REVIEW ARTICLE

Protein damage and methylation-mediated repair in the erythrocyte

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INTRODUCTION

Natural proteins can be subject to non-enzymic molecular alterations, including deamidation of asparaginyl and glutamyl residues, aspartyl racemization, glycation of α- and ε-free amino groups, and oxidation of methionyl, cysteinyl, tryptophanyl and histidyl residues. The rate at which these alterations occur and their specific localization within a protein appear to be strongly influenced by specific protein structural determinants, as well as by protein microenvironment.

The occurrence of structural modifications does not necessarily imply a functional alteration, although it should be pointed out that: (1) even a single apparently minor change can trigger a significant functional impairment, at least in some proteins, just as happens in cases where a single point mutation, leading to an amino acid substitution, profoundly affects physicochemical and functional properties of the macromolecule. As a matter of fact a non-enzymic protein modification may be able to modify either protein charge (e.g. deamidation) or structure (e.g. racemization); this can be particularly detrimental to protein function if a crucial residue involved in catalysis or in ligand binding is affected. (2) In long-lived proteins the accumulation of time-dependent spontaneous molecular damage may be quantitatively and statistically more relevant to protein function. In this case the affected residues could be considered as built-in self-destruct timers, ticking away toward an inevitable end: old functionally impaired molecules.

Taking into account all the possible structural and functional implications, we propose that these non-enzymic protein modifications, as a whole, should be referred to as 'protein fatigue' in analogy to the so-called 'metal fatigue', a phenomenon which is well known to aerospace engineers [1], consisting of the accumulation of fatigue damage, such as cracks, in metal structures as a consequence of compressive or tensile stresses. Even small stress cycles can contribute significantly to damage accumulation [1]. The analogy with 'protein fatigue' is, in our opinion, evident, since the effects of structural damage in proteins is time-dependent; the effects of individual stresses may be cumulative, but a damaging event, small as it may be, such as the alteration of a single amino acid residue, may still induce significant functional impairment. Unfortunately, at present we are not quite able to predict the patterns of accumulation and rate of increment of protein damage and its functional effects, as the engineers can in the case of 'metal fatigue'.

Among the several different spontaneous protein reactions which take place, we will focus our attention on those which give rise to abnormal isomerized L-isoaspartyl or racemized D-aspartyl residues. Relevant to this issue is the isolation and characterization of a protein carboxyl methyltransferase, named protein L-isoaspartyl/D-aspartyl methyltransferase (PCMT type II; EC 2.1.1.77), which specifically recognizes and modifies these damaged aspartyl sites in protein substrates. This specific enzymic methylation is presently envisioned as a step in a mechanism by which these altered proteins are repaired and/or committed to degradation.

CLASSIFICATION OF PROTEIN CARBOXYL METHYLTRANSFERASES

PCMT type II belongs to a large group of protein carboxyl methyltransferases, enzymes which catalyse the *S*-adenosylmethionine (AdoMet)-dependent methylation of protein free carboxyl groups [2,3]. Four classes of these enzymes have been identified so far and classified according to the nature of the modified residue.

(i) Glutamyl side chains are specifically methyl esterified by an L-glutamyl protein methyltransferase (PCMT type I; EC 2.1.1.80), which is only present in chemotactic bacteria. The function of this methylation reaction is restricted to modulation of bacterial chemotaxis through the formation of glutamyl-γ-methyl esters of specific glutamyl residues in chemoreceptor proteins [3,4].

(ii) PCMT type II is a ubiquitous housekeeping enzyme that, as previously mentioned, specifically recognizes D-aspartyl and L-isoaspartyl residues which are modified yielding D-aspartyl-β-methyl ester and L-isoaspartyl-α-methyl ester respectively. Many proteins are recognized and modified *in vitro* by this enzyme, while the natural *in vivo* substrates have been identified in specific cells and tissues including human erythrocytes [3,5].

(iii) PCMT type III methyl esterifies the free α-carboxyl group of the C-terminal cysteinyl residue in membrane-related proteins in eukaryotes. This methylation is considered to be an intermediate step in the anchorage of these proteins to the membrane [3,6].

Most recently a new type of PCMT has been identified by Xie and Clarke [7] in eukaryotic cells. This enzyme catalyses the methyl esterification of C-terminal leucine residues of cytosolic polypeptides and may have a regulatory function with respect to the methyl-accepting proteins.

This review article deals with the role of PCMT type II in the post-biosynthetic processing of damaged proteins, with special regard to the human erythrocyte model.

SOURCE OF ALTERED ASPARTYL RESIDUES IN PROTEINS

The major covalent modification which accounts for the occurrence of L-isoaspartyl and D-aspartyl residues in proteins is deamidation of intrinsically labile L-asparaginyl residues [8–11]. The mechanism by which deamidation occurs implies the formation of a cyclic succinimidyl intermediate, the opening of

Abbreviations used: AdoHcy, *S*-adenosylhomocysteine; AdoMet, *S*-adenosylmethionine; BOC, butyloxycarbonyl group; BS-RNAase, bovine seminal ribonuclease; peptide α-16, BS-RNAase$^{62-76}$(isoAsp^{67}); peptide α-17, BS-RNAase$^{62-76}$(Asp^{67}); EGF, epidermal growth factor; PCMT type II, protein L-isoaspartyl/D-aspartyl methyltransferase.

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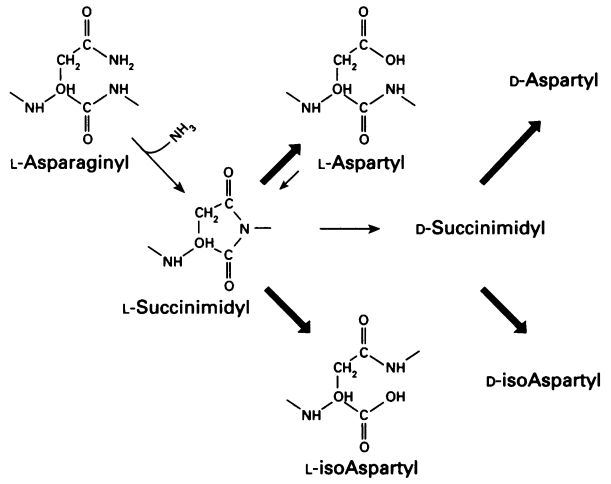

Figure 1 Mechanism for the formation of altered aspartyl residues in proteins

Deamidation of asparaginyl residues represents the quantitatively most important source of altered aspartyl residues in proteins. The mechanism proceeds through the intramolecular attack of the α-nitrogen of the Asn^{+1} residue to the β-carbonyl group of the Asn residue, leading to the formation of an L-succinimidyl intermediate which is itself unstable. The opening of the succinimide ring, on either side of the nitrogen atom, yields normal L-aspartyl or L-isoaspartyl respectively. The formation of the L-succinimidyl derivative may also occur directly from a normal L-aspartyl residue. The mechanism also explains the formation of D-aspartyl and D-isoaspartyl residues through racemization of the L-succinimidyl into a D-succinimidyl residue. Bold arrows indicate faster reactions.

which, on either side of the imide nitrogen, generates both a normal peptide bond or an atypical isopeptide bond, where the aspartyl is linked through its β-carbonyl group to the residue which follows in the peptide chain (Figure 1). The ratio of the two peptides is always in favour of the 'iso' form and it is generally 7:3 [8–11].

Both the formation of succinimidyl intermediate and its cleavage occur spontaneously, and are strictly dependent on the sequence flanking the asparaginyl site, as well as on the local conformation and flexibility of the polypeptide chain [12–14]. The influence of salts, buffers and solvents on the kinetics and mechanism of succinimide ring formation have also been explored using model peptides [15–18].

The effect of the nature of the Asn^{+1} residue on the rate of succinimide formation has been amply investigated [8,10,12,13,19]. The deamidation rate, as a function of sequence determinants, has been measured using short model peptides in which conformational effects are minimized. It has been shown that the highest rates occur when Asn is followed by a residue with a small non-bulky side-chain, such as Gly, Ala or Ser. Most Asn residues found to be susceptible to deamidation in proteins, *in vivo*, are in fact at the level of Asn-Gly and Asn-Ser sequences. The presence of different residues at position Asn^{+1} in a number of naturally deamidated proteins may suggest that alternative mechanisms for deamidation are also operative [13]. According to Wright, the analysis of the nearest-neighbour frequency for Asn residues in 1076 proteins supports the existence of a selection in favour of non-polar bulky amino acids and is against the occurrence of small and reactive ones, indicating that deamidation "...is a post-translational event which is selected against in the course of evolution..." [13].

On the other hand, it has been shown that not all Asn-Gly sequences in proteins are inevitably committed to deamidation, since not all secondary or three-dimensional structure arrangements are permissive. For example, Bornstein and Balian showed that succinimide formation from Asn^{67}-Gly^{68} in ribonuclease A is allowed only after protein unfolding [20]. Similarly, in mouse epidermal growth factor (EGF) the sequence Asn^{15}-Gly^{16} is protected from deamidation because it is part of a β-turn constricted between two adjacent disulphide bridges [14]. This result is consistent with Clarke's conclusion that "...asparaginyl residues generally exist in native proteins in conformations where the peptide bond nitrogen atom cannot approach the side chain carbonyl carbon to form a succinimide ring..." [12].

The indication that in many cases asparaginyl residues in natural proteins may be protected against deamidation by conformational constraints supports the notion that such deamidation is potentially detrimental to protein structure and function. For example, Asn deamidation has been shown to significantly affect the biological activity of human EGF [21,22], human growth hormone-releasing factor [23], calmodulin [24,25], lysozyme [26], and haemoglobin Providence [27].

It has also been shown that Asn deamidation may be one of the mechanisms by which enzyme subforms are generated *in vivo*, such as in the cases of rabbit liver hydroxymethyltransferase [28], bovine seminal ribonuclease (BS-RNAase) [11], and human triosephosphate isomerase [29].

The mechanism of Asn deamidation, reported in Figure 1, can also explain the formation of D-aspartyl residues, through racemization of the L-succinimide derivative, followed by the opening of the D-succinimidyl ring that will yield both D-aspartyl or D-isoaspartyl residues. The identification of D-aspartyl residues in erythrocyte and eye lens proteins will be discussed later.

It has been predicted that at least a subset of altered aspartyl residues may arise from the isomerization of normal L-aspartyl residues, which also occur through the succinimide intermediate (Figure 1) [18]. Such an unstable L-aspartyl residue has been found in glucagon [30] and in human growth hormone [31].

The hypothesis that the presence of L-isoAsp residues could be a consequence of errors occurring during protein biosynthesis has found no experimental validation [32].

PCMT TYPE II, AN ENZYME WHICH RECOGNIZES ALTERED ASPARTYL RESIDUES

Structure and physicochemical properties

PCMT type II is a ubiquitous enzyme, which has been identified in both prokaryotic and eukaryotic organisms [33]. A remarkable amount of information is available today on the physicochemical and structural characterization of PCMT type II from different mammalian sources, brain and blood being the tissues endowed with the highest enzyme activities [33].

The human enzyme has been purified to homogeneity from erythrocytes and extensively characterized regarding its physicochemical features, as well as its protein sequence and isoenzyme pattern [34–38]. The enzyme acts as a monomer of 25 kDa and it is mainly cytosolic. A membrane-bound form of the enzyme has been reported in rat brain [39,40].

Two major isoenzymes of type II PCMT have been identified in human erythrocytes [35,36]; the isoenzyme pattern is strikingly reproducible in other tissues, including brain and testis from several other mammalian species, such as ox, horse, rabbit, rat and mouse [35]. In particular, the two isoenzymes, purified to homogeneity from human erythrocytes, differ in their isoelectric point values by about 1 pH unit [35,36]. At least one additional minor form, with pI value intermediate between the two major isoenzymes, was identified in human erythrocytes [36]. The complete amino acid sequence of isoenzyme I from human

Figure 2 Sequence of human PCMT type II

The primary structure of PCMT type II isoenzyme I from human erythrocytes is shown as established by Ingrosso et al. [37], by chemical sequence studies. The sequence of isoenzyme II is also reported for comparison. Sequence data for isoenzyme II have been obtained in part by chemical sequencing of purified enzyme from human erythrocytes [34,38]. Confirmation and further refinements of this structure have been deduced from a human cDNA clone [43]. Dots refer to invariant residues in the two isoenzymes. At least two putative polymorphic sites at position 22 (I/L) of isoenzyme I [37], and 199 (I/V) of both isoenzyme I [37] and II (see [38] and [43]) have been reported. Additional genomic polymorphism has been suggested to occur at position 205 of isoenzyme II [43]. The most important differences between the two isoenzymes have been detected in the C-terminal regions (grey shading) [38,43]. The nature of the acetyl-alanyl residue as the N-terminal blocking group has been demonstrated for human isoenzyme I [37]. Motifs I, II and III (red shading) refer to the regions of sequence similarity with other AdoMet-dependent enzymes [37], underlined residues are conserved with respect to the consensus regions identified by Kagan and Clarke [52]. A hyphen between two residues indicates that a gap must be inserted in order to optimize the alignment.

erythrocytes was determined by Ingrosso et al. [37] (Figure 2). This structure includes 226 residues with an N-terminal acetyl-Ala. Alternative residues were detected at positions 22 (Ile/Leu) and 119 (Ile/Val); these variations have been attributed to the existence of two or more allelic variants in analogy to other erythrocyte enzymes [37]. The human isoenzyme I shows more than 95% sequence identity with the homologous bovine brain [41] and rat brain [42] enzymes. The two human isoenzymes differ in the last C-terminal residues (Figure 2) accounting for the different measured pI values [38,43]. Interestingly the C-terminal region -RWK of human isoenzyme I is replaced, in isoenzyme II, by -RDEL, a sequence which is identical to a mammalian endoplasmic reticulum retention signal, and may have functional meaning in cells other than erythrocytes [43]. It has been established that both isoenzymes are encoded by a single gene [38,43], which maps to human chromosome 6 (6q22.3–6q24 region) [44]. The mouse gene has been localized in chromosome 10 (position 8.2 ± 3.5 cM proximal to the *Myb* locus) [44]. This region of the mouse chromosome is syntenic to the human 6q24 region.

The mechanism leading to the formation of the two human isoenzymes has been clarified recently as an alternative splicing of a single gene transcript [43].

A disulphide linkage has been reported to occur, in the bovine brain enzyme, between Cys^{42} and Cys^{94} [41]; these cysteine residues are conserved in both human isoenzymes [37].

A PCMT of type II is also widely distributed in eubacteria [45]. The prokaryotic enzyme has been purified from *Escherichia coli* as a single 24 kDa polypeptide chain. Its sequence, deduced from DNA cloning, shows more than 30% sequence identity with the human isoenzymes [46]. This high degree of structural similarity indicates that PCMT type II is involved in crucial cell function(s) [46].

Binding sites for the methyl donor AdoMet

Research efforts from different groups have been focused on the elucidation of the substrate specificity of PCMT type II, since it has been considered crucial to the understanding of the physiological role(s) of this reaction.

As far as the kinetic mechanism is concerned, a rapid equilibrium random Bi Bi mechanism has been proposed, involving the formation of a ternary complex (enzyme–AdoMet–methyl acceptor substrate), the interconversion of which represents the rate-limiting step of the reaction [47].

The binding sites for AdoMet have been the object of studies in our laboratory. We utilized a number of analogues and derivatives of the methyl donor and of the product inhibitor *S*-adenosylhomocysteine (AdoHcy) [48]. This approach allowed us to identify the amino and carboxyl groups of the methionine moiety, as well as the 6-amino group of adenine, as being essential for the recognition of AdoMet [49] (Figure 3).

The nature of the enzyme residues involved in AdoMet binding, according to the model proposed in Figure 3, has not yet been unambiguously elucidated. However, the clarification of the primary structure of PCMT type II led to the recognition of three regions of structural similarities between PCMT type I and a group of several enzymes that recognize AdoMet or its derivatives as substrates (see Figure 2) [37]. Motif I had been recognized previously in a conspicuous number of DNA methyltransferases [50,51]. Recent data, obtained by screening a large collection of methyltransferase sequences, led to the conclusion that these regions represent conserved structural motifs, which are useful in the identification and characterization of enzymes which bind either AdoMet or AdoHcy [52]. The functional meaning of these structural similarities does not appear to be confined to methyltransferases alone, since they have been found also in AdoMet

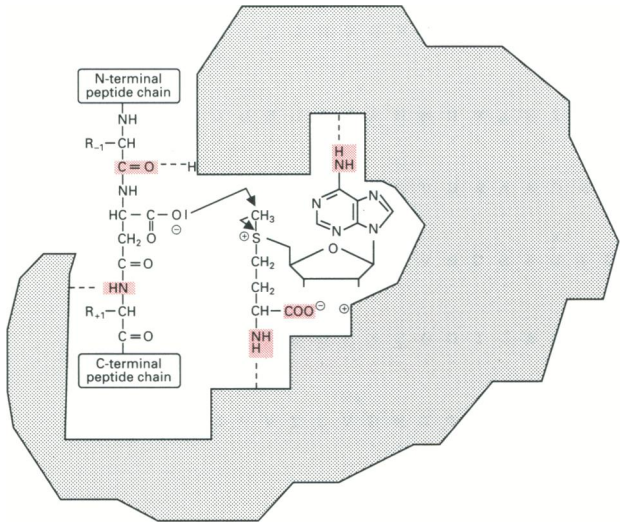

Figure 3 Proposed interaction sites of PCMT type II with AdoMet and the peptide substrate

Grey area represents the putative active site of the enzyme PCMT type II. Binding sites on both AdoMet and the methyl-accepting peptide are shaded in red. Details are provided in the text.

decarboxylase and AdoHcy hydrolase [37,52]. Motif I, defined as a nine-residue block with the consensus sequence (V/I/L)(L/V)(D/E)(V/I)G(G/C)G(T/P)G, appears to be similar to the nucleotide-binding region of protein kinases, as well as of other nucleotide-binding proteins [52]. The consensus sequence for motif II is (P/G)(Q/T)(F/Y/A)DA(I/V/Y)(F/I)(C/V/L), while motif III is defined by the consensus sequence LL(R/K)PGG(R/I/L)(L/I)(L/F/I/V)(I/L) [52].

It is now clear that these motifs may be of importance in the three-dimensional arrangement of the nucleotide-binding site and can be useful in both the recognition of methyltransferases from open reading frames and in the identification of residues involved in the catalysis [37,52].

Although the three-dimensional structure of PCMT type II has not yet been established, data now available on other methyltransferases confirm the potential meaning of the described consensus regions in the binding of ligands. For example, the crystal structure of catechol-*O*-methyltransferase has been elucidated recently [53], thus confirming the location of two regions, homologous to methyltransferase motifs I and III respectively, the first one involved in AdoMet binding and the other one just preceding an Mg^{2+} binding site. In particular the presence of an invariant aspartyl residue, identified in region II of PCMT type II as well as in other AdoMet-binding enzymes, suggests a potential functional role for this residue ([52], see also ref. [54] for a review on sequence similarity studies on methyltransferases).

The 8-azido derivative of AdoMet has been proposed as a tool for the identification of the amino acid residues involved in enzymic catalysis [55]. The application of such a technique to the characterization of the PCMT type II binding site for AdoMet was recently attempted [56], and led to the apparent isolation of a stable adduct of radiolabelled 8-N_3-AdoMet with a decapeptide including residues 113–121. However, it appears that the definitive identification of the putative affinity-labelled residue(s) needs to be confirmed by peptide sequencing, eventually in comparison with data from crystallography.

Structural requirements of the methyl-accepting polypeptide

As far as the characterization of the methyl-accepting residue is concerned, new information has become available since the early 1980s.

It has been clearly established that both natural and synthetic isoaspartyl-containing peptides can be stoichiometrically methyl esterified *in vitro*, with K_m values ranging from 10^{-7} to 10^{-4} M. Conversely, the D-aspartyl-containing peptides so far tested have been found to be poor substrates of PCMT type II, with K_m values, at their best, in the range of $(2.7–4.8) \times 10^{-3}$ M [57,58]. On the other hand evidence for methylation of erythrocyte proteins at the level of D-aspartyl residues has been reported [59–62], as will be discussed further.

The structural requirements of the methyl-accepting substrates have been established by assaying, *in vitro*, *ad hoc* designed oligopeptides, both as substrates and inhibitors of purified PCMT type II. It has been demonstrated that: (i) a hydrogen atom on the amide linkage following the isoaspartyl residue is essential for both recognition and catalysis, since peptides containing either sarcosine [63] or proline [57] at the $isoAsp^{+1}$ position were not recognized as substrates; (ii) the isoAsp residue is not recognized by the enzyme unless its α-amino group is involved in a carboamide bond [57,63,64], thus explaining the lack of substrate activity of natural polypeptides containing L-isoAsp as the N-terminal residue [14]; (iii) the presence of a bulky side-chain residue at the $isoAsp^{-1}$ position enhances its substrate activity [57]; (iv) higher K_m values for peptides containing negatively charged residues at position(s) $isoAsp^{+1}$ and/or $isoAsp^{+2}$ and/or $isoAsp^{+3}$ indicate that the binding of these peptides with the enzyme is unfavoured [57]; (v) the shortest substrate peptide for PCMT type II is the dipeptide t-BOC-isoAsp-Gly [57]; however, its K_m value is one order of magnitude higher than that of the tripeptide t-BOC-isoAsp-Gly-Gly [63,64].

These data, as a whole, are consistent with the model of the binding sites of PCMT type II for both the methyl donor and the methyl-accepting substrates, as reported in Figure 3.

The effect of other structural constraints on the methyl-accepting capability has also been investigated. In ovalbumin, $isoAsp^{101}$-lysozyme and in calcium-free $isoAsp^{56}$-calbindin, higher-order structure does not affect their methyl-accepting capability [57]. On the other hand, neither $isoAsp^{67}$-BS-RNAase [64,65], nor recombinant human $isoAsp^{11}$-EGF [22], are recognized by PCMT type II in their biologically active conformations. Similarly it has been suggested that in recombinant human growth hormone, aged *in vitro*, conformational constraints may prevent methyl esterification of some of its isoaspartyl residues [31]. Therefore the three-dimensional structure of an isoAsp-containing protein plays a crucial role as a specificity determinant for PCMT type II.

THE ERYTHROCYTE MODEL

As already mentioned, the time-dependent spontaneous reactions altering the covalent structure of proteins might occur at such rates that significant damage to long-lived proteins accumulates during the lifetime of the cells. These events can be particularly dramatic in the case of cells and tissues in which protein turnover is extremely low or virtually absent. In this respect human erythrocytes represent an excellent model system in which to study the relationships between post-translational spontaneous modifications of proteins and cell aging, as well as the presence of enzymic systems evolved to prevent or reduce the accumulation of aberrant proteins and the functional consequences of such damage [66–68].

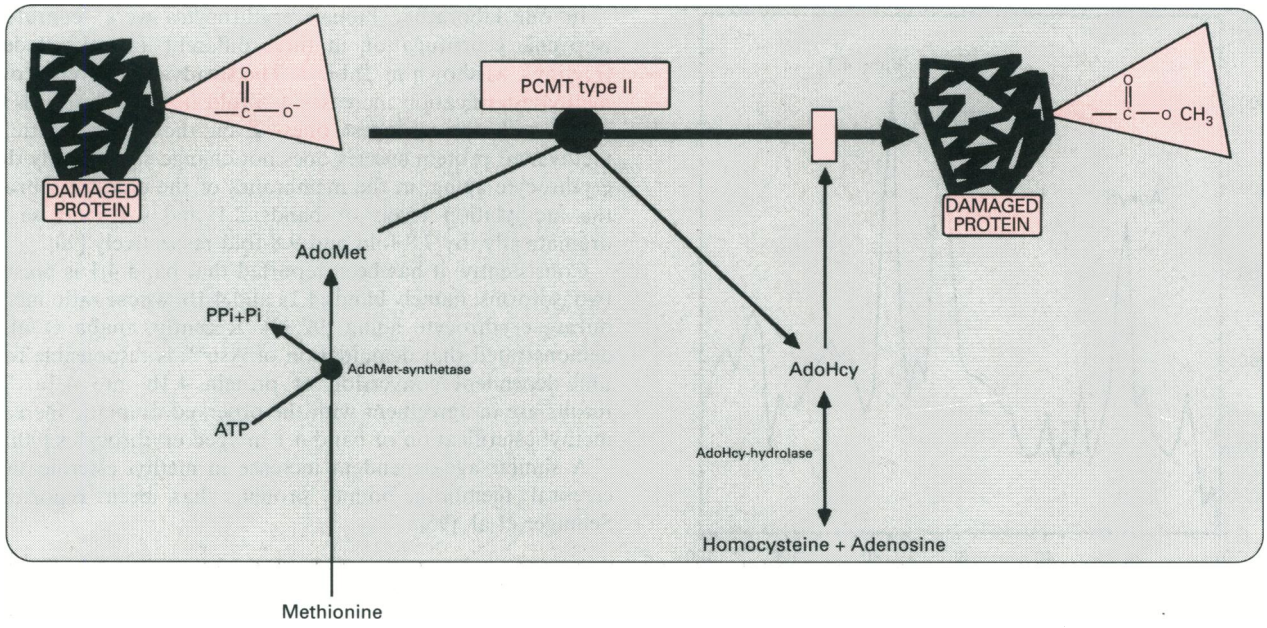

Figure 4 Enzymic methyl esterification of proteins and AdoMet metabolism in intact human erythrocytes

The scheme indicates the methylation process and the metabolic pathways for the biosynthesis of the methyl donor AdoMet and the removal of its demethylated product AdoHcy.

Studies performed *in vitro* showed that the specific activity of the human erythrocyte PCMT type II is among the highest reported for this enzyme in mammalian tissues [69]. Moreover, in these cells membrane proteins represent preferential substrates for this enzyme *in vitro* [70–72].

In order to identify the natural substrates of erythrocyte PCMT type II, a number of approaches have been employed, using mature intact erythrocytes as a model system [61,62,73–75].

As shown in Figure 4, when intact erythrocytes are incubated in the presence of methyl-labelled methionine, the amino acid is rapidly taken up and converted into AdoMet, through the action of a cytosolic AdoMet synthetase, which has been purified and characterized by Oden and Clarke [76]. Endogenous protein substrates are actively methyl esterified by PCMT type II, while the demethylated product of AdoMet, AdoHcy (a competitive inhibitor of all AdoMet-dependent methyltransferases), is rapidly removed by its enzymic hydrolysis to adenosine and homocysteine [77,78]. The latter enzyme plays a key role in the methyl-esterification process, in that all AdoMet-dependent methyltransferases are regulated *in vivo* by the [AdoMet]/[AdoHcy] ratio [79,80].

The erythrocyte AdoMet concentration, ranging from 2.5 μM [81] to 3.5 μM [82], is in the same order of magnitude as the apparent K_m value of PCMT type II for the methyl donor substrate (2 μM) [83]. This relatively low intracellular AdoMet concentration, compared with other tissues [84], is balanced by the fact that the enzymic methyl esterification of proteins represents virtually the only AdoMet-dependent reaction operative in mature erythrocytes, accounting for more than 90 % of utilization of the sulphonium compound [82]. However, it is possible to hypothesize that any situation leading to a depletion of AdoMet and/or an increase of AdoHcy could affect the PCMT type II-catalysed reaction, by altering the [AdoMet]/[AdoHcy] ratio. In this respect, recent data from our laboratory demonstrated an impairment of membrane protein methyl esterification, associated with a significant increase in AdoHcy concentration, in erythrocytes from uraemic patients [81].

PCMT type II natural substrates

Several proteins are methylated in intact erythrocytes, even though to a highly substoichiometric extent [60,61,85]. As for cytosolic proteins, carbonic anhydrase and haemoglobin have been found to be modified to the extent of one methyl group/1.65×10^4 polypeptide chains and three methyl groups/10^6 polypeptide chains, respectively [61]. In haemoglobin 80 % of methyl esters are associated with the β-chain [61,86]. In the α-chain one methylation site has been detected within the sequence 1–30, including two potential sites of formation of altered aspartyl residues such as Asp^6 and Asn^9 [87].

However, the major methyl-accepting proteins are the cytoskeletal proteins, ankyrin (band 2.1) and band 4.1, as well as the integral membrane protein band 3 (Figure 5) [68,73–75].

Besides the methyl-esterified membrane proteins initially described, we recently identified methyl esters of band 4.2 [81], a protein highly homologous to tissue transglutaminase but devoid of any enzymic activity [88], which is believed to play a crucial role in the stability of erythrocyte cytoskeleton.

Protein methylation in cell aging

It has been reported that in the human erythrocyte, after 120 days in circulation, membrane proteins should contain at least one racemized Asx residue per molecule [67,89]. The racemization rate calculated for the cytosolic proteins is much lower. Therefore, the major methyl-accepting substrates in erythrocytes are just the ones that accumulate altered aspartyls, i.e. membrane proteins. These data are consistent with the age-dependent increases in methyl acceptability of membrane proteins, observed independently by Galletti et al. [90] and Barber and Clarke [91].

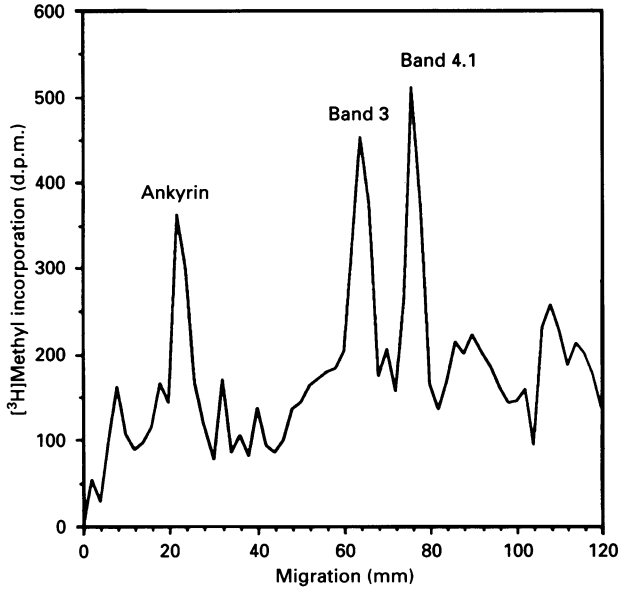

Figure 5 Methyl esterification pattern of membrane proteins in intact human erythrocytes

Membrane proteins, methyl esterified in intact erythrocytes in the presence of labelled methionine, as detailed in [81] and [90], were analysed by SDS/PAGE. The methyl groups incorporated in each individual band were evaluated by counting the radioactivity extracted from 2-mm-thick slices of the gel. The assignments of radioactivity peaks to membrane protein species were obtained by comparison with samples run in parallel and stained with Coomassie Brilliant Blue.

In our laboratory human erythrocytes were separated by isopycnic centrifugation in three different age-related density fractions, as shown in Table 1. The steady-state level of overall methyl esterification increases 4–5-fold in the oldest cells compared with the youngest ones. Even though the pattern of methylated protein species does not change significantly during erythrocyte aging, in the membranes of the oldest erythrocytes the methylation levels of bands 2.1 and 4.1 increase more dramatically, by 7.8-fold and 9.8-fold respectively [90].

Consistently, it has been reported that band 4.1 is present in two isoforms, namely bands 4.1a and 4.1b, whose ratio increases during erythrocyte aging [92,93]. Recently, Inaba et al. [94] demonstrated that deamidation of Asn^{502} is responsible for the time-dependent conversion of protein 4.1b into 4.1a. These results are in agreement with the observed dramatic increase in methyl esterification of band 4.1 in aged erythrocytes [90].

A similar age-dependent increase in methyl esterification of cerebral membrane-bound proteins has been reported by Sellinger et al. [95].

Identification of the methyl-esterified residues

The question is now: what kind of altered aspartyl residues are methylated in intact erythrocyte membrane proteins? Are these D-aspartyl or L-isoaspartyl residues? Initial evidence for enzymic methylation of D-aspartyl residues was provided by the isolation of labelled D-aspartate-β-methyl esters from membrane protein hydrolysates [59,60]. More recently the stereoconfiguration analysis of the aspartyl residues released from proteolytic digestion of membrane proteins methylated in intact erythrocytes

Table 1 Increased methyl esterification of membrane proteins during human erythrocyte aging

Erythrocytes were separated into age-related fractions (A–C) by isopycnic centrifugation. Membrane protein methyl esterification was performed on intact cells by incubation in the presence of [^{3}H-methyl]methionine. Membrane proteins were then isolated and analysed by SDS/PAGE. Gels were cut into 1 mm slices and radioactivity incorporated into individual protein bands was measured after extraction from the gel slices. The assignment to each proper membrane protein band was performed by comparison with gel runs of parallel samples stained with Coomassie Brilliant Blue and densitometrically scanned. S.D., standard deviation. For details see ref. [90].

Fraction	Distance from gradient top (%)	Relative density (d)	Total [^{3}H]methyl incorporation		Polypeptide bands							
					2.1		3		4.1		4.5	
			Amount*	Increase†	Amount*	Increase†	Amount*	Increase†	Amount*	Increase†	Amount*	Increase†
A	5–10	< 1.050	1935 ± 360	–	130 ± 29	–	153 ± 43	–	216 ± 63	–	342 ± 60	–
B	40–50	1.080–1.090	4675 ± 600	2.40	262 ± 40	2.00	447 ± 65	2.90	488 ± 76	2.25	788 ± 100	2.30
C	85–95	< 1.123	9500 ± 619	4.90	1016 ± 203	7.80	731 ± 148	4.80	2031 ± 265	9.40	1220 ± 79	3.60

* Incorporation expressed in c.p.m./0.1 mg of protein.
† Increase (fold).

Table 2 Highly racemized proteins as substrates *in vitro* of PCMT type II

Experimental details are given in refs. [96,97]. Abbreviation: n.d., not determined.

	Total aspartate (nmol/mg)	Extent of racemization		D-Aspartate content (nmol/mg)	[^{14}C]Methyl incorporation (nmol/mg)
		D/D + L (%)	D/L (%)		
Lenticular water-soluble proteins	67.7	4.16 ± 0.20	–	2.8	2.4
Lenticular water-insoluble proteins	40.1	16.76 ± 0.19	–	6.7	4.7
Tooth dentin	n.d.	–	4.9	n.d.	19.37
Tooth enamel	n.d.	–	6.8	n.d.	10.04

showed that at least 22% of the methyl groups, associated with bands 2.1 and 4.1, are D-aspartyl-β-methyl esters [58]. Indirect indication of D-aspartyl as an important methylation site in age-damaged proteins had also been inferred from studies by Manna et al. [96,97], utilizing highly racemized eye lens proteins. When these proteins were methylated *in vitro* in the presence of purified PCMT type II, the number of methyl groups incorporated was of the same order of magnitude as the D-aspartate content (Table 2).

The relatively low yield of D-aspartate-β-methyl esters from methyl esterified erythrocyte membrane proteins, and the evidence that an L-isoaspartyl residue is by far the major methyl esterification site widely characterized *in vitro*, may indicate that L-isoAsp residues are also predominantly methylated by PCMT type II in the membrane proteins of intact erythrocytes. Unfortunately, the direct demonstration of the presence of L-isoaspartyl-α-methyl esters in erythrocyte membrane proteins may be hampered by the relative difficulty with which proteolytic enzymes cleave isopeptide bonds. It is also possible to hypothesize that D-aspartate-β-methyl esters are more stable than L-isoaspartyl-α-methyl esters, either in the intact cell or during isolation procedures, thus complicating the identification and characterization of the latter residues.

Enzymic methyl esterification of calmodulin

The calcium-binding protein calmodulin is one of the major methyl-accepting substrates so far identified in intact erythrocytes [98]. Methylation was found to occur at residues Asp^{2} and $Asp^{78/80}$, which are not part of any Ca^{2+}-binding domain. Moreover additional methyl esterified Asx residues have been identified in the sequence region 90–105, corresponding to calcium-binding loop III, residues 93–105 and region 110–141, which includes calcium-binding loop IV (residues 129–140).

Studies carried out using calmodulin purified from bovine brain revealed the key role of calcium ions in the generation of methylatable residues. As a matter of fact when calmodulin is methylated *in vitro*, upon preincubation in the presence of EGTA, methylation occurs at the same residues methyl esterified in intact erythrocytes [99]. Conversely, when this protein is methyl esterified, after preincubation in the presence of millimolar Ca^{2+}, methylation occurs only at the level of Asp^{2} and $Asp^{78/80}$ [100]. These results suggest that the presence of calcium prevents the formation of methyl-accepting altered aspartyls by reducing calmodulin conformational flexibility at the level of Ca^{2+}-binding sites. The low Ca^{2+} concentration in human erythrocytes ($< 0.1\ \mu M$) may explain the occurrence of methylatable sites at the level of III and IV calcium-binding domains observed in intact erythrocytes [98].

PROTEIN METHYL ESTERIFICATION OF ALTERED ASPARTYL RESIDUES IN OTHER MODEL SYSTEMS

Cells and tissues other than erythrocytes have also been utilized as model systems for studying the biological role(s) of PCMT type II. In general these models provide an example of protein aging *in vivo*, and/or they are suitable for investigating the post-biosynthetic processing of methyl esterified proteins.

The eye lens

The mammalian eye lens has been utilized for studying the aging process both at cellular and molecular levels. The lens is composed of layers of anucleated fibre cells, concentrically arranged by age, which derive from the epithelium located at the anterior part underneath the capsule. New fibres are always laid down at the periphery (lens cortex), so that progressively older fibres are found as one moves axially toward the centre of the tissue (lens nucleus). Lens fibre maturation is accompanied by loss of nucleus and organelles, therefore protein synthesis and turnover rapidly ceases after cell differentiation. Since the tissue never sheds its cells, lens proteins reach exceptionally high ages, accumulating a large number of age-related modifications including Asx racemization and deamidation [101–105].

Initial evidence indicated the presence of an active PCMT type II and its endogenous protein substrates in crude soluble extracts of bovine [106] and human [107] eye lenses. Methyl acceptability of endogenous protein substrates was found to increase as the eye lens ages, in parallel with the age-dependent increase of protein damage [107]. Furthermore, a high specific activity of PCMT type II was detected in both normal and yellow cataractous lenses, but not in brown cataractous lenses, where a very low enzyme activity could be measured [107].

Later on in our laboratory, intact bovine lenses, cultured *in vitro*, were utilized for studying both AdoMet metabolism and protein carboxyl methylation of type II. When these lenses were incubated for 8 h in the presence of [^{14}C]methyl-labelled methionine, about 23% of the total radioactivity taken up was found associated with AdoMet, and protein methyl esters could account for approximately 4% of total radioactivity incorporated into the lens [97]. Further studies have shown that protein methyl esterification in lens nucleus unexpectedly decreased significantly, in spite of the rise of molecular damage in the proteins of this cell fraction [108]. This result was explained by the decreased activity of both enzymes AdoMet synthetase and AdoHcy hydrolase, which affects PCMT type II activity through a decrease in [AdoMet]/[AdoHcy] ratio.

Xenopous laevis oocytes

Another interesting model system investigated by O'Connor et al. is the oocyte of the amphibian *Xenopous laevis*, in which an active PCMT type II has been detected [109,110], showing the same substrate specificity for deamidated and racemized polypeptides as the homologous human erythrocyte PCMT type II. Two forms of the enzyme have been detected with molecular masses of 27 kDa and 34 kDa respectively. The first one is present in both nucleus and cytoplasm, the second one only in the cytoplasm [109]. A ubiquitous subcellular distribution of the endogenous substrates has been reported [109,110], calmodulin being one of the best methyl-accepting substrates [111]. This protein is methyl esterified in intact oocytes to an extent (2×10^{-4} esters/mol of protein) very close to that found in erythrocytes [111].

Interestingly it has been shown that proteins methylated *in vivo*, such as nuclear proteins, are poor substrates for PCMT type II when assayed *in vitro*. Conversely, proteins which were known to be inaccessible to the methytransferase *in vivo*, are highly methylated *in vitro*. These results suggest that PCMT type II can prevent the accumulation of altered aspartyl residues in proteins in intact cells [109].

Oocytes can be microinjected with exogenous compounds, the metabolism of which can be investigated under conditions mimicking those existing *in vivo*. This technique has been utilized to load oocytes with detectable amounts of peptides and proteins containing altered aspartyl residues. Isoaspartyl-peptides showed inhibitory activity toward methyl esterification of co-injected ovalbumin, which parallels the ability of these peptides to act as substrates *in vitro* [112].

HeLa cells

HeLa cells have also been utilized as a model system, in order to detect modifications in the levels of protein methyl esterification in response to stress conditions favouring protein damage, such as heat shock. An increased rate of protein methyl esterification, without any induction of methyltransferase synthesis, was detected as a result of the heat shock treatment [113]. These results indicate that: (1) PCMT type II is a housekeeping enzyme, since its gene transcription level is not affected by stress-inducing agents; (2) the methyltransferase is normally not saturated with endogenous substrates, confirming the findings obtained with different systems [79,111,112,114] (the physiological meaning of these observations will be discussed in the next section); and (3) the temperature-dependent increase in protein methylation could reflect either the formation of new methyl-accepting sites in protein substrates, or the increased accessibility of pre-existing sites due to protein unfolding and denaturation [113].

These results, as a whole, confirm the broad specificity of PCMT type II toward polypeptides containing altered aspartyl residues and are in agreement with the proposed biological role(s) of this enzyme.

BIOLOGICAL ROLE(S) OF PCMT TYPE II

The enzyme PCMT type II was first identified in 1965 by Axelrod and Daly in bovine pituitary gland [115] and named 'methanol-forming enzyme', since it was thought to catalyse the hydrolysis of AdoMet to methanol and AdoHcy. Later, in 1969, Kim and Paik [116] and Liss et al. [117], independently, described an enzymic activity catalysing the formation of methyl esters of free carboxyl groups in protein substrates which are hydrolysed yielding methanol. The two enzymic activities, which share methanol as a common end product, were subsequently found to be identical [83].

The apparent reversibility of the reaction, which actually reflects the instability of the methyl esters, was initially interpreted as an on/off mechanism by which a variety of cellular events could be regulated (reviewed by Paik and Kim in 1980, [2]). However, none of the hypothesized regulatory functions has been unequivocally demonstrated, although they have been intensively explored, particularly in the erythrocyte. As a matter of fact, the activity of anion transport protein band 3 was found to be unaffected by its enzymic methyl esterification [75]. In addition, inhibition of protein carboxyl methylation in intact erythrocytes, by AdoHcy, failed to show any changes in the shape, osmotic fragility or deformability properties of the cells [79,118]. Based on this body of evidence, Clarke and O'Connor in 1983 suggested that: "Regulatory roles for methylation in the erythrocytes, if they exist at all, are most likely secondary to the major functions [of this enzyme]" [119].

Three major results have been crucial for the understanding of the physiological role of PCMT type II: (1) its broad substrate specificity; (2) the substoichiometry of methylation reaction; and (3) the identification of altered aspartyl residues as the methylation sites. These facts led to the hypothesis that this methyltransferase plays a major role in the processing of age-damaged proteins. The methylation reaction can indeed be involved, either as part of a mechanism for the repair of structurally altered L-isoaspartyl/D-aspartyl-containing proteins, or as a signal for selective recognition of such damaged proteins for their further catabolism.

PCMT type II-mediated repair of isopeptide bonds

The concept of protein repair is not new, since a number of mechanisms have been characterized, which are capable of minimizing spontaneous molecular damage. For example a peptidyl prolyl *cis–trans*-isomerase, which *in vitro* catalyses the refolding of urea-denatured ribonuclease A, has been identified [120]. Similarly, it has been shown that methionine sulphoxide residues in proteins may be enzymically reduced back to normal methionine residues [121]. Molecular chaperones, which include several heat-shock and stress proteins, can be considered a further example of protein repair. Besides their role in the folding of *de novo* synthesized polypeptides, this class of proteins can be involved also in maintaining the folded state of partially denatured proteins under stress conditions [122].

The role of PCMT type II in the repair of isopeptide bonds, first proposed by Aswad [123], has been demonstrated by independent research groups, using isoaspartyl-containing peptides and proteins as model systems [24,25,58,64,65,124–127].

In 1988 our group described the PCMT type II-mediated repair of the pentadecapeptide α-16 from tryptic hydrolysis of naturally deamidated BS-RNAase [64,65]. The peptide α-16 [BS-RNAase$^{62-76}$(isoAsp^{67})] was incubated in the presence of purified PCMT type II and AdoMet and the assay mixture was analysed at different times. As shown in Figure 6, after 12 h of incubation a significant conversion of the isopeptide into its normal counterpart, peptide α-17 [BS-RNAase$^{62-76}$(Asp^{67})], was observed. In a time-course experiment, the characterization of the reaction intermediates, as the methyl ester and the succinimidyl derivatives of peptide α-16, allowed us to draw up the mechanism for the repair process depicted in Figure 7: (1) enzymic methylation of the α-carboxyl group of the isoAsp^{67} residue; (2) hydrolysis of the methyl ester, yielding methanol and the succinimidyl intermediate; and (3) cleavage of the cyclic intermediate to both normal α-17 and the original α-16 peptides, in the ratio 3:7. Therefore, since only 30% of the isopeptide is converted into

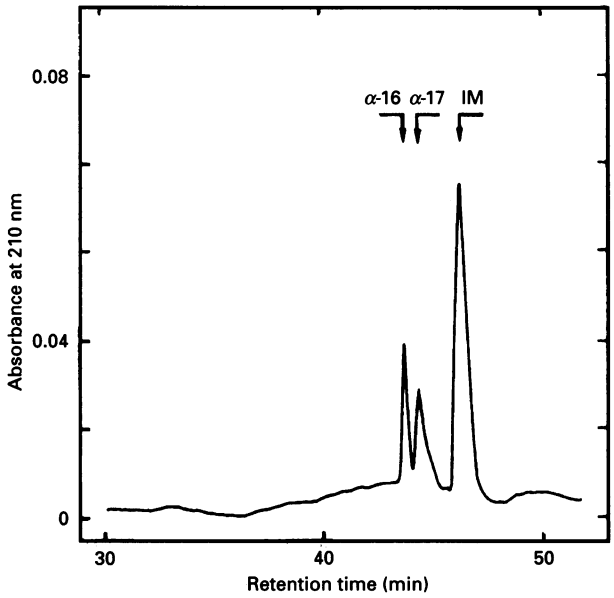

Figure 6 PCMT type II-mediated conversion of an isoaspartyl-containing peptide into its normal aspartyl counterpart

Peptide α-16 [BS-RNAase$^{62-76}$ (isoAsp^{67})] was incubated for 12 h in the presence of purified PCMT type II and methyl-labelled AdoMet. The assay mixture was analysed by reverse-phase h.p.l.c. For further details see [65]. Abbreviations: IM, succinimidyl derivative of peptide α-16; α-17, normal peptide [BS-RNAase$^{62-76}$ (Asp^{67})]. Reproduced with permission from [65]. Copyright 1988 American Chemical Society.

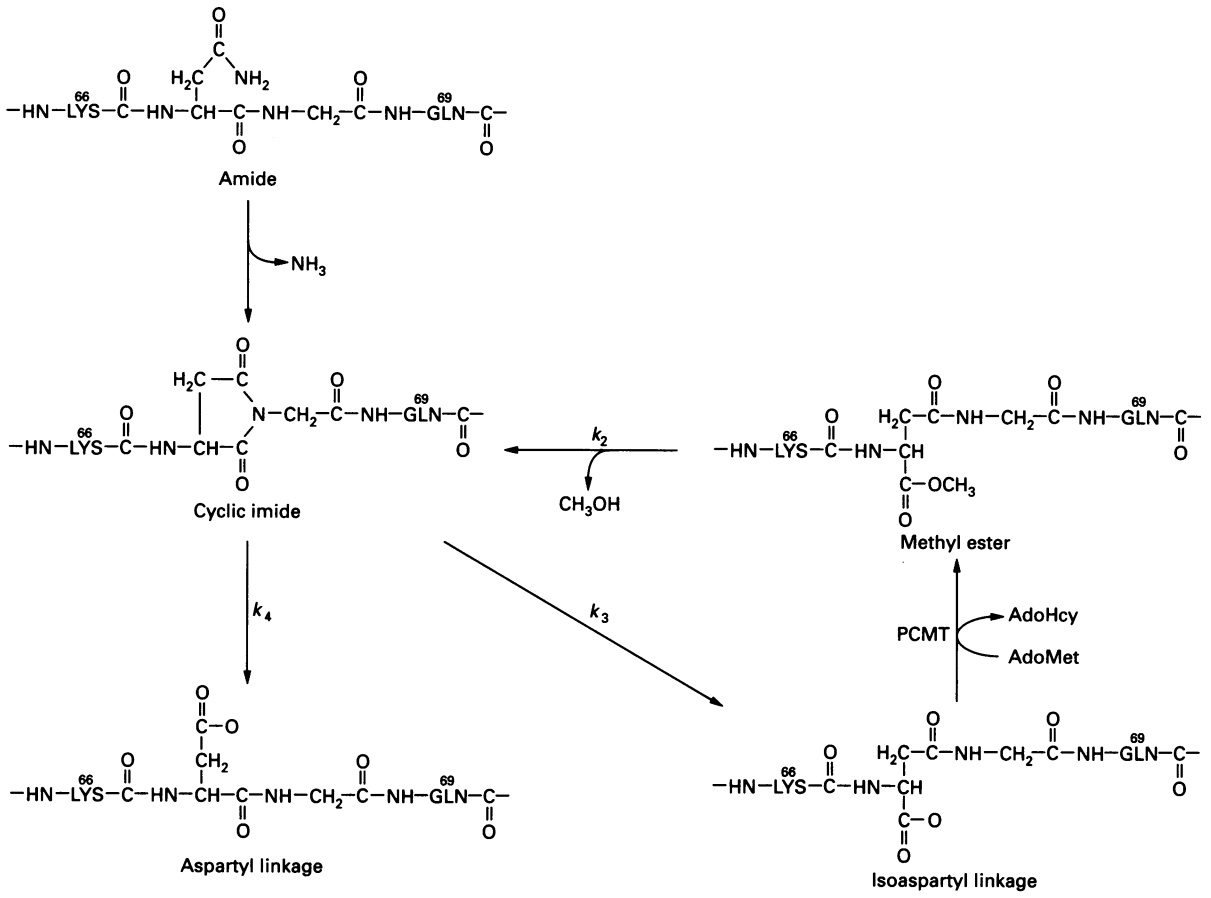

Figure 7 Repair mechanism of an isopeptide bond in an isoaspartyl-containing peptide

Peptide α-16 [residues 62–76 (isoAsp^{67})] from BS-RNAase, is generated through deamidation, *in vivo*, of Asn^{67}. Enzymic methyl esterification of this peptide is followed by non-enzymic rapid hydrolysis of the methyl group yielding methanol and the succinimidyl derivative. The spontaneous opening of the succinimidyl ring leads to the formation of either the normal peptide α-17 [residues 62–76 (Asp^{67})], or the initial isoaspartyl-containing peptide. Repeated methylation/demethylation cycles allow quantitative conversion. Rate constants k_2, k_3 and k_4 are defined in [65]. Reproduced with permission from [65]. Copyright 1988 American Chemical Society.

normal α-17 at each methylation step, the effective repair seen after incubation for 12 h occurred through repeated methylation cycles.

An elegant demonstration of the effectiveness and utility of this repair function was provided by Aswad's group, with the calcium-binding protein calmodulin [24,25]. Incubation of this protein, for 30 days under physiological conditions, induced deamidation of Asn residues which generates two calmodulin forms which can be resolved by gel electrophoresis under non-denaturing conditions: the Asp form (form B), which is not a substrate for PCMT type II, and the isoAsp form (form A), which becomes stoichiometrically methyl esterified *in vitro*. Form A shows only 10% of the activity of calmodulin in the stimulation of protein kinase, while form B more closely resembles the native protein, exhibiting 40% of its original activity. Methyl esterification of form A, *in vitro*, results in a significant repair of isopeptide bonds, restoring about 50% of the activity of the native protein.

Methylation of calmodulin has also been reported to occur in intact erythrocytes, as already discussed, with a stoichiometry of 2.5×10^{-4} methyl groups incorporated/polypeptide chain [98]. This result suggests that either this protein can be partially protected, *in vivo*, against extensive deamidation and/or that the deamidated molecules are continuously repaired and therefore the low stoichiometry of methyl esterification, measured in intact cells, reflects the steady-state level of the intermediates of the repair process [98].

The role of PCMT type II in the repair of damaged proteins in erythrocytes and other tissues, particularly where protein turnover is reduced or virtually absent, requires that this enzyme remains active during the life span of these cells. Indeed, its specific activity does not change in either aged erythrocytes [90] or in the nuclear part of eye lens [108]. Moreover, it has been recently reported that PCMT type II is able to catalyse the automethylation of age-damaged sites in its own sequence [128], at the level of deamidation-susceptible site(s) [37]. Therefore, it is possible to infer the existence of self-repair of the enzyme, which is needed to maintain the efficiency of the repair system itself, particularly in long-lived cells.

The data so far discussed demonstrate that: (1) L-isoaspartyl polypeptides are recognized and modified by PCMT type II, either *in vitro* or in intact cells; (2) methylation-mediated repair of these L-isoaspartyl sites does occur *in vitro*; (3) enzymic methylation of a certain subset of erythrocyte proteins does occur, in intact cells, at the level of D-aspartyl residues.

Efforts have been made, recently, to evaluate the quantitative

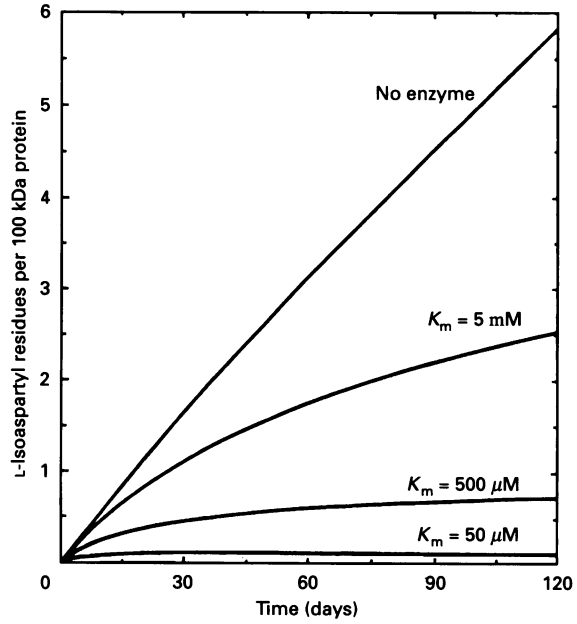

Figure 8 Mathematical simulation of the ability of PCMT type II to prevent the accumulation of L-isoaspartyl residues in erythrocyte proteins

A computer model was set up by Lowenson and Clarke [57] to calculate the accumulation of L-isoaspartyl residues, *in vivo*, in a typical 100 kDa protein, during the 120-day erythrocyte life span. The four curves refer to accumulation of L-isoAsp residues in the absence and in the presence of the methyltransferase with K_m values for the protein substrate ranging from 5×10^{-5} to 5×10^{-3} M. Reproduced from [57] with the permission of the American Society for Biochemistry and Molecular Biology.

contribution of protein methylation in the repair of damaged aspartyl residues *in vivo*. To address this question mathematical models have been developed, which simulate the processes of formation of altered aspartyls and their PCMT type II-dependent repair [56,57,129].

Lowenson and Clarke considered, as a typical example for a computer simulation, a 100 kDa erythrocyte protein of average composition (52 Asp and 35 Asn residues), in which 1 % of Asx residues are labile ($t_{\frac{1}{2}}$ = 50 days), 25 % are intermediate ($t_{\frac{1}{2}}$ = 400 days), and 74 % are stable ($t_{\frac{1}{2}}$ = 2000 days). They calculated that, in the absence of any repair mechanism, the rate of spontaneous formation of L-isoaspartyl residues would generate about six such residues/protein molecule after 120 days of erythrocyte life span. The presence of such a number of isoaspartyl sites in a protein can be predicted to significantly affect both its structure and function. In the presence of an active repair mechanism, the number of these altered aspartyl residues would decrease to a value ranging from 2.53 to 0.0011/protein molecule, depending on the K_m value of PCMT type II for the individual protein substrates (ranging from 5×10^{-3} to 5×10^{-5}) (see [57] and Figure 8).

The repair process of an L-isoaspartyl residue, as depicted in Figure 7, certainly appears to allow the quantitative conversion of an isopeptide bond into its normal counterpart, but would not be able to restore the original Asn residue. In this sense, a 'true repair' is possible only in the case of L-isoaspartyl residues originating from isomerization of L-aspartyl residues. It should be pointed out, however, that in some instances the restoration of an α-peptide bond is *per se* sufficient to re-establish a significant part of the biological activity lost in the deamidation process [24,25]. The existence of a hypothetical 'amidating enzyme', able to regenerate the original amide group of an Asn residue, has not been demonstrated yet.

Can D-aspartyl residues be repaired by PCMT type II?

As for methylation of D-aspartyl residues, the processing of D-aspartyl-β-methyl esters is still unclear. However, two major hypotheses have been proposed.

(1) The enzymic methyl esterification could be the first step in the restoration of the normal L-configuration through the action of a postulated racemase-esterase [59]. It should be pointed out, however, that no racemase-esterase has been identified so far.

(2) Enzymic methyl esterification could contribute to a significant reduction of D-aspartyl residues in proteins, through a mechanism analogous to the repair of L-isoaspartyl sites. This mechanism would consist of spontaneous methyl ester hydrolysis, yielding the D-succinimidyl derivative, whose opening would generate a substantial amount of D-isoaspartyl residues. This hypothesis is supported by computer simulation analyses using mathematical models. According to this proposal, methylation of D-aspartyl residues may contribute to reducing by 2–5-fold the accumulation of these residues during the 120-day erythrocyte life span [58]. These models predict that D-isoaspartyl residues may accumulate within erythrocyte proteins, as a product of D-aspartyl repair. Unfortunately no data are available, at present, regarding either the abundance of D-isoaspartyl residues in proteins, or the ability of PCMT type II to recognize these residues [58]. The presence of such D-isoaspartyl sites is indeed expected to be less detrimental to protein structure and function than both D-aspartyl and L-isoaspartyl sites, as has been proposed by Murray and Clarke [10]. These authors, by studying molecular models, observed that the position of the free carboxyl group space of a D-isoaspartyl carboxyl residue is in fact similar to that of a normal L-aspartyl residue.

Potential role(s) in protein degradation

In 1974 Robinson and Rudd proposed that deamidation of Asn and/or Gln residues in proteins may represent a biological clock, determining the mean lifetime of certain proteins *in vivo* [130]. As we previously discussed it is now clear that a substantial amount of Asn residues are actually protected against deamidation, due to protein structural constraints [13]. However, an inverse correlation has also been reported between protein Asn + Gln content and their stability [131]. In this respect it has been proposed that at least for a subset of deamidated proteins the formation of the methyl ester derivative may act as a signal for their selective proteolytic degradation [61].

Alternatively, it is possible to speculate that PCMT type II-mediated conversion of an isopeptide bond into a normal one would facilitate the complete hydrolysis of peptides derived from deamidated proteins. The latter hypothesis is supported by the following observations: (1) isopeptide bonds are not readily cleaved by proteases [132]; (2) (isoaspartyl-containing oligopeptides have been actually isolated from human urine, suggesting that they may represent the final product of proteolytic cleavage at the level of an isopeptide bond [133]; (3) short peptides are, *in vitro*, preferential substrates for methylation, in that they can more easily adopt the right alignment with the enzyme binding site [63]; (4) a post-proline endopeptidase has been identified in human erythrocytes, which cleaves succinimidyl-containing peptides at a rate 10-fold higher than that of prolyl peptides [134].

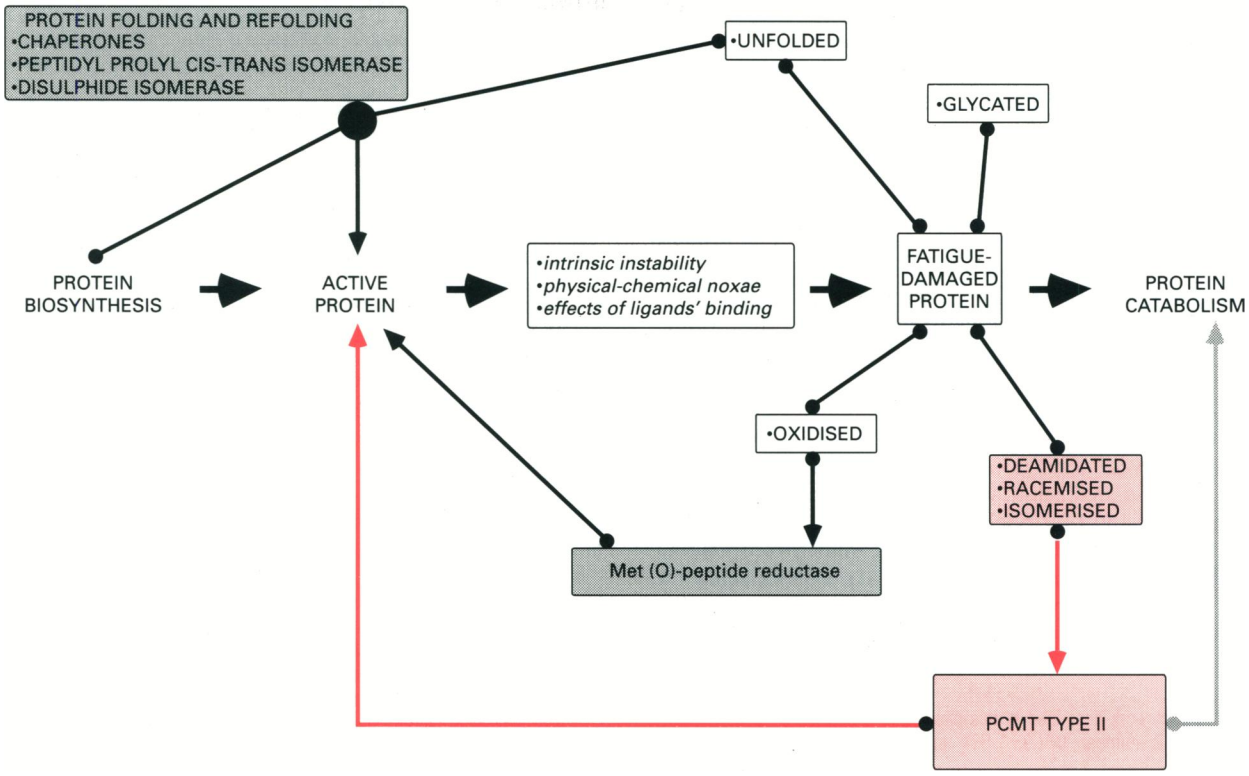

Figure 9 Overall hypothesis on the role of the enzyme PCMT type II in the post-biosynthetic processing of fatigue-damaged proteins

The scheme illustrates the factors involved in the spontaneous generation of fatigue damage in proteins (white boxes). In evidence, red shading illustrates the enzyme PCMT type II and its role in the repair of altered aspartyl residues due to Asn deamidation and/or Asp racemization/isomerization reactions. The hypothesized role of PCMT type II-catalysed methyl esterification, as a signal for recognition and commitment to catabolism of damaged polypeptides, is shown by a grey arrow. Other key proteins which recognize different types of fatigue damage are also shown (grey shaded boxes).

Regulation of PCMT type II activity

In order to ensure the adaptation of the repair system to increased formation of altered aspartyl residues in proteins under certain conditions, it appeared to be of some importance to establish whether or not PCMT type II is saturated *in vivo* by its substrates. Two considerations should be taken into account in this respect. First, membrane protein methylation increases severalfold in human erythrocytes during cell aging, thus paralleling the predicted increase of the damaged aspartyl sites in proteins [90,91]. Secondly, the rate of methyl esterification of exogenous deamidated calmodulin could be increased up to 8-fold by increasing the concentration of the protein microinjected into *Xenopous laevis* oocytes. This indicates that the methylation system is in fact not saturated *in vivo* by the endogenous methyl-accepting substrates [111].

An important aspect concerns the regulatory role of the intracellular [AdoMet]/[AdoHcy] ratio in the activity of PCMT type II and its possible influence on the efficiency of the repair mechanism(s). For example the reduction of protein methylation in the nuclear aged part of cultured bovine lenses is likely to be due to a decreased AdoMet/AdoHcy concentration ratio [108]. Moreover a condition leading to increased AdoHcy concentration has been reported to actually occur *in vivo* in patients with chronic renal failure [81]. In these subjects we observed a significant reduction of erythrocyte membrane protein methyl esterification, due to a dramatic rise in intracellular AdoHcy concentration [135].

CONCLUSIONS AND PERSPECTIVES

Recent developments in protein microcharacterization techniques allowed the identification of protein deamidation and isomerization processes at Asx residues. This in turn provides crucial information on both the substrate specificity and the biological role of PCMT type II, which had been misinterpreted for many years.

Figure 9 summarizes the overall hypotheses on the biological role(s) of PCMT type II with respect to its function in the maintenance of protein stability and/or in protein degradation.

Future investigations should be focused on the following problems: (i) conditions able to influence the rate of occurrence of protein fatigue damage of Asx residues *in vivo*; (ii) where and how structural alterations, induced by the appearance of abnormal aspartyl residues, affect each specific protein function; and (iii) the extent of repair carried out *in vivo* by PCMT type II-catalysed methylation.

A comprehensive understanding of the functional implications of protein fatigue related to Asx damage, which in the erythrocyte and in the eye lens parallels cell aging, is instrumental to the understanding of the metabolic fate of such altered polypeptides. At present not all the effects of protein fatigue connected to Asx damage are known. We have summarized some examples of typical protein functions, such as enzyme activities, which can be affected; however, the influence of protein fatigue on different properties, including immunogeneity, appear to be worth investigating. In this direction, recent studies indicate that certain

immunological properties of short peptides may actually be influenced by aspartyl racemization [136].

Finally we ought to mention some prospective biotechnological applications of PCMT type II. Protein fatigue does not only occur when these macromolecules are in the living organisms, but also when they are shelf stored [22,137]. Today's industry has the capability of producing unlimited amounts of recombinant proteins, whose intrinsic instability can be responsible for the formation of deamidated/isomerized side products. Interestingly, useful selective chemical methods have been developed recently by Carter and McFadden for detecting either β-isoaspartyl residues [138] or their succinimidyl precursors [139]. However, L-isoaspartyl content in many aged proteins may be quantitatively below the detection limit of these methods [138]. The use of PCMT type II, as a tool for characterization of deamidated/isomerized Asx in proteins, has been proposed, and it appears to be suitable even when isoaspartyl content is low, but still sufficient to cause significant structural and functional protein alterations [22,31,130,140]. Such utilization of PCMT type II has been successfully attempted in the case of human recombinant EGF [22] and human growth hormone [31], thus opening the way to a wide range of potential applications, including quality control of protein products for human nutrition and disease therapy.

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