OF PIMT ACTIVITIES DEDUCED FROM WHOLE GENOME SEQUENCES

As the data continues to accumulate from whole-genome sequencing projects, it is clear PIMT orthologs are distributed throughout all phylogenetic kingdoms and domains. PIMT can therefore be classified as an ancient enzyme, but not as one whose activity is essential for life, since orthologs are missing is some species. Information about PIMT gene names, locus identification and database accession numbers is give in Table I for the PIMTs that have been studied in detail. More information about the evolution of PIMTs is available for prokaryotic sequences than for eukaryotic sequences, because of the larger number of completed genome sequences. Prokaryotic orthologs of PIMT, known as *pcm* sequences, form the Cluster of Orthologous Group (COG) 2518 [99]. The phylogenetic distribution of *pcm* sequences within the prokaryotes is completely unique and unlike that of any other COG, so PIMT function appears to have evolved independently of other metabolic enzymes. Supporting this conclusion, *pcm* sequences are rarely observed in gene fusions.

Within the prokaryotes, *pcm* orthologs are much more widely distributed in the archaea than in the eubacteria, perhaps reflecting the ability of archaea to live under extreme conditions that might be expected to cause structural damage to proteins. Multiple *pcm* genes are present in the genomes of some archaea, such as *Archaeoglobus fulgidis* and *S. tokodaii*, although it is not clear if both genes encode functional proteins. *A. fulgidis* has multiple gene duplications, including *pcm*, which show differential codon usage and are therefore thought to have arisen from lateral gene transfer [100]. Lateral gene transfer of an archaeal *pcm* sequence is also the most likely origin of the *pcm* gene in *T. maritima*, a hyperthermophilic eubacterium [101]. Sequence comparisons using the BLAST algorithm [102] indicate that the closest orthologs to the *T. maritima* sequence are those from *A. fulgidis* and other archaea, followed by orthologs from the eubacteria.

PIMT-encoding sequences are less widely distributed within the eubacteria than in other domains, and few generalities can be made about their distribution within the eubacteria. Potential PIMT-encoding sequences are present in about half of the eubacterial genomes sequences to date, including *E. coli*, *Rhizobium meliloti* and many opportunistic pathogens. Orthologs of *pcm* are generally not detected in mycoplasma and other obligate pathogens, such as *Treponema* and *Borrelia*. The absence of PIMT in obligate pathogens is not surprising, since these organisms rely on their hosts for many essential functions. One exception is the ulcer bacterium, *Helicobacter pylori* , an obligate parasite of the human gut. *H. pylori* may have retained PIMT function because its ecological niche is an acidic environment containing high concentrations of urea, which could reduce the structural stability of its proteins.

PIMT-encoding sequences are nearly universally distributed in the eukaryotes. The only exceptions identified to date are *Encephalitozoon cuniculi*, a parasitic microsporidium, and yeasts from the order *Saccharomyces*. PIMT orthologs are readily identified in genomes from all other orders of yeast, suggesting that the ancestral yeast PIMT-coding sequence was lost before the genome duplication and subsequent specialization that accompanied the evolution of the Saccharomycetes [103,104]. PIMT-encoding sequences have been maintained in the genomes of all multicellular eukaryotes studied to date. Higher plants are unique in possessing duplicated genes that appear capable of encoding PIMT activities (Section VII.B.). Animal genomes contain a single copy of a PIMT-encoding sequence, but alternative transcript splicing is used in mammalian species to generate multiple isozymes (Section VII.A.).

Figure 4 shows a sequence comparison of the PIMT-encoding sequences from several model organisms and other species in which PIMTs have been studied in detail. The phylogenetic tree derived from the alignment is shown in Fig. 5. The most highly conserved regions of the PIMT sequence include a core methyltransferase fold common to many AdoMet-dependent methyltransferases [96] and additional PIMT-specific regions implicated in the binding of isoaspartyl substrates [18,19].

V. BIOCHEMISTRY OF PROTEIN ISOASPARTYL METHYLATION *IN VIVO*

The analysis of protein isoaspartyl methylation reactions in intact cells is a complex undertaking, due to the poor permeability of cells to AdoMet as well as the many different kinds of methylated molecules in cells. Consequently, the most complete information about isoAsp methylation in intact cells has been obtained from a few cell types, such as human erythrocytes and *Xenopus* oocytes, which offer unique advantages for studies of protein isoAsp methylation.

A. Human erythrocytes

Erythrocytes were a fortuitous choice for methylation studies for several reasons. First, the incorporation of methyl groups into protein methyl esters from L-[methyl-^{3}H]methionine is not obscured by the incorporation of radioactivity into newly synthesized proteins or methylated nucleic acids. Secondly, PIMT is the major methyltransferase in erythrocyte cytoplasm [28,55], ensuring that a large fraction of the radioactivity is incorporated into protein [^{3}H]methyl esters [47]. In addition, the stoichiometries of individual methylation reactions can be calculated because the biochemical composition of the red cell membrane had already been well-defined [105]. Many of the first studies that established the characteristics of PIMT-catalyzed reactions were carried out in Steve Clarke's laboratory at about the same time that his group identified the unusual nature of PIMT substrates.

1. Characteristics of protein isoAsp methylation in intact cells

A heterogeneous group of endogenous methyl-accepting proteins is observed when human erythrocytes are incubated with high concentrations of L- [methyl-^{3}H]methionine, the permeable precursor to [^{3}H]AdoMet in erythrocytes [106]. Both membrane and cytosolic proteins are methylated by PIMT [47,51,52,107]. Figure 6 shows a stained gel (left) and fluorogram (right) of cytosolic (lane A) and membrane (lane B) proteins obtained from a typical labeling experiment [108]. The membranes (lane B) in this experiment are "pink ghosts," which retain some of the loosely associated hemoglobin (Hb). This membrane-bound Hb can be released from the membrane with either 100 mM NaCl (lane C) or a nonionic detergent (lane D). In the cytosolic fraction, protein methyl esters are readily detected with both α-globin and β-globin and with an unidentified 35,000 kDa protein. Cytosolic Hb has a very low methyl-accepting activity, but its sheer predominance obscures the detection other cytosolic methyl-accepting proteins, which include CaM [109,110]and many other proteins [47]. In the membrane fraction (lane B), both integral and peripheral proteins have been identified as methyl-acceptors. Some of the highest levels of methyl-accepting activity are associated with the cytoskeletal proteins, ankyrin and band 4.1 [51,52]. Band 3, the anion transporter protein, is also methylated in intact cells [111,112]. The heavy exposure of the fluorogram in Fig. 6 makes it difficult to resolve Band 3 methylation in the membrane fraction (lane B), but this is apparent in the detergent extract of membranes (land D). Proteolytic mapping indicates that the physiological methylation sites in Band 3 are distributed throughout its sequence [111]. A number of other

unidentified methyl-accepting accepting proteins are also detected in the membrane fraction. Interestingly, the small fraction of Hb that associates with membranes is more highly methylated than cytosolic Hb (see below).

The calculated stoichiometries for individual methylation reactions vary over several orders of magnitude, but all of the reactions are markedly substoichiometric. Bands 2.1 (ankyrin) and 4.1 represent some of the most active methyl-accepting proteins in red cells, yet only about 1% of Band 4.1 polypeptides are methylated at steady state [52]. Methylation stoichiometries are much lower for other erythrocyte proteins. Only one in every thousand Band 3 molecules and one in every 300,000 Hb molecules is estimated to be methylated at steady state [47]. These low stoichiometries are partly explained by the unusual nature of the methylated residues. The spontaneous processes responsible for the generation of D-Asp and L-isoAsp residues (Fig. 2) are relatively slow, and isomerized variants would be expected to comprise only a small fraction of any one polypeptide.

Another factor underlying the low stoichiometries of methylation detected in intact cells is the efficient metabolism of isoAsp residues by PIMT. Biosynthetic labeling studies indicate that erythrocyte protein methyl esters are metabolically labile [47,51]. When cells are incubated continuously with L-[methyl-^{3}H]methionine, the rate of [^{3}H]methanol production from ester turnover is close to an order or magnitude higher than the rate of radioactive label incorporation into protein methyl esters, indicating that the average life time of a protein methyl ester is very short. Pulse-chase analysis confirms that individual protein methyl esters turn over with half-times ranging from minutes to hours.

Other lines of evidence also indicate that that isoAsp-containing proteins are maintained at very low levels in erythrocytes due to their active metabolism by PIMT [47,113]. When erythrocyte membranes are used as the substrate for PIMT and [^{3}H]AdoMet *in vitro*, methyl groups are not incorporated into the physiological methyl-acceptors unless the cells had been preincubated with methylation inhibitors for several hours prior to membrane isolation [55]. In addition, the methyl-accepting activity of erythrocytes in PIMT-knockout mice is significantly higher than that of normal mice [23,114].

2. Age-dependent increases in protein methylation

The protein repair process may become less efficient as cells age, and this decline may contribute to the higher levels of methyl-accepting activity observed in older cells [52,113,115]. Circulating erythrocytes have a narrowly-defined lifespan of 120 days. As erythrocytes age, membrane blebbing reduces the surface area and disrupts the normal process of membrane transport, causing cells to become more dense. This property can be exploited to separate cells into rough age fractions by centrifugation on density gradients. Fractions enriched in older cells have 3-4 times higher levels of methylated proteins than younger cells in biosynthetic labeling experiments [52,113,115]. The spectrum of methylated proteins does not change with age, however, with individual proteins demonstrating fairly uniform increases in methyl-accepting activity. At the same time that the number of protein methyl esters is increasing in older cells, the specific activity of PIMT is declining, because erythrocytes are unable to synthesize replacements for denatured enzymes *de novo*. Thus, PIMT should become increasingly saturated with substrates in older erythrocytes, which could potentially lead to less effective protein repair.

The reduced resistance of older erythrocytes to oxidative damage may contribute to higher levels of methyl-accepting proteins in older cells. It is well-established that levels of

oxidant defense enzymes decline in older erythrocytes. Failure to resist oxidative damage is manifest in Heinz bodies, aggregates of oxidatively-damaged Hb that associate with the membrane and are hypothesized to play a role in the removal of aged blood cells from the circulation [116]. As shown in Fig. 6, the 1-2% of Hb that associates with erythrocyte membranes is also significantly enriched in methyl-accepting sites. The specific methyl-accepting activity of membrane Hb is about 10-fold higher than that of cytosolic Hb [108]. Reactive oxygen species (ROS) may contribute to this higher methyl-accepting activity, because even a short incubation of Hb with acetylphenylhydrazine produces a dramatic increase in Hb's methyl-accepting capacity . [108]. Other experiments with intact erythrocytes have also implicated ROS in the generation of methyl-accepting sites. Erythrocytes exposed to the ROS generators, *t*-butyl peroxide or hydrogen peroxide, have elevated rates of methylation relative to control cells [117]. Even larger increases in methyl-accepting activity are observed in cells with an inherited deficiency in glucose-6-phosphate dehydrogenase that sensitizes erythrocytes to oxidative stress [118]. The mechanism by which ROS increase methyl-accepting sites is unclear. Chemically, ROS are not expected to react directly with Asn or Asp residues to generate isoAsp residues. The production of isoAsp residues is more likely to be a secondary effect of chemical damage to other amino acids, which alters the three-dimensional structure of the protein.

B. Nonerythroid mammalian cells

Biosynthetic labeling studies with L- [methyl-^{3}H]methionine have established that the basic features of carboxyl methylation reactions are similar in erythrocytes and nonerythroid cells, although fewer biochemical details are available in nonerythroid cells. In these experiments, the base lability of methyl esters has been used to differentiate carboxyl methylation reactions from the background of incorporation of radioactivity from L-[methyl-^{3}H]methionine into polypeptide backbones and into base-stable linkages with other amino acids. In both brain tissue slices [119,120] and cultured cells [121] [122], the substrates for carboxyl methylation in are heterogeneous. In platelets, methyl esters are associated with more than thirty different proteins resolved by SDS-PAGE [121]. Protein methyl esters in platelets are metabolically labile, as shown by the rapid and continuing evolution of [^{3}H]methanol during culture. Similarly, a diverse set of methyl-accepting proteins has been identified in neuroblastoma cells by SDS-PAGE [122]. Individual substrates for PIMT have not been identified in nonerythroid cells, because additional purification steps would be required to separate methyl-accepting proteins from other proteins with similar electrophoretic mobilities. Another complicating factor arises from the fact that simple base treatment does not allow one to distinguish protein Asp methyl esters from esters formed by other enzymes, such as those formed during the C-terminal processing of CAAX-box proteins [123] and C-terminal esters in protein phosphatase 2A [124].

C. *Xenopus* oocytes

Erythrocytes are highly specialized cells that have lost their ability to synthesize new protein as well as their intracellular organelles. To understand isoAsp methylation reactions in more typical cells, protein isoAsp methylation has been analyzed in *Xenopus laevis* oocytes, which offer some unique advantages for these studies. Fully-grown oocytes are roughly 1.2 mm in diameter and are easily microinjected with impermeable molecules and macromolecules. Oocytes have an exceptionally large nucleus, the germinal vesicle, that can be manually isolated with minimal contamination [125], and other organelles can be purified by standard differential

centrifugation techniques. Finally, oocytes have been extensively used to study quantitative aspects of protein synthesis and translational regulation [126], making it possible to appreciate the importance of protein carboxyl methylation in the context of overall protein metabolism.

1. Kinetic analysis of protein methylation

Xenopus oocytes can be easily injected with impermeable substrates for PIMT, including [^{3}H]AdoMet, isoAsp-containing peptides and protein substrates. Direct injection of [^{3}H]AdoMet allows the investigator to monitor methylation reactions without the background incorporation into proteins encountered using L-[^{3}H-methyl] methionine as the metabolic precursor. Kinetic analyses of [^{3}H]AdoMet utilization in oocytes established that protein carboxyl methylation reactions represent approximately one-third of the oocyte AdoMet utilization [127]. The calculated rate of protein carboxylmethylation is very similar to that of protein synthesis for both the aggregate of oocyte proteins [127] and for an individual oocyte protein, CaM [128]. Together, the results indicate that PIMT-catalyzed reactions are metabolically significant in oocytes, even though cells are able to replace damaged proteins by *de novo* synthesis.

2. Subcellular compartmentation of PIMT and methyl-accepting proteins

As in other cells, the substrates for PIMT in oocytes are heterogeneous [127]. Diverse groups of methyl-accepting proteins are found in both the nucleus and cytoplasm, which are quite distinct from each other. Methylation of nuclear proteins could occur in either the nucleus or cytoplasm, because the specific activities of PIMT in the nucleus and cytoplasm are identical. It is likely that PIMT diffuses freely through the nuclear pores, because it is smaller than the exclusion limit for nuclear pores in oocytes [129] and it lacks a nuclear localization signal (NLS). Unfortunately, it has not been possible to identify the individual methyl-accepting proteins in oocytes, because the proteins cannot be labeled to a sufficiently high specific activity with a single injection of [^{3}H]AdoMet.

Some isoAsp-containing proteins in oocytes are not methylated by PIMT because they are sequestered in cytoplasmic organelles. The largest organelles in oocytes are yolk platelets, which undergo a massive increase in size and number during oogenesis [125]. The process of oogenesis occurs over several months, during which time, yolk protein is accumulated by receptor-mediated endocytosis and processed into semi-crystalline arrays within yolk platelets, where they are inaccessible to cytoplasmic PIMT. Figure 7 shows an experiment in which yolk platelets were isolated from oocytes ranging in size from 0.6 to 1.2 mm in diameter and used as substrates for PIMT *in vitro* [62]. Both of the major yolk proteins, phosvitin and lipovitellin, are methylated by PIMT as well as other minor yolk proteins. The specific methyl-accepting of yolk proteins increases about four-fold between the 0.6 mm (lane 1) and 1.2 mm (lane 6) oocyte. The data in Fig. 7 seriously underestimate the real rate at which methyl-accepting sites accumulate in yolk proteins, however, because newly-synthesized protein is continually added to yolk plates by endocytosis. Nonetheless, the data indicate that methyl-accepting sites can accumulate rapidly in a physiological compartment where PIMT is not present. By contrast, the specific methyl-accepting activity of cytosolic proteins does not increase over the same physiological time span, consistent with a repair function for PIMT in oocytes.

3. Alternative processing of isoAsp proteins

The large size of *Xenopus* oocytes has facilitated their use as a microinjection model for analyzing the metabolism of defined isoAsp-containing substrates for PIMT *in vivo*. The oocyte

PIMT is not saturated with endogenous substrates and demonstrates considerable reserve capacity in microinjection experiments. Rates of methylation increase by up to an order of magnitude following the injection of isoAsp peptides or proteins [128,130,131]. At the same time, however, a significant fraction of injected isoAsp-containing peptide and protein substrates are proteolytically degraded, suggesting that carboxyl methylation and proteolysis represent alternative pathways for the metabolism of isoAsp-containing proteins. This last conclusion was strengthened by the demonstration that an isomerized variant of CaM was protected from proteolytic digestion by incubation with PIMT and AdoMet prior to injection [131]. The proteolytic activity responsible for the degradation of isomerized CaM was identified as the proteasome, based on its sensitivity to lactacystin [73]. Interestingly, degradation of isomerized CaM does not appear to require ubiquitination, because purified proteasomes are able to degrade isomerized CaM directly.

VI. ACCUMULATION OF ISOASPARTYL AND RACEMIZED PROTEINS DURING AGING

An implication of the repair hypothesis is that isoAsp and D-aspartyl sequences will accumulate over time in proteins sequences that cannot be modified by PIMT *in vivo*. Accordingly, one would expect that methyl-accepting sites would accumulate rapidly in extracellular protein sequences that are not normally accessible to PIMT. Only small amounts of PIMT have been detected in plasma, presumably originating in damaged cells [132]. Methyl-accepting sites in intracellular proteins would also be expected to increase if PIMT activities became unable to cope with the burden of damaged proteins, due either to aging or enhanced rates of protein damage. Considerable experimental evidence supports both of these predictions, as discussed below. Although correlative, the data suggest that the absence of PIMT-catalyzed repair could have pathological implications for age-related diseases.

A. Collagen

Collagen is the major extracellular matrix protein in vertebrates and might therefore be expected to accumulate isoAsp residues over time. Interestingly, the first description of isoAsp generation via a succinimide intermediate involved the α1 chain of collagen I [133]. More recent experiments point to a complex chemistry that generates potential substrates for PIMT. The Asp residue in the C-terminal telopeptide $A_{1209}HDGGR_{1214}$ of collagen α1 [134] is derivatized to a mixture of D-Asp and (D/L)-isoAsp variants (Fig. 2), which can be distinguished using isoform-specific antibodies. Due to the normal turnover of bone tissue, the quantities of the various peptides isoforms can be monitored in urine samples obtained from patients. Comparing the isoform profile of individuals ranging in age from 0.2 to 74 years, investigators observed a steady decline in the normal isoform and a corresponding increase in damaged isoforms with donor age [134]. In another experiment, samples obtained directly from cortical bone were found to have higher proportions of isomerized variants than samples from trabecular bone, which turns over more slowly [135], suggesting that older cells are the source of the isomerized variants. Based on these results, it has been suggested that the collagen isoform profile could provide a useful clinical indicator of bone growth and turnover.

A rat model has been used to assess the functional consequences of isoAsp formation in collagen. The specific methyl-accepting activity of rat tail collagen increases dramatically between three and twenty weeks of age [136]. Collagen isolated from the tails of 20-week old

rats had about one-third the ability of collagen from 3-week old rats to support the motility of fibroblasts in a cell migration assay. Exhaustive methylation of the 20-week collagen with PIMT prior to the migration assay restored much of the lost activity, suggesting that isoaspartyl formation and/or racemization can disrupt the extracellular matrix and interfere with normal remodeling processes.

B. Crystallins

The mammalian eye lens has provided a useful physiological model for studying age-related changes in protein repair. The oldest fiber cells at the center of lens nucleus are as old as the organism, and they are surrounded by layers of progressively younger fiber cells in the lens cortex. The predominant lens proteins are the crystallins, a group of developmentally-regulated proteins responsible for lens transparency. Crystallins are remarkably stable proteins that accumulate over time a large number of chemical modifications caused by oxidation, crosslinking, deamidation, racemization and other processes [137]. At least some of the D-Asp and L-isoAsp residues that have been detected chemically in crystallins are expected to be substrates for PIMT *in vivo*, because PIMT catalyzes the methylation of all crystallin classes *in vitro* [138,139]. Detailed chemical information is available for one potential methyl-accepting site, Asp-151 of αA crystallin [140], which is highly prone to both racemization and isomerization. In this experiment, tryptic peptides corresponding to residues 146-157 were isolated from the lens tissues of newborns and adults aged 30, 60 and 80 years, and the proportion of each isoform was determined using standard chemical methods. The investigators found that the fraction of normal peptide decreased steadily over time, representing only 42% of the total at 80 years, while the D-isoAsp isomer showed the greatest increase with age, representing 35% of the total at 80 years. The D-isoAsp isoform was found to be particularly concentrated in the lens nucleus [141] by immunochemical localization with an isoform-specific antibody.

The pattern of crystallin isoforms and the changes observed during aging are in good agreement with transformations outlined in Fig. 2, if protein repair becomes less efficient during aging. Measurements of PIMT and endogenous methyl-accepting proteins in lens tissues are, in fact, consistent with this possibility. In a comparative study of human lenses varying in age from 3 to 89 years, a negative correlation was observed between PIMT and lens protein methyl-accepting activities. PIMT levels declined steadily with age, while endogenous methyl-accepting activity showed the opposite trend [138,139]. At any age, PIMT specific activity was significantly lower in the lens nucleus than in the cortex, while the opposite relationship was observed for methyl-accepting activity. The functional consequences of declining PIMT are unclear. Exceptionally low levels of PIMT have been observed in cataractous lenses [139,142], but any relationship with cataract formation is strictly correlative.

C. Amyloid

Extracellular L-isoAsp residues and D-Asp residues have also been identified in the amyloid deposits from patients with Alzheimer's disease [143-147] [148] and may play a role in the formation of these peptide aggregates. The major component of amyloid plaques is the β-amyloid peptide Aβ derived from the processing of the Alzheimer's precursor protein (APP). Various kinds of evidence suggest that racemization and isomerization occur at all three Asp residues in Aβ, located at positions 1, 7 and 23. Structural analysis of the Aβ peptides isolated from neuritic plaques and the parenchymal vasculature reveals mixtures of racemized and

isomerized Asp residues at both positions 1 and 7 [143,144,146], suggesting that the residues arise through the succinimide chemistry shown in Fig. 2. The presence of the Aβ(isoAsp7) variant in both neuritic plaques and the cerebrovascular amyloid has been confirmed cytologically using isoform-specific antibodies [147,148] [149]. Isomerized variants of Asp-23 have not been detected biochemically in amyloid, but this could reflect the fact that only a portion of the amyloid deposits can be solubilized sufficiently for biochemical characterization. Immunochemical experiments with peptide-specific antibodies indicate that Aβ(isoAsp23) is present in both neuritic plaques and the brain microvasculature [148,149]. Staining is particularly intense in the cores of amyloid deposits, suggesting that Asp-23 variants of Aβ are highly insoluble.

Potential roles for isomerized and racemized Asp residues in amyloid formation have been addressed using synthetic peptides corresponding to the various Aβ variants. Modifications to Asp-23 appear to be the most deleterious. Synthetic Aβ1-42(isoAsp23) showed a much greater tendency to aggregate than either Aβ1-42(isoAsp7) or normal Aβ1-42. Aggregation in this experiment was also highly correlated with neurotoxicity with cultured neurons [150]. Racemization of Asp23may also increase its aggregation properties. In a second study, the D-Asp23 variant of Aβ1-35 was much more prone to aggregation and fibril formation than either Aβ1-35(D-Asp7) or unmodified Aβ1-35 [145]. Interestingly, Asp-23 is the site of the Iowa D23N mutation associated with inherited early onset dementia, and the substitution of an Asn residue for Asp-27 increases the propensity of synthetic Aβ peptides to undergo isomerization [149]. It is not clear that PIMT could play a role in preventing Alzheimer's disease, since the isomerization reactions that promote amyloid formation most likely occur in the mature protein, when the relevant amino acids are not accessible to PIMT.

VII. Physiological studies of PIMT function

PIMT activities are widely distributed across phylogenetic domains (Section IV.), suggesting that PIMT plays a fundamental role in cellular protein metabolism. Nonetheless, cellular requirements for PIMT activity may vary widely. Significant variations in PIMT concentrations have been observed between different cell types within an organism and in a single cell type responding to physiological and environmental stimuli. This section reviews our current understanding of PIMT regulation in several species. The regulatory elements responsible for physiological changes in PIMT activities remain largely unknown. For the most part, PIMT appears to be constitutively produced in cells, although the data suggest that PIMT concentrations may increase in response to the burden of structurally damaged proteins.

A. Mammals

1. Cellular and subcellular distribution

PIMT is ubiquitously expressed in mammalian tissues, although PIMT activities vary widely among tissues and between cell types. In rodents, PIMT specific activities are particularly high in brain and testis, while much lower levels of enzyme activity are present in liver and kidney [25]. Immunohistochemical analyzes of brain slices indicate that PIMT concentrations are significantly higher in neuronal cells than in glial cells, although oligodendrocytes and astroglial cells both stain positively with anti-PIMT antisera [151-153]. Biochemical measurements are consistent with the immunochemical results, because PIMT specific activities in the developing rat brain parallel its population by mature neurons [152]. PIMT activity may be important for neuronal function. Brain tissue contains many different substrates for PIMT,

including important neuronal proteins such as synapsin [57,154,155] and tubulin [156]. The loss of PIMT function in transgenic mice (see below) results in neuronal death and the development of fatal epileptic seizures, while overexpression of PIMT in primary cortical neurons is able to protect cells against Bax-induced apoptosis [157].

Within the testis, PIMT activities are present at significantly higher levels in germ cells than in somatic cells. Rodent testes depleted of germ cells as a consequence of either mutation or X-ray irradiation have reduced levels of PIMT [158,159], and during prepuberal development, PIMT specific activities increase in parallel with the population of the seminiferous tubules with spermatogenic cells and mature sperm [158-161]. The changes in PIMT activity during spermatogenesis have been followed using staged cell populations isolated from dissociated seminiferous tubules. [158,159]. These experiments indicate that PIMT is upregulated during the final haploid phase of spermatogenesis. It is tempting to speculate that high concentrations of PIMT in mature sperm may have important implications for fertility, because mature sperm are translationally inactive cells that may be stored in the epididymis and vas deferens for several weeks prior to ejaculation. Sperm isolated from both the caput (proximal) and caudal (distal) epididymides have been shown to possess both PIMT and methyl-accepting proteins [162,163]. Unfortunately, it has not been possible to directly address the functional important of PIMT to male fertility in transgenic mice, because PIMT-deficient mice die before they are fully fertile [24].

2. Gene structure and transcriptional regulation

A single gene encoding PIMT is present in mammalian genomes. The first cloned *Pcmt* gene was isolated from a rat brain cDNA library by Sato *et al*. [15], who also reported the presence of multiple transcripts in rat brain mRNA. Subsequent experiments established that the mouse *Pcmt1* [16] and human *PCMT1* [164] genes are organized into eight exons that are transcribed and spliced into at least three transcripts of varying lengths [15]. An alternative splice site has been identified in the seventh exon [33,35,165], which is used to produce the coding sequences for isozymes with distinct C-termini (Section II.A.). The 5'-flanking sequences of the mouse [16] and human [164] genes have features that are typical of constitutively expressed genes. Both sequences are GC-rich, with CpG islands that start approximately 400 bp upstream from the transcription start site and extend through exon 1 and intron 1. The methylation status of the CpG islands is not known, but their presence suggests that the genes may be subject to silencing by DNA methylation. The genes lack conventional TATA boxes, and transcription begins at multiple start sites [35,164]. Most of the consensus transcription factor binding sites that can be identified (using stringent criteria) are binding sites for widely-expressed transcription factors, such as Sp1, ETF and AP1. Interestingly, the sequences also contain potential antioxidant [166] and unfolded protein [167] response elements, but there is no evidence that *Pcmt1* transcription responds to these stimuli (O'Connor, unpublished data). A minimal sequence for the mouse promoter has been functionally defined by monitoring reporter gene expression in cultured NIH/3T3 cells [35]. This 407 bp sequence extends from the transcription start sites upstream through the GC-island, including the transcription factor binding sites. Overall, the features of the *Pcmt1* promoter are consistent with the wide expression of PIMT in mammalian tissues. Additional tissue-specific enhancers undoubtedly exist and contribute to the tissue-specific differences in *Pcmt1* regulation, but these factors have not yet been identified.

The regulation of PIMT levels appears to occur primarily at the transcriptional level in mammals, since transcript levels closely parallel measured PIMT specific activities. High levels of *Pcmt1* transcripts are detected in mouse brain and testis, while much lower transcript levels are detected in preparations of liver RNA [16,161]. Brain and testicular tissues also show parallel increases in PIMT enzymatic activities and *Pcmt1* transcript levels during early development, coincident with increased numbers of neurons and spermatogenic cells, respectively [158,161]. The size distribution of *Pcmt1* transcripts is similar in all tissues except the testis. The major testicular transcript is a smaller 900 bp transcript with a unique 3'-untranslated region derived from alternative splicing in intron 7 [24,35]. This transcript is positively regulated during the differentiation of pachytene spermatocytes to round spermatids. It is possible that this unique 3'-untranslated region is important for translational regulation during the late stages of spermiogenesis when the spermatid nucleus has condensed and become transcriptionally inactive. Like the protamine and testis-specific actin mRNAs that are translated in late spermatids [168], *Pcmt* transcripts are distributed in both the polysomal and non-polysomal fractions obtained by ultracentrifugation [24].

3. Functional analysis in transgenic models

Loss of PIMT function in mice produces phenotypes that appear to primarily affect the nervous system. Two different strains of mice generated by homologous recombination demonstrate virtually identical phenotypes [23,114]. PIMT-deficient mice appear fairly normal at birth, although the animals are slightly smaller than wild-type. Histologically, most tissues appear normal and the overall neuroanatomy is normal. Reproducible abnormalities are detected, however, in some pyramidal neurons in layer V of the precentral cortex, granule cells in dentate gyrus, and some astrocytes in the hippocampus [114]. A few weeks after birth, brains of knockout mice become enlarged and brains continue to grow at higher rates than in wild-type mice [114,153]. The underlying biochemical disturbances accompanying these morphological changes may involve alterations in insulin signaling through PI3K/Akt pathway. Western blots and immunochemical data indicate that the insulin and IGF-1 receptors are up-regulated in the knockout hippocampus, and components of the PI3K/Akt signaling pathway are more highly phosphorylated [153].

The biochemical changes in knockout mice become manifest in several behavioral disorders. PIMT-deficient mice fail to mate and they experience epileptic seizures that increase in severity before mice finally die from fatal seizures between 22 and 60 days after birth [23,114,169]. Mutant animals also display exaggerated sensitivity to convulsive agents, reduced memory and altered behaviors in several quantitative assays [170,171]. Electrophysiological measurements with isolated brain slices indicate that synaptic transmission within the hippocampus is abnormal [170], possibly accounting for the behavioral disorders displayed by the mice. Neurons appear to be the cell type most affected by the loss of PIMT function, because the epileptic phenotype is partially rescued by PIMT transgenes in which the neuron-specific *enolase* promoter controls *Pcmt* expression [172]. Rescued mice can live up to five times longer than knockout mice, and they accumulate fewer unmodified isoAsp-containing proteins in their brain tissue. More complete rescue is observed when either of the PIMT isozymes is expressed in knockout mice under the control of the stronger, but less specific, *prion* promoter [155]. The epileptic phenotype is also partially rescued when an adenovirus containing the PIMT1 coding sequence is injected into the ventricles of embyonic mice [37]. In all of the rescue experiments, there is a good correlation between the degree of phenotype rescue and protein repair.

Non-neural tissues appear to be less affected by the loss of PIMT activity, even though methyl-accepting proteins also accumulate in these tissues. No histological differences are apparent in the non-neural tissues of PIMT-deficient mice and no consistent phenotypes have been observed. Potential testicular defects have been examined in considerable detail [24], because of the high PIMT concentrations normally detected in testis (see above). Molecular analyses indicate that spermatogenesis occurs normally in the absence of PIMT activity. Sperm-specific markers appear at the normal times during development and acrosome reaction-competent sperm can be recovered from the epididymides of knockout mice [24]. It has not been possible to perform quantitative analyses on the fertilization-competence of PIMT-deficient sperm, since the animals die from seizures about the same time that they reach sexual maturity. In addition, PIMT-deficient mice fail to mate. Knockout mice rescued with a neuron-specific *enolase:Pcmt* transgene are partially fertile [172], suggesting that behavioral factors contribute to the infertility of knockout mice. Additional experiments are required to exclude direct effects of PIMT depletion on sperm function, because the neuron-specific *enolase* promoter has been reported to be partially active testis [202].

Overall, there is a strong correlation between the severity of mouse phenotypes and the accumulation of unprocessed isoAsp-containing proteins [23,114,172]. As predicted from the scheme in Fig. 2, PIMT-deficient mice also have higher levels of racemized proteins [201], which are also unrepaired. Lack of protein repair may not be sufficient to totally account for the phenotypes observed in PIMT-deficient mice, however, because the ratio of AdoMet to AdoHcy is unexpectedly elevated in the brains of PIMT-deficient mice [173]. AdoMet is known to have pharmacological properties [174] and it is also involved in the synthesis of other neurotransmitters [175], raising the possibility that epigenetic factors contribute to the phenotypes displayed by PIMT-deficient mice.

B. Plants

1. Distribution of PIMT activities in higher plants

Data from several studies have established that PIMT activities are widely distributed among all the major divisions of the plant kingdom [27,40,58,59]. In a comparative study [59], functional PIMT activity was detected in crude extracts prepared from 45 different species, representing 23 families of both seedless and seed-bearing plants. Fairly similar levels of PIMT activity were observed in seeds obtained from coniferous species and from both monocotyledonous and dicotyledonous angiosperms. Overall, lower levels of PIMT activity were detected in extracts prepared from mosses and ferns than in extracts prepared from seed-bearing plants. PIMT activity was not detected in several algal species, but this could reflect the limitations of using enzymatic assays, rather than genomic information, to identify sources of PIMTs. Biochemical measurements are probably best construed as providing only rough estimates of PIMT activity, because some plant extracts contain substances that can interfere with biochemical measurements of PIMT activity [58,59].

In higher plants, PIMT specific activities are often concentrated in seeds. It has been suggested that PIMT activity is important for maintaining the structural integrity of embryonic proteins during long-term storage in the seed [27]. Consistent with this hypothesis, PIMT is positively regulated during seed development [17] and is more concentrated in embryonic tissue than in the endosperm [59]. Depending on the species, seeds can be stored for considerable periods of time, retaining the ability to germinate and produce progeny. An extreme example is the sacred lotus plant, whose seeds can retain the ability to germinate for centuries. PIMT

appears to be very stable in lotus seeds, as shown by the recovery of robust PIMT activity from seeds estimated to be 95 and 416 years old [176]. In a more typical example, PIMT activity was detected in barley seeds aged for 17 years, even though the seeds were no longer viable. Higher levels of PIMT activity were detected in younger seeds stored for 5 or 11 years, which still maintained partial viability [59]. The older seeds also demonstrated higher methyl-accepting activity than younger seeds, suggesting that the seed PIMT may become progressively more saturated with potential substrates over time.

PIMT is not restricted to the seed tissue of higher plants. Enzymatic activity is also detected in extracts prepared from nonreproductive tissues. Considerable variability is observed, however, in the tissue distribution of PIMT between plant species. Relatively high levels of PIMT are present in corn stems and leaves and in carrot roots and leaves. By contrast, rice and wheat have only low levels of activity in the same tissues [177]. In *Arabidopsis*, PIMT enzymatic activity was not detected in tissues other than seeds, although *PIMT* transcripts were readily identified in RNA samples prepared from these tissues [38]. Additional studies will be needed to determine if this apparent contradiction reflects post-transcriptional regulation of PIMT levels in nonreproductive tissue or if the difference is caused by the presence of substances in tissue extracts that interfere with PIMT assays.

2. Gene structure and transcriptional regulation

The regulation of PIMT production in plants is expected to be at least as complex as in animals, because higher plants may contain more than one gene that encodes PIMT. Large scale genome sequencing and microarray projects involving *Arabidopsis* and cereal grains have already produced a wealth of information about *PIMT* gene organization and transcript processing that will need to be reconciled with biochemical information in the future. Information from the sequencing and microarray projects is available online in databases maintained by the Institute for Genomic Research (www.tigr.org) and the Arabidopsis Information Resource (www.arabidopsis.org). These projects have identified paralogous *PIMT* sequences on two different chromosomes in the genomes of *Arabidopsis*, rice, and wheat. Like many other plant genes, the two *PIMT* genes most likely arose by ancestral gene duplication. The duplicated genes have undergone considerable sequence divergence, yet the predicted proteins within a species are more than 85% similar. There is good evidence that both genes are transcribed. Full-length cDNA and EST clones have been reported for both *PIMT* genes in *Arabidopsis* and rice. EST sequences have also been reported for both wheat genes, although only one full-length cDNA sequence has been isolated [27].

PIMT genes in plants are organized into four exons which may used for alternative splicing. The most detailed information about *PIMT* transcription is available for *Arabidopsis*. *PIMT1* transcripts are more abundant than *PIMT2* transcripts, but *PIMT2* may be subject to more complex regulation. Alternative splicing between exons 1 and 2 of *PIMT2* primary transcripts generates transcripts for two distinct proteins, each of which is predicted to have a NLS [39]. The NLS is functional in *Arabidopsis* cells, but their significance is unclear, since PIMTs are expected to diffuse freely through nuclear pores [62]. NLS sequences are not present in other *PIMT2* sequences reported in the public databases or in transcripts of the *PIMT1* gene . It is not clear if alternative processing occurs during *PIMT1* transcription. Two transcripts hybridize with *PIMT1* probes on northern blots [38], but further analysis is needed to determine if this is the result of alternative processing of *PIMT1* transcripts or cross-hybridization of *PIMT2* transcripts with the *PIMT1* probe.

There is good evidence for the transcriptional regulation of PIMT activities in higher plants during development and in response to environmental stress. In wheat, *PIMT* transcription increases during caryopsis development coincident with increases in PIMT activity. PIMT transcription also increases during salt stress and desiccation [17]. The phytohormone, abscisic acid (ABA), is hypothesized to be the common regulator in these transitions. ABA is known to promote seed development, and it has been implicated as well in plant salt responses. Consistent with this hypothesis, ABA treatment of 4-day old wheat seedlings induces *PIMT* transcription. In all cases, there is good correspondence between data obtained from northern blots and enzymatic assays, suggesting that regulation of PIMT levels in wheat occurs primarily at the transcriptional level.

ABA has also been implicated in regulation of *PIMT* transcription in *Arabidopsis*. The 5'-flanking sequences of both the *PIMT1* [38] and *PIMT2* [39] genes contain putative ABA response elements . Transcription of both genes increases during seed development and in response to ABA treatment. Some differences are observed, however, in the responses of the two genes to environmental stress. Transcription of *PIMT2* increases in response to both salt stress and dehydration, while *PIMT1* is minimally affected by the stresses.

3. Functional importance of PIMT in plants

Functional analyses of PIMT in plants are strictly correlative at present. Based on the distribution of PIMT activities and the positive regulation of *PIMT* transcript by ABA, it is likely that PIMT is important for maintaining protein function during long-term storage or under stress conditions. Potential roles for PIMT in plant survival can be tested in transgenic plants, because transgenic *Arabidopsis* lines deficient in single *PIMT* genes are already available in stock collections. The development of transgenic models will be complicated by the fact that *PIMT* genes are duplicated in higher plants, and it may be necessary to inactivate both genes in order to analyze PIMT function.

C. *Drosophila*

1. Distribution of PIMT activities in *Drosophila*

The small size and short lifespan of *Drosophila* have made it possible to analyze PIMT levels throughout the complete lifespan. PIMT can be detected biochemically in the early embryo before embryonic transcription is initiated, suggesting that maternal transcripts are translated in the oocyte and early embryo [178]. PIMT levels then remain essentially constant during embryogenesis,before dropping to their lowest levels in larvae, and subsequently increasing during pupal development. The highest levels of PIMT are found in the adult fly. PIMT specific activity increases sharply during the first day after eclosion to the adult level, where it is maintained at an essentially constant level for the duration of the lifespan [179,180].

2. Gene structure and regulation of PIMT activity

The *Drosophila Pcmt* gene consists of four exons and is located in region 83B of the third chromosome. There is no evidence for alternative splicing during the processing of *Pcmt* transcripts, since a single 1.6 kb transcript is detected on northern blots of *Drosophila* RNA [179]. The abundance of this transcript is always proportionate to the biochemical PIMT activity, suggesting that PIMT is primarily regulated at the transcriptional level in flies. The regulatory factors that control *Pcmt* transcription are unknown. The upstream flanking region of the *Pcmt* gene possesses few consensus binding sites for known transcriptional regulators in

Drosophila. Transcription initiates approximately 200 bp upstream from the initiation codon in the proximity of a TATA box and an arthropod capsite consensus element [181]. The absence of other binding sites for tissue- and stage-specific transcription factors seem to be consistent with constitutive regulation of the gene.

The only changes that have been noted in *Pcmt* expression occur in response to heat. Flies respond to a series of five 15-min heat shocks at 34 °C by elevating PIMT levels approximately 50% [180]. In addition, the PIMT specific activity is slightly higher in flies raised at 29 °C than in flies raised at 25 °C (O'Connor, data not shown). These small temperature-dependent increases in PIMT expression do not appear to be mediated by the well-characerized *Drosopohila* heat-shock transcription factor [182], however, because no binding sites can be detected in the *Pcmt* flanking sequence.

3. Functional analysis in transgenic models

No loss-of-function *Pcmt* mutants are currently available in *Drosophila*, and high throughput RNAi screens have not identified any phenotypes associated with *Pcmt* silencing. Functional studies have been restricted to overexpression experiments, which suggest that PIMT plays an important role in mitigating the aging process. When the binary GAL4-UAS system [183] is used to drive constitutive overexpression of PIMT activity in flies, the median lifespan is increased by 30-40% [180]. The longevity effect is temperature-dependent. Lifespan is extended when flies are raised at 29 °C , but not at 25 °C, suggesting that PIMT activity can become limiting under conditions where rates of protein isomerization are expected to increase. PIMT is one of very few proteins that extend the *Drosophila* lifespan when over-expressed *in vivo*. Other overexpressed proteins that increase the *Drosophila* lifespan include Cu/Zn superoxide dismutase [184-186], mitochondrial Mn-superoxide dismutase [187], heat shock proteins [188], and methionine sulfoxide reductase [189]. A common feature of all these proteins is that they are involved in preventing or repairing damage to protein structures.

D. *Caenorhabditis elegans*

1. PIMT activities during C. elegans development

The nematode *C. elegans* is a popular model organism in which to study both early development and aging. The *pcm-1* gene in *C. elegans* encodes a PIMT of 225 amino acids. The enzyme has been overexpressed in *E. coli* and shown to catalyze the methylation of isoAsp peptide substrates, although much less effectively than human PIMT [45]. Unlike mammalian PIMTs, the *C. elegans* enzyme does not recognize ovalbumin as a substrate, suggesting that considerable divergence has occurred in the enzyme active site. Biochemical analyses of PIMT activity during the *C. elegans* lifespan are difficult because of the organism's small size, but PIMT is reported to be enriched in dauer larvae, a long-lived quiescent state that worms can enter in response to overcrowding or nutritional deprivation. The PIMT specific activity measured in dauer larvae is approximately twice that of mixed-stage populations, in good agreement with transcriptional data (see below). Dauer larvae can survive for periods as long as 70 days in this altered metabolic state, which perhaps increases the metabolic requirement for PIMT to maintain protein integrity.

2. Gene structure and transcriptional regulation

The *pcm-1* gene is located on chromosome 5 and organized in 7 exons [45]. It is not clear if alternative processing generates multiple transcripts in *C. elegans*, because data on *pcm-1*

transcription have been obtained exclusively from genome-wide microarray experiments. These data reveal only small fluctuations in the concentration of *pcm-1*transcripts during development. In a carefully synchronized study of early development [190], *pcm-1* transcript levels remained essentially constant from the oocyte through the larval stages. While the *pcm-1* transcripts in oocytes are of maternal origin, embryonic transcription subsequently maintains *pcm-1* transcripts at a steady state. Transcript levels do not change significantly during the adult lifespan [191], but are approximately two-fold higher in dauer larvae [192,193], in good agreement with the biochemical measurements [45].

In microarray experiments, the transcription profile of *pcm-1* does not cluster with those of other genes. A broader view is required to begin to detect associations between *pcm-1* transcription and that of other genes. Such a compendium of gene expression has been constructed for *C. elegans* genes based on 553 microarray experiments from 30 different laboratories [194]. The experimental data used in the analysis included experiments involving different developmental stages, growth conditions, aging, stress responses and mutant strains. The outcome of the analysis was the identification of groups of genes, or "mountains," whose expression was highly correlated. Using this statistical data, *pcm-1* was placed in Mount 6, consisting of 909 genes that are transcribed at elevated levels in neuronal cells [194]. Mount 6 includes a broad spectrum of proteins with diverse functions, and much additional work will be required to establish functional relationships between them. The tendency for Mount 6 transcripts to be elevated in neuronal cells is particularly interesting in light of the elevated PIMT concentrations in mammalian neurons, suggesting that the functional importance of PIMT to neuronal function is conserved during evolution. Because of its comparative simplicity, *C. elegans* may provide a useful model in which to investigate physiological roles for PIMT in the nervous system.

3. Functional analysis of PIMT

PIMT-deficient worms have been generated using transposon mutagenesis [22]. The mutant strains were isolated by screening for the spontaneous excision of a Tc1 transposon located in the fourth intron of the *pcm-1* gene. Imprecision excision of the transposon generated a deletion encompassing exons 2 through 5. Homozygous PIMT-deficient worms were viable and developed normally, but showed several subtle phenotypes. In mixed cultures with wild-type worms, the frequency of the null allele decreased slowly over time. The difference between the wild-type and mutant worms was a slight one, however, because the null allele was still detected after 65 generations. A second phenotype was observed during dauer phase. The mean survival time for mutant worms in dauer phase was 24.5 days, compared to 27 days for the wild-type control. The results suggest that the fitness of mutant worms is slightly less than that of wild-type worms, but the differences are subtle. It will be important to extend these studies in the future to exclude any contributions of genetic drift or genetic background to the phenotypes.

E. Bacteria

1. Protein isoAsp methylation in *E. coli*

The functional significance of bacterial PIMTs has been studied most thoroughly in *E. coli*, because of the techniques available for genetic manipulation. The *E. coli* PIMT sequence is 31% identical to the human PIMT sequence, and the enzyme catalyzes methylation of the same isoAsp substrates as human PIMT, but with reduced efficiency [195]. The K_ms of *E. coli* PIMT for isoAsp peptides and ovalbumin are significantly higher than those of mammalian PIMTs with

the same substrates. Surprisingly, *E. coli* PIMT may also be less effective than mammalian PIMT at modifying bacterial methyl-accepting proteins. This possibility was serendipitously discovered during the analysis of *E. coli* that had been genetically engineered to overexpress rat PIMT [196]. Multiple proteins in extracts prepared from both wild-type and overexpressing *E.coli* were able to act as substrates for purified mammalian PIMT, but the specific methyl-accepting activities of proteins from PIMT-overexpressing bacteria were significantly lower than those from wild-type bacteria, suggesting that overexpressed rat PIMT had previously modified isoAsp residues *in vivo*. The most prominent methyl-accepting protein was identified as ribosomal protein S11, which was methylated to a stoichiometry estimated as 0.5 mol/mol S11. The results suggest that isoAsp sites are inefficiently methylated in intact *E. coli*, in contrast to their efficient methylation in higher eukaryotes. In addition, the results raise the possibility that L-isoAsp residues in S11 are generated by a catalytic mechanism , both because of the speed with which they arise and because their fractional representation is greater than that predicted for a spontaneous mechanism of isoAsp generation (Fig. 2).

2. Gene structure

The regulation of *pcm* transcription has not been studied in detail. Genome annotation predicts that the *pcm* gene is part of an operon that includes four other coding sequences. The predicted coding sequences partially overlap one other, such that the *pcm* coding sequence would be situated at the 3'-end of a polycistronic transcript. The proximal gene, *SurE*, encodes a protein with nucleotidase activity that has also been described as a survival protein. *SurE* has been previously shown to interact genetically with *pcm* [197], but the mechanism is obscure. The functions of the other proteins in the operon have not been identified. The *E. coli* operon organization has a restricted distribution within the bacteria. It will be interesting to determine if *pcm* is transcribed as part of a polycistronic mRNA and if the relationship between the proteins encoding by the operon is coincidental or functional.

3. Functional analysis of PIMT in bacteria

The functional importance of bacterial PIMT has been tested in mutant strains. A strain lacking PIMT activity was constructed by replacing the endogenous *pcm* gene with a chloramphenicol resistance gene [198]. Gene replacement did not affect the growth or stress resistance of vegetative cells, but produced phenotypes that were manifest only under stationary phase culture conditions. Mutant cells showed a reduced resistance to several stresses that could conceivably destabilize protein structures, including methanol, paraquat, and high salt. In all cases, the mutant phenotypes could be complemented by a wild-type *pcm* gene, indicating that the loss of PIMT function was responsible for the phenotypes. The *pcm* mutation also conferred a competitive disadvantage to stationary phase mutants co-cultured with normal cells. Interestingly, the mutant phenotypes have been recently shown to be restricted to cultures maintained under alkaline conditions [199], which are known to promote the generation of protein isoAsp residues by the spontaneous mechanism shown in Fig. 2. In agreement with this prediction, extracts prepared from cells grown at pH 8 or 9 had elevated methyl-accepting activity in direct proportion to the alkalinity of the culture medium.

Other evidence that *E. coli* PIMT might be involved in maintaining native protein structures was obtained in a genetic screen designed to identify multicopy suppressors of protein aggregation and inclusion body formation [200]. In this screen, the *E. coli* tester strain overexpressed a three-domain fusion protein, consisting of the preS2 and S' domains of the

hepatitis B surface antigen N-terminal to β-galactosidase. The fusion protein is soluble in *E. coli* cytoplasm at 37 °C, but denatures and forms insoluble aggregates at 43 °C. Transformation of the tester strain with a genomic segment containing the *pcm* coding sequence suppressed fusion protein aggregation at 43 °C. In addition, PIMT overexpression also protected β-galactosidase from thermal denaturation in extracts prepared from the transformed cells. The mechanism by which PIMT suppresses aggregation requires further investigation. The fusion protein is a highly nonphysiological substrate produced at high levels in the cytoplasm, and its aggregation has not actually been linked to isoAsp formation. It will also be important to determine if a direct link exists between the suppression of fusion protein aggregation and PIMT catalytic activity.

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FOOTNOTES

[1]Abbreviations: PIMT: protein L-isoaspartyl, D-aspartyl *O*-methyltransferase; AdoMet: S-adenosylmethionine;AdoHcy: S-adenosylhomocysteine; CaM: calmodulin; NLS: nuclear localization signal; ROS: reactive oxygen species

Table I
Accession information for PIMTs in online databases

Species	Gene name	TrEMBL/Swiss Protein	Database Identifier	PDB Accession	Literature References
E. coli	*pcm*	P24206	b2743		195
P. furiosus	*pcm*	Q8TZR3	PF1922	1JG1-1JG4	19
T. maritima	*pcm*	Q56308	TM0704	1DL5	18
S. tododaii	*pcm*	Q972K9	ST1123	1VBF	93
S. pombe	*pcm2*	Q9URZ1	SPAC869.08		
C. elegans	*pcm-1*	Q27873	C10F3.5		45
D. melanogaster	*Pcmt*	Q27869	CG2152	1R18	20
A. thaliana	*PIMT1*	Q42539	At3g48330		38
A. thaliana	*PIMT2*	Q8GXQ4	At5g50240		39
T. aestivum	*PCM*	Q43209			27
M. musculus	*Pcmt1*	P23506	MGI:97502		16
H. sapiens	*PCMT1*	P22061	HGNC:8728	1I1N, 1KR5	43, 94, 164

Accession numbers and gene names were obtained from various online databases. There are numerous links between the databases, so key URLs are given below. Detailed information for bacterial species and higher plants can be obtained at The Instiitute for Genomic Research (www.tigr.org). Information for human, mouse and *S. pombe* can be accessed through the Sanger Center (www.sanger.ac.uk) or the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). Species-sepcific databases include information for *Drosophila* (flybase.bio.indiana.edu), *C. elegans* (www.wormbase.org) and *Arabidopsis* (www.arabidopsis.org). Other useful URLs are those for TrEMBL/Swiss Protein (us.expasy.org/sprot) and the Protein Data Bank (www.rcsb.org).

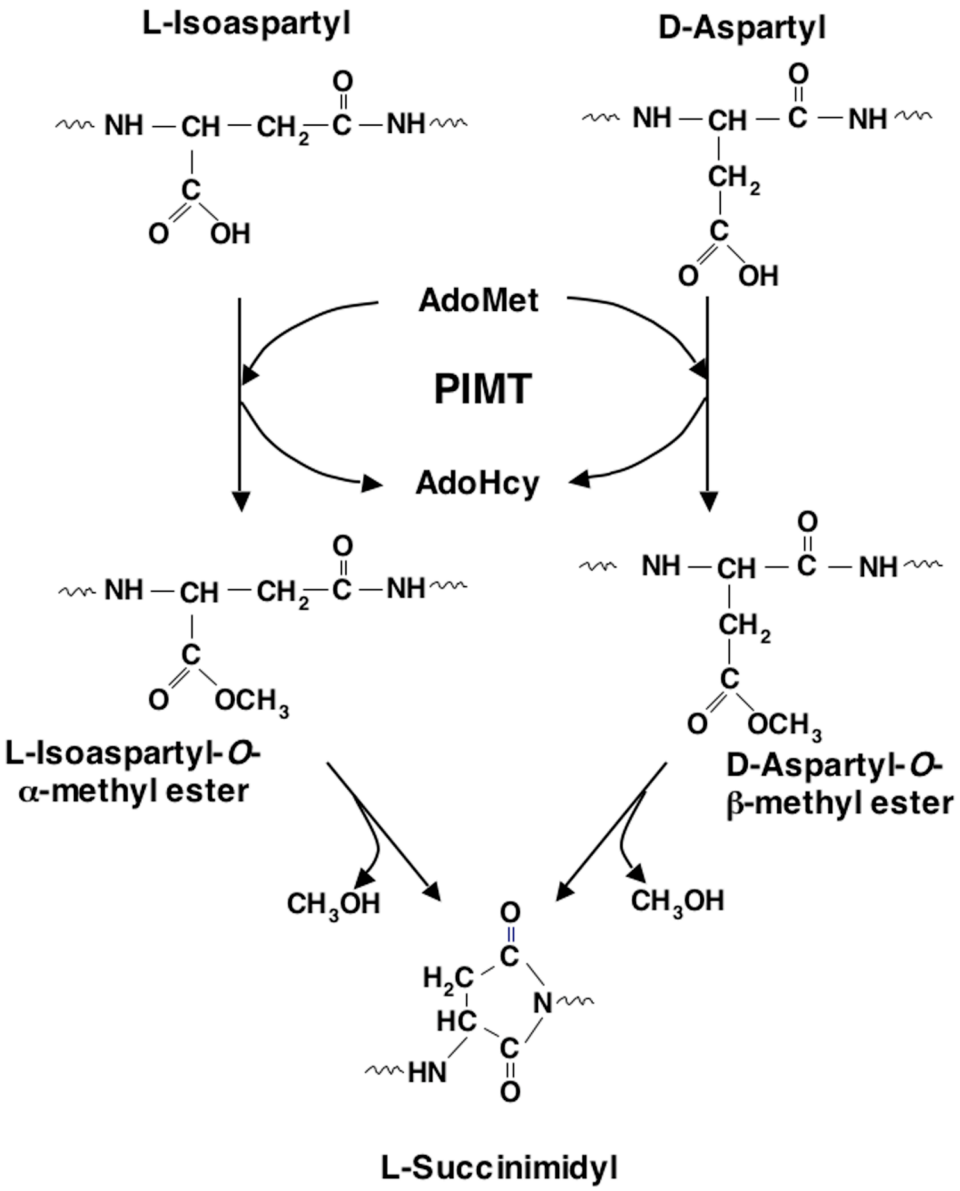

Figure 1. Enzymatic reaction. PIMT catalyzes the transfer of methyl groups from AdoMet to protein L-isoaspartyl and D-aspartyl residues. Methyl esters spontaneously hydrolyze to form a succinimidyl structure.

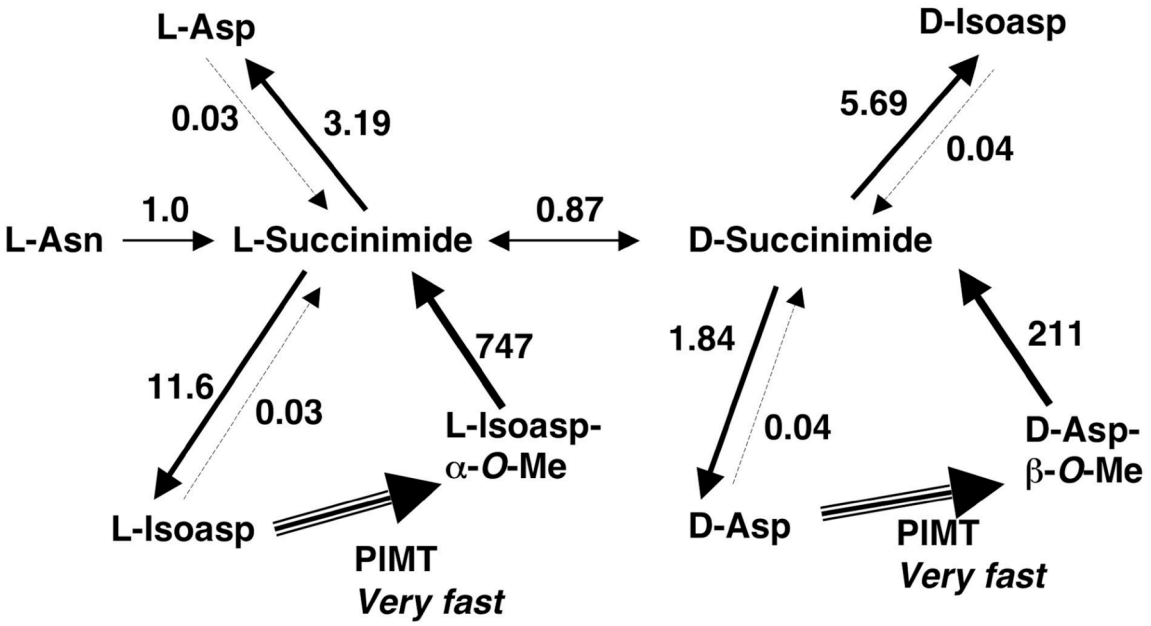

Figure 2. Spontaneous and enzymatic conversions involving the peptide VYP(X)HA, where X represents an Asn, Asp, isoAsp or a methyl ester. Relative rate constants for the various interconversions were calculated from the experimental data in references [50] and [53].

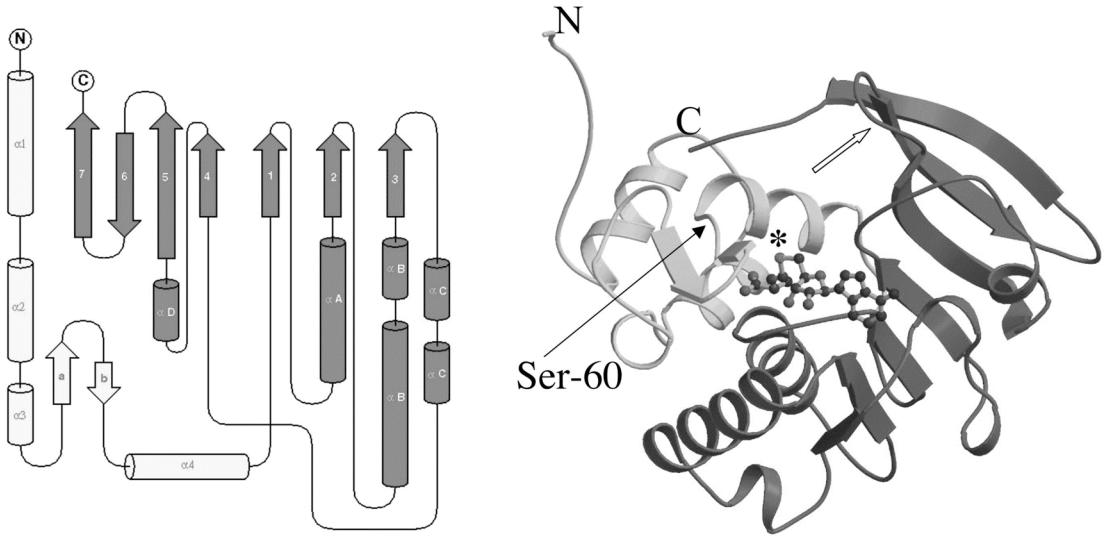

Figure 3. Topology diagram and three-dimensional structure of *Drosophila* PIMT (PDB accession number 1R18). The methyltransferase fold common to Class I AdoMet-dependent is shown in dark shading, and the N-terminal subdomain characteristic of PIMTs. is shown in light shading [20]. An asterisk shows the position of the sulfur atom in AdoHcy. The open arrow indicates a loop whose position is significantly different in AdoMet and AdoHcy co-crystals [19]. The position of Ser-60 essential for catalysis is indicated.

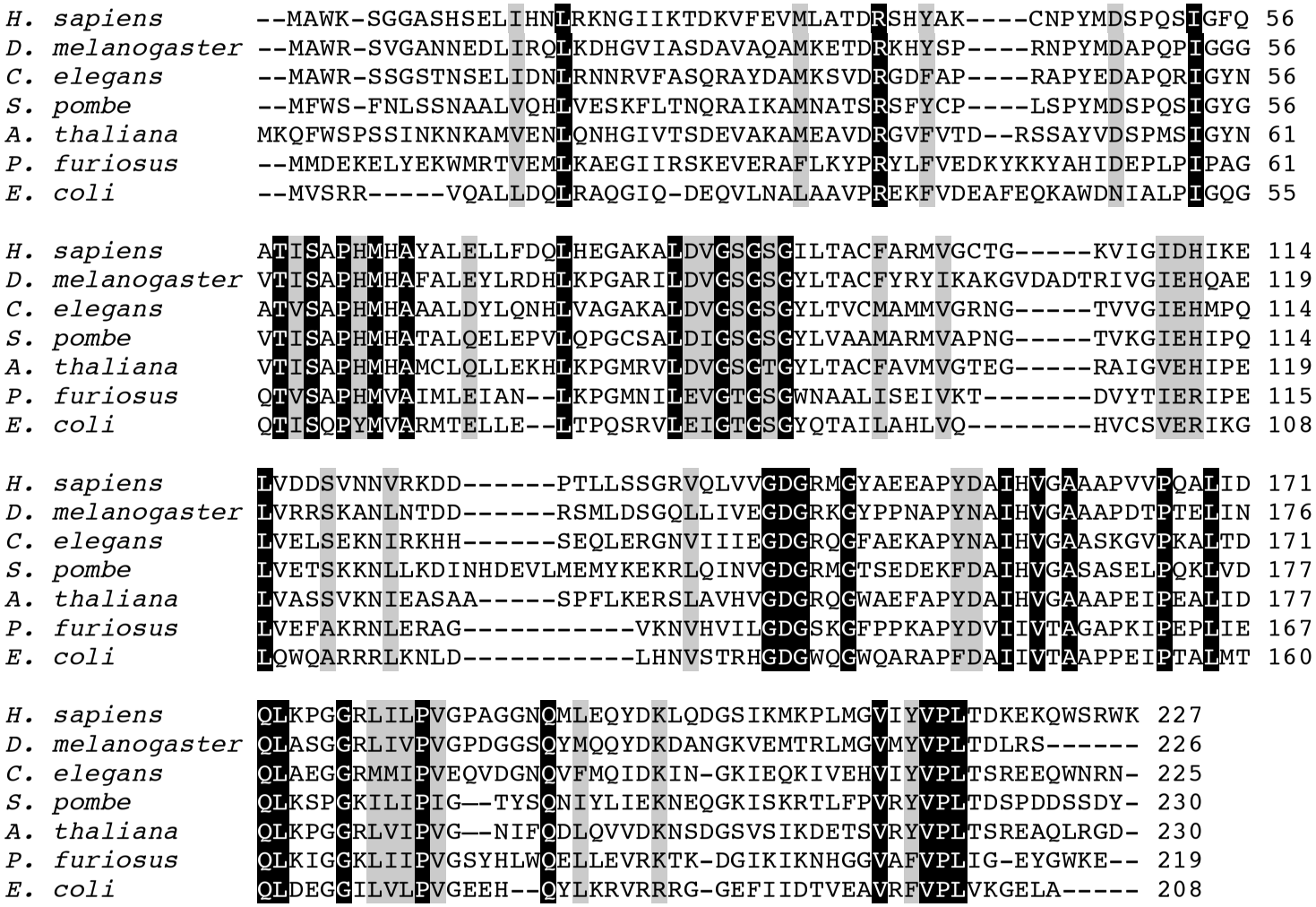

Figure 4. Alignment of PIMT sequences using the Clustal W algorithm (http://www.ebi.ac.uk). Invariant residues are shown by white letters on a black background. Strongly conserved regions are shaded.

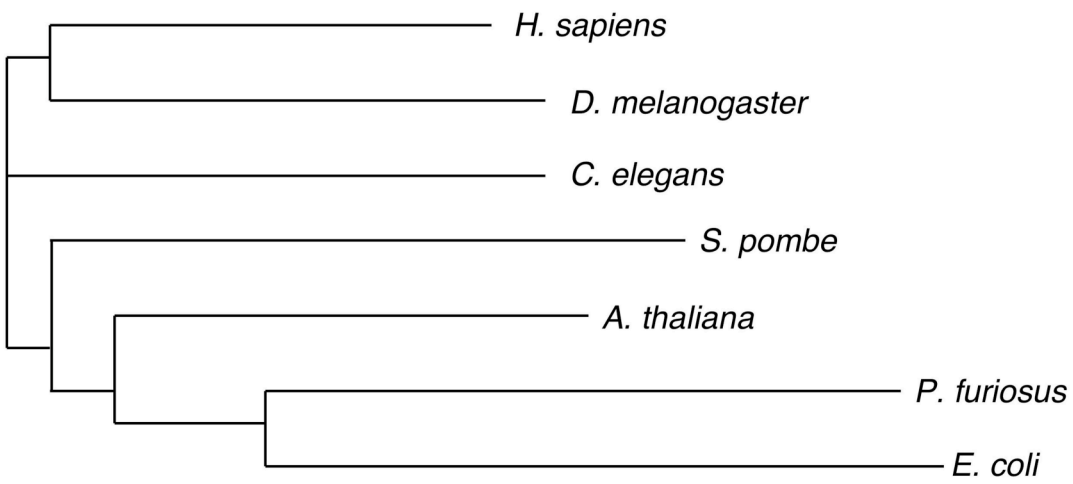

Figure 5. Phylogenetic tree for PIMTs constructed from the alignment data in Fig. 4 using the phylip algorithm from the European Bioinformatics Institute (http://www.ebi.ac.uk).

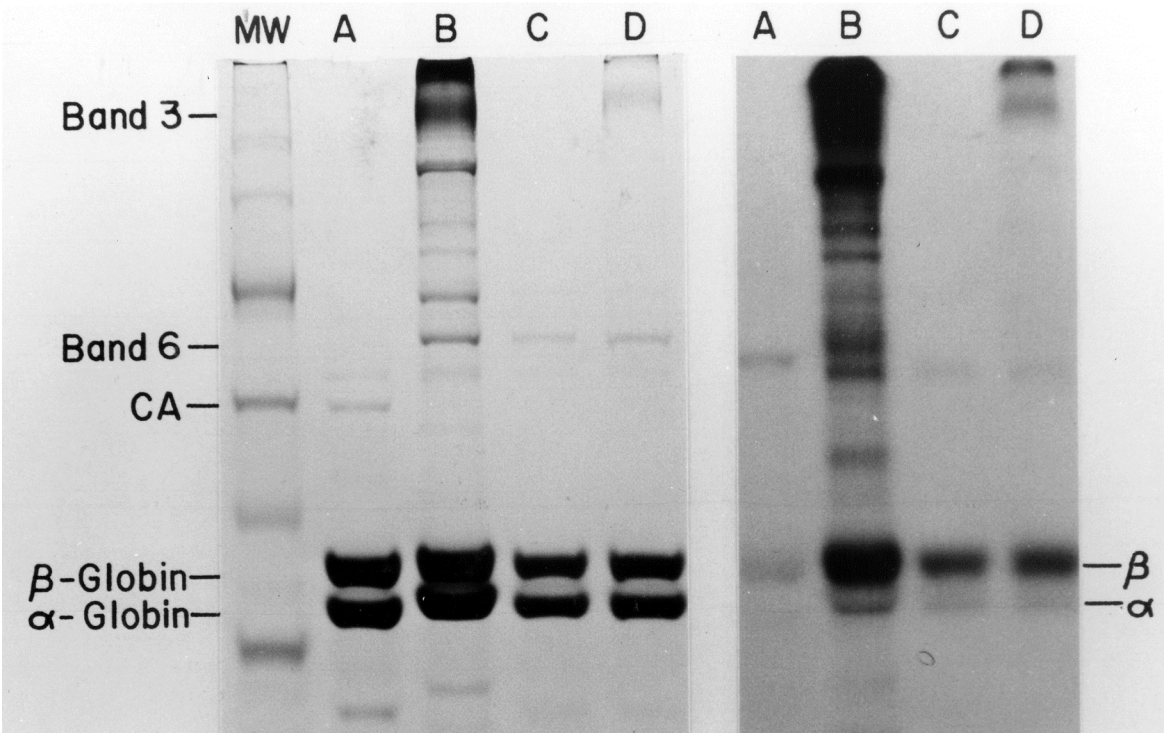

Figure 6. Methylation of membrane and cytosolic proteins in human erythrocytes. Stained gel (left) and fluorogram (right) of proteins prepared from erythrocytes incubated with L-[methyl-^{3}H]methionine and separated by SDS-PAGE at pH 2.4. Cytosolic proteins (lane A), membrane proteins (lane B), proteins extracted from membranes with 100 mM NaCl (lane C) or 0.5% Nonidet P-40 (lane D). Reproduced from Figure 1 in O'Connor and Yutzey [108].

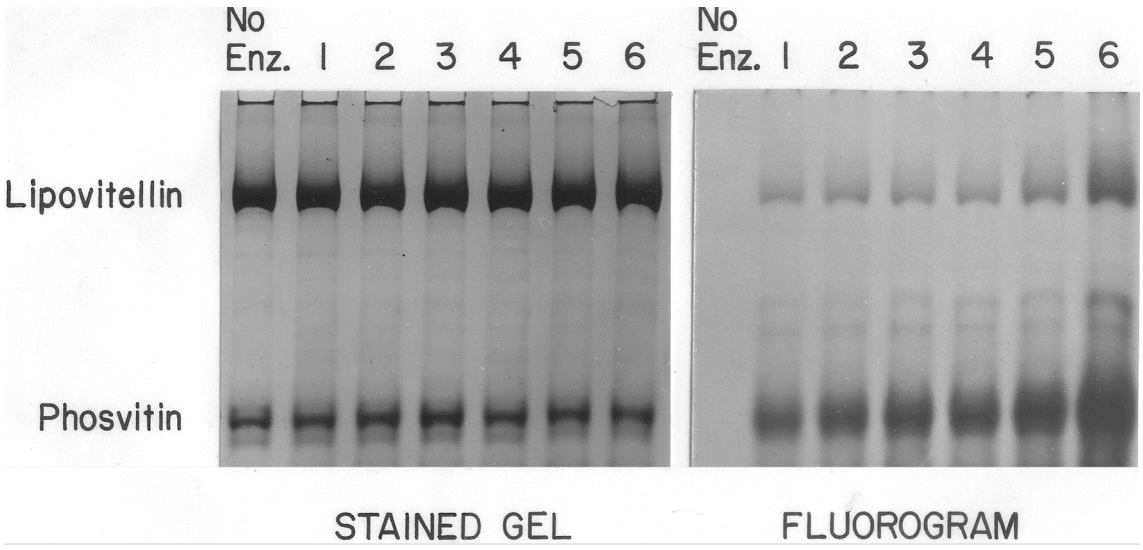

Figure 7. Methyl-accepting activity of yolk proteins obtained from oocytes at different stages of oogenesis. Yolk proteins were isolated from vitellogenic oocytes ranging in diameter from 0.6-0.7 mm (lane 1), 0.7-0.8 mm (lane 2), 0.8-0.925 mm (lane 3), 0.925-1.075 mm (lane 4), 1.075-1.2 mm (lane 5) and 1.2 to 1.25 mm (lane 6) and used as the substrate for PIMT and [^{3}H]AdoMet. Methylated proteins were separated by SDS-PAGE at pH 2.4 and methylated proteins were visualized by fluorography. Reproduced from Fig. 5 in O'Connor [62].