D-Aspartate Content of Erythrocyte Membrane Proteins is Decreased in Uremia: Implications for the Repair of Damaged Proteins

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Abstract. The authors of this article have demonstrated that erythrocytes from patients affected by either chronic renal failure or ESRD, conditions associated with erythrocyte membrane disorders, show reduced levels of methyl esterified membrane proteins because of elevated *S*-adenosylhomocysteine concentration. The enzyme proteine L-isospartyl (D-aspartyl) *O*-methyltransferase, responsible for the bulk of this methyl esterification, is implicated in the repair of proteins containing isomerized and racemized aspartyl residues, which arise from L-asparaginyl and L-aspartyl residues. The presence of these altered residues, spontaneously generated during protein aging, can adversely affect protein function. The amount of D- and L-aspartyl residues (and their isomerized derivatives) in erythrocyte membranes from hemodialysis patients was determined. The total level of D-aspartyl derivatives (D-Asx) actually was found to be lower than in controls. In contrast, neither the abundance of several other amino acids, nor of total non-Asx D-amino acids, differs between patients and controls. Mathematical simulation of relevant reactions supports the hypothesis that these effects reflect the lessening of the normal D-isoaspartyl residue accumulation that occurs as a side reaction in the methyltransferase-induced repair process. This evidence is the first that D-Asx content is influenced *in vivo* by L-isoaspartyl (D-aspartyl) *O*-methyltransferase activity and can be significantly altered in a disease where this activity is inhibited, thus representing a red flag in a disrupted circuit. (J Am Soc Nephrol 8: 95–104, 1997)

Human red blood cells (RBC) provide an excellent model for studying nonenzymatic protein damage, its possible repair, and its effect on cellular function and aging (1,2). We have been interested in the influence of racemization processes on erythrocyte proteins in the spontaneous reactions that convert the L-amino acids incorporated during ribosomal biosynthesis to D-amino acids (3–5). In proteins, aspartic acid and asparagine (Asx) residues appear to be particularly susceptible to racemization reactions (4,5). This susceptibility results, at least in part, from the spontaneous formation of racemization-prone succinimide residues from Asx residues (4–6). D-succinimide residues are readily hydrolyzed to give a mixture of D-isoaspartyl (D-isoAsp) and D-aspartyl (D-Asp) residues in a ratio of approximately 3:1 (7). The presence of these racemized residues may affect protein function and cell physiology. For example, β-amyloid protein from the brains of patients with Alzheimer's disease (AD) has been shown to contain high levels of D-Asp and D-isoAsp residues (8–10). Because these residues can significantly reduce the solubility of β-amyloid *in vitro* (8,11), they may influence the accumulation of β-amyloid in the brain, which appears to be critical in the development of AD. Other proteins containing high levels of D-Asp residues include dentine in teeth (12) and the crystallins of the eye lens (13). Although particularly abundant in the aggregated proteins in cataracts, the role of D-Asx residues in the progression of this disease, as in AD, is yet to be elucidated.

Protein damage is especially important in RBC because *de novo* protein biosynthesis is permanently switched off, making replacement of "defective" molecules impossible (1). Interestingly, a remarkable number of pathologies exist in which RBC membrane proteins are altered in some way. This presumably reflects the fact that the RBC membrane and the underlying cytoskeleton are organized into a highly integrated network responsible for regulating exchanges with the extracellular environment, as well as maintaining cell shape and deformability (14).

We have recently been studying the spontaneous formation of abnormal aspartyl residues in RBC membrane proteins and their contribution to so-called "protein fatigue damage" (1,2). Of particular interest is the role of the L-isoaspartyl (D-aspartyl) *O*-methyltransferase (PCMT; EC 2.1.1.77), which, by methylating certain damaged Asp residues in RBC membrane proteins, is thought to lead to their repair to the original L-Asp

Received July 22, 1996. Accepted September 10, 1996.
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1046-6673/0801-0095$03.00/0
Journal of the American Society of Nephrology

form (7, 15–17). However, we found that in RBC from patients with either chronic renal failure or ESRD, conditions characterized by prominent derangement of RBC membrane function (18,19), methyl esterification of membrane proteins is significantly impaired (20).

We have now investigated the composition of RBC membrane proteins from patients undergoing chronic hemodialysis therapy for ESRD to determine the relationship between methyl esterification and the content of racemized residues using an analytical method that drastically reduces the additional racemization inevitably induced by hydrolysis procedures to permit quantitation of the low levels of D-Asx in these proteins.

Methods

Patients

Chronic hemodialysis patients, along with a group of age- and sex-matched healthy control subjects, were selected.

The mean age of the patient group was 41 ± 15 years (range 21–73), the RBC count was 3.6 ± 0.4 × 10^6 cells/μl, hemoglobin was 10.5 ± 0.4 g/dL, and the hematocrit was 32 ± 2.9%. In our dialysis unit, the Kt/V, calculated in each patient at monthly intervals, was found to be not less than 1.4 (which represents our previously set target value) in the 6 months prior to the experiment. Dialysis prescribed to our patients was conventional—three sessions per week, 4 hours per session—performed with a bicarbonate dialysis solution and with parallel-plate, no-reuse dialyzers. All patients received intravenous erythropoietin therapy three times weekly to maintain a target hematocrit of 31% to 33%, as well as oral iron supplementation according to their iron status. All patients responded to erythropoietin as assessed by the increase in reticulocyte count performed at the start of therapy.

Patients were all under stable clinical conditions at the time of this study. Causes of renal failure included chronic glomerulonephritis, polycystic kidney disease, and chronic pyelonephritis. Patients affected by systemic illnesses, such as hypertension antecedent to renal failure, diabetes mellitus, systemic lupus erythematosus, or cardiac disease, were excluded from the study. Blood levels of phosphate and systemic arterial pressure were controlled pharmacologically. Patients did not receive folic acid or vitamin supplementation, nor inhibitors of phosphodiesterases, for at least 3 months prior to the study.

Materials

D-aspartate oxidase (EC 1.4.3.1) was purified from the hepatopancreas of *Octopus vulgaris* (40 U/mL, 5 mg/mL) (21), D-amino acid oxidase (EC 1.4.3.3) was purified from hog kidney (15 U/mL, 5 mg/mL) (22), and peptidyl D-amino acid hydrolase (EC 3.4.13.17) was purified from the intestine of *Loligo vulgaris* (50 U/mL, 5 mg/mL) (23). Pronase (lyophilized, 7 U/mg) was purchased from Boehringer-Mannheim Biochemica (Mannheim, Germany). Leucine amino peptidase (150–300 U/mL, 10 mg/mL), tyramine, and all other biochemicals were from Sigma Chemical (St. Louis, MO; Poole, Dorset, UK). The following synthetic peptides containing D-Asp residues—1: Val-Tyr-Pro-(D-Asp)-His-Ala; 2: Ala-(D-Asp)-Thr; 3: Lys-Ala-Ser-Ala-(D-Asp)-Leu-Ala-Lys-Tyr—were synthesized as previously described (24). (All amino acids are in the L-configuration unless specifically designated D-). The corresponding D-isoAsp-containing analogs of these peptides were obtained through the chemical procedure described by McFadden and Clarke (7). This procedure gives an approximate ratio of 3:1 isoaspartyl:aspartyl-containing peptides.

Erythrocyte Membrane Preparation

Blood was obtained after an overnight fast in all patients and immediately before the dialysis session (before passage through the hemofilter) of the uremic patients. Membranes were obtained from fresh blood samples using the method of Perna *et al.* (20). Protein content of the creamy white membrane pellets was determined by the method of Bradford (25).

Hydrolysis of Membrane Proteins

Erythrocyte membrane proteins were hydrolyzed according to the procedure of D'Aniello *et al.* (26), which was developed to minimize the background racemization prevalent in other techniques.

Membrane pellets (3 mg) were dissolved in 4 mL of 6 M HCl (Carlo Erba, Milan, Italy; high-grade purity) and incubated at 37°C for 24 h. At this temperature, soluble and insoluble proteins are hydrolyzed to peptides and amino acids, but racemization does not detectably occur (26). After elimination of the HCl through evaporation, the hydrolysate was resuspended in 2 mL of 0.1 M Tris HCl, pH 8.2. The pH of the suspension was checked and adjusted when necessary to within the optimal range for pronase activity (pH 7.5–9.0). Pronase (200 μg in 20 μL of 0.1 M Tris HCl, pH 8.2), 10 μL of 1 M $CaCl_2$, and 10 μL of Triton X-100 were added, and the mixture was incubated at 50°C until the ninhydrin assay for free amino acid content showed no further increase (about 5 h). The pronase was then removed by centrifuging the solution through a filter with a 10,000 Da cutoff (Centricon 10; Amicon, Beverly, MA), and $MgCl_2$ was added (from a 1 M stock) to a final concentration of 5 mM.

At this point, most of the D-amino acids are presumably still contained within small peptides. These amino acids are released by a combination of leucine amino peptidase and peptidyl D-amino acid hydrolase. Leucine amino peptidase was prepared from 500 μL of an ammonium sulfate suspension by centrifugation at 15,000× *g* for 5 min, followed by resuspension of the pellet in 500 μL of 0.1 M Tris HCl, pH 8.2. Peptidyl D-amino acid hydrolase was prepared by dissolving the purified protein in 100 μL of 0.1 M Tris HCl, pH 8.2, reaching a 5 mg/mL solution. Leucine amino peptidase (30 μL) and peptidyl D-amino acid hydrolase (10 μL) were added to the filtered protein digests. These mixtures were filtered using a sterile syringe through a 0.45 μm-pore membrane into Vacutainers (Becton Dickinson, Rutherford, NJ) and then incubated at 37°C overnight. Aseptic conditions were maintained to prevent contamination by bacteria that could alter the results because they consume amino acids and also contain racemases. After incubation, the samples were again filtered and analyzed.

Hydrolysis of Synthetic Peptides

The synthetic peptides, prepared as described above (see Materials), were split into two aliquots; one was hydrolyzed chemically, and the other was treated with the mild procedure used with membranes, except that Triton X-100 was omitted. The chemical procedure was as follows: about 1 mg of each peptide was dissolved in 1 mL of 6 N HCl and incubated at 110°C for 24 h *in vacuo*. After elimination of the HCl by evaporation, the hydrolysate was dissolved in 0.5 mL of 0.1 M Tris HCl, pH 8.2. Amino acid analysis was performed, with two different methods—HPLC and enzymatic (described below)—on aliquots of both the chemical and enzymatic hydrolysates. The results obtained were used to determine the extent of recovery of the amino acids generated by the enzymatic hydrolysis procedure.

Determination of Total D+L-Amino Acids

Total D+L-amino acids were determined with ninhydrin reagent according to the method of Doi *et al.* (27).

Determination of Individual D+L-Amino Acids

Determination of each amino acid was performed using HPLC separation of *O*-phthalaldehyde (OPA)-derivatized amino acids, as described by Godel *et al.* (28), with slight modifications. Briefly, 20 μL of OPA-derivatized samples were injected into a Beckman System Gold Solvent Module model 126, equipped with a 25 cm × 4.6 mm Supelco C18 reverse-phase column (DuPont, Boston, MA), and a Waters fluorescence detector (set at 334 nm excitation; 445 nm emission). The column was equilibrated with 100% buffer A: 15 mM citrate buffer, pH 7.0, containing 7.5% acetonitrile (vol/vol). Buffer B was 70% (vol/vol) acetonitrile in citrate buffer. Amino acids were eluted with a 16-min linear gradient from O % to 15% buffer B over 16 min, followed by a 22-min linear gradient from 15% to 31% buffer B, and a final 6-min isocratic elution at 31% buffer B. The flow rate was 1.2 mL/min throughout the analysis.

Determination of Total D-Amino Acids

Total D-amino acids freed from proteins were quantified using a fluorometric method based on the combined use of D-aspartate oxidase and D-amino acid oxidase. These two enzymes together oxidize almost all free D-amino acids. In fact, D-aspartate oxidase oxidizes D-Asp, D-Glu, and their derivatives, as well as *N*-methyl-D-Asp, whereas D-amino acid oxidase oxidizes all other D-amino acids (29). These enzymes, upon oxidizing D-amino acids, produce hydrogen peroxide, which, in the presence of peroxidase, reacts with tyramine to give a highly fluorescent product (26). Aliquots (200 μL) of the peptide or RBC protein hydrolysates were mixed with 100 μL of 10 mM tyramine (in 0.1 M Tris HCl, pH 8.2), 10 μl of peroxidase (diluted 1:10 in 0.1 M Tris-HCl, pH 8.2, to give 250 U/mL), 5 μl of D-aspartate oxidase (5 mg/mL), or 5 μL of D-amino acid oxidase (5 mg/mL). After incubation at 37°C for 30 min, 1 mL of 0.1 M Tris-HCl, pH 8.2 was added. Fluorescence was measured at 325 nm excitation and 415 nm emission using the maximal sensitivity of the fluorometer, against the same assay mixture from which the oxidases were omitted. A calibration curve was prepared using D-aspartate (or D-alanine in the D-amino acid oxidase assays) as a standard, at concentrations ranging between 0.0 and 0.1 nmol/mL of assay mixture.

Specific Quantitation of D-Asp

Specific quantitation of D-Asp was performed using two methods:

1. *HPLC method.* This method was used for determination of D-Asp in synthetic peptides. It employed derivatization with OPA and *N*-acetyl-L-cysteine, and HPLC separation of the resulting diastereomers according to Aswad (30).
2. *Enzymatic fluorimetric method.* This method was used for the determination of D-Asp in both membrane protein and synthetic peptide hydrolysates. It is based on fluorimetric determination of D-Asp by measuring NAD^+ (31) according to the following reactions: in the presence of D-aspartate oxidase:

$$\text{D-Asp} + O_2 + H_2O \rightarrow \text{oxaloacetate} + H_2O_2 + \text{ammonia}.$$

Then, in the presence of malate dehydrogenase:

$$\text{oxaloacetate} + \text{NADH} + H^+ \rightarrow \text{malate} + NAD^+.$$

For the assay, aliquots of the hydrolysates (10–50 μL) were diluted with water to a final volume of 50 μL and mixed with 100 μL of an assay mixture made with 2 mL of 0.2 M Tris HCl, pH 8.2, 20 μL of malate dehydrogenase (5.500 U/mL), 20 μL of lactate dehydrogenase (6.000 U/mL), 3 μL of purified D-aspartate oxidase (5 mg/mL), and 100 μl of 10 mM NADH (dissolved in 0.2 M Tris HCl, pH 8.2). Following incubation at 37°C for 30 min, the reactions were stopped by the addition of 50 μL of 6 M HCl; incubation at 37°C for 10 min destroys unreacted NADH, but not the NAD^+ generated by the enzymatic reaction. Addition of 550 μL of 6 M NaOH, with incubation for 60 min at 37°C, converts the NAD^+ to a highly fluorescent product measured at 360 nm excitation and 460 nm emission. The concentration of D-Asp is calculated utilizing an *ad hoc* standard curve. This method can detect as little as 0.01 nmol of D-Asp, even in the presence of 1000 nmol of L-Asp (26). Lactate dehydrogenase was added to the assay mixture to measure the small amount of oxaloacetate that spontaneously becomes pyruvate (31).

Determination of Glutamate Oxaloacetate Transaminase Activity

Because RBC age has been described as decreased in uremia (32), we measured glutamate oxaloacetate transaminase (GOT) activity, as previously described (20), as a marker of cell age (33).

Statistical Analysis

Statistical analysis was performed using an unpaired *t* test. All calculations were performed using the software package StatWorks™, running on an Apple Macintosh IIfx personal computer. All results are presented as the mean ± SD.

Mathematical Modeling of the Reactions Occurring within Aging Erythrocytes

The changes in protein composition arising from the reactions that spontaneously degrade L-Asx residues and the enzymatic methylation that leads to their partial repair can be mathematically modeled (24). Briefly, these reactions are written as equations and solved using estimates of the initial reactant concentrations (L-Asp, L-Asn), the first order rate constants for the nonenzymatic steps, and the Michaelis-Menten constants for the methylation reactions (see Results). The products arising during the first minute are then used as reactants for the reactions occurring during the next minute; these iterative calculations, performed on a VAX 11–780 computer using a Fortran compiler, are repeated 172,800 times to model the full 120-day average life span of the human erythrocyte.

Results

Effect of Uremia on the D-Asp and D-isoAsp Content of Erythrocyte Membrane Proteins

One of the apparent consequences of chronic renal failure and ESRD is an increase in RBC membrane disorders. We have examined the composition of RBC membrane proteins from uremic hemodialysis patients in a search for alterations that may contribute to these disorders. Membrane proteins purified from uremic and nonuremic (control) individuals were hydrolyzed to their component amino acids using mild chemical and enzymatic digestions at 37°C. Interestingly, we found that the content of racemized aspartyl residues in these proteins is significantly lower in uremic patients than in the controls. Expressed either as an absolute (nmol D-Asx/mg protein) or relative (D-Asx/[D-Asx+L-Asx]) value, the amount of racemized residues differs threefold between the two groups (Table 1).

To establish that these values truly represent the composition of the membrane proteins *in vivo* rather than artifacts of the analytical procedure, the efficiency of the hydrolytic method used was examined with synthetic D-Asp- and D-isoAsp-containing peptides. To simulate the variety of sequences present in cellular proteins, three peptides of different lengths and

Table 1. D-aspartic acid released from membrane proteins of erythrocytes from eight uremic patients and eight control individuals by a mild acid/enzymatic hydrolysis procedure.[a]

D-Asx (nmol/mg)		D-Asx/D-Asx+L-Asx (%)		GOT (U/g Hb)	
Controls	Uremics	Controls	Uremics	Controls	Uremics
A					
1.28 ± 0.28	1.60 ± 0.39	0.35	0.38	8.50 ± 1.060	12.33 ± 1.423
4.66 ± 1.07	0.51 ± 0.29	1.29	0.14	6.45 ± 0.716	6.60 ± 0.943
2.75 ± 0.48	0.45 ± 0.28	0.80	0.11	7.18 ± 0.800	5.51 ± 0.689
4.50 ± 0.86	2.80 ± 0.75	1.24	0.63	6.78 ± 0.510	6.29 ± 0.474
1.42 ± 0.39	0.36 ± 0.18	0.39	0.12	10.46 ± 0.803	9.39 ± 3.561
3.16 ± 0.31	0.99 ± 0.36	0.86	0.29	7.86 ± 0.524	8.58 ± 0.572
2.84 ± 0.26	0.24 ± 0.12	0.77	0.15	7.64 ± 0.456	5.58 ± 0.797
3.10 ± 0.92	0.37 ± 0.09	0.84	0.14	6.74 ± 0.533	7.72 ± 2.083
B (mean ± SD)					
2.96 ± 1.22	0.92[b] ± 0.88	0.82 ± 0.34	2.25[c] ± 0.18	7.70 ± 1.36	7.75 ± 2.32

[a] Results are given both as D-Asx content and percentage of D-Asx/D-Asx+L-Asx. The specific activity of cytosolic GOT, a marker of erythrocyte age, is also given. Part A shows the results obtained from triplicate determinations in individual subjects. Part B shows the mean ± SD within each group. D-Asx, D-aspartyl derivatives; L-Asx, L-aspartyl derivatives; GOT, glutamate oxaloacetate transaminase.
[b] $P < 0.005$ *versus* control.
[c] $P < 0.001$ *versus* control.

compositions were digested using the mild enzymatic conditions. As shown in Table 2, the efficiency of release of D-Asx residues from these peptides differed greatly. We found that the tripeptides Ala-(D-Asp)-Thr and Ala-(D-isoAsp)-Thr were fully digested. On the other hand, about 75% of the D-Asx content was released as free aspartic acid from the nonapeptides Lys-Ala-Ser-Ala-(D-Asp)-Leu-Ala-Lys-Tyr and Lys-Ala-Ser-Ala-(D-isoAsp)-Leu-Ala-Lys-Tyr, whereas only about 20% of the D-Asx from the hexapeptides Val-Tyr-Pro-(D-Asp)-His-Ala and Val-Tyr-Pro-(D-isoAsp)-His-Ala was recovered, possibly because the Pro adjacent to the D-Asx is poorly recognized by most proteases. More important, however, D-Asp and D-isoAsp in equivalent sequences are released with equal efficiency by the mild hydrolytic procedure. Quantitation of racemized residues by chemical derivatization and by the utilization of D-aspartate oxidase gave similar results, indicating that these methods are comparable (Table 2). Thus, although some D-Asx residues in the RBC proteins may be poorly released by this method, the lower amount of D-Asx residues in the uremic patients does not appear to result solely from the possibility that these cells have a higher D-isoAsp:D-Asp ratio than control cells.

Because D-Asx accumulates in RBC membrane proteins in a time-dependent manner, the age of the cells is another factor that can affect D-Asx content. Furthermore, it has been reported that chronic renal failure can lead to reduced life span of circulating RBC (32). To determine the relative ages of the cell populations used in the current study, GOT activity was measured, as previously described (20), as a marker for cell age (33). Surprisingly, we found no significant differences in the GOT activity between uremic and control RBC (Table 1). Therefore, the reduction in D-Asx content observed in RBC

Table 2. The efficiency of release of D-Asp and D-isoAsp residues from synthetic peptides using a mild acidic and enzymatic procedure, expressed as the percentage of total recovery obtained after high temperature chemical hydrolysis of the same peptide[a]

Peptide	D-Asp release (% of total D-Asx residues present)			
	HPLC analysis		Enzymatic analysis	
	D-isoAsp	D-Asp	D-isoAsp	D-Asp
V-Y-P-(D-Asx)-H-A	15.53 ± 4.50	17.51 ± 3.49	21.07 ± 4.24	23.75 ± 3.64
A-(D-Asx)-T	103.28 ± 5.70	106.06 ± 3.61	102.67 ± 5.71	100.43 ± 6.85
K-A-S-A-(D-Asx)-L-A-K-Y	76.36 ± 3.51	70.10 ± 5.66	73.94 ± 7.71	84.04 ± 4.25

[a] D-Asx residues in the hydrolysates are quantitated both by HPLC following OPA derivatization and by the enzymatic procedure (detailed under Methods). The D-isoAsp peptides preparation also contains the D-aspartyl analog in amounts of about 20%–25% of the total peptide. Results are expressed as the mean ±SD of two hydrolysis experiments. D-Asp, D-aspartyl; D-isoAsp, D-isoaspartyl; D-Asx, D-aspartyl derivatives; HPLC, high-pressure liquid chromatography; OPA, O-phthalaldehyde.

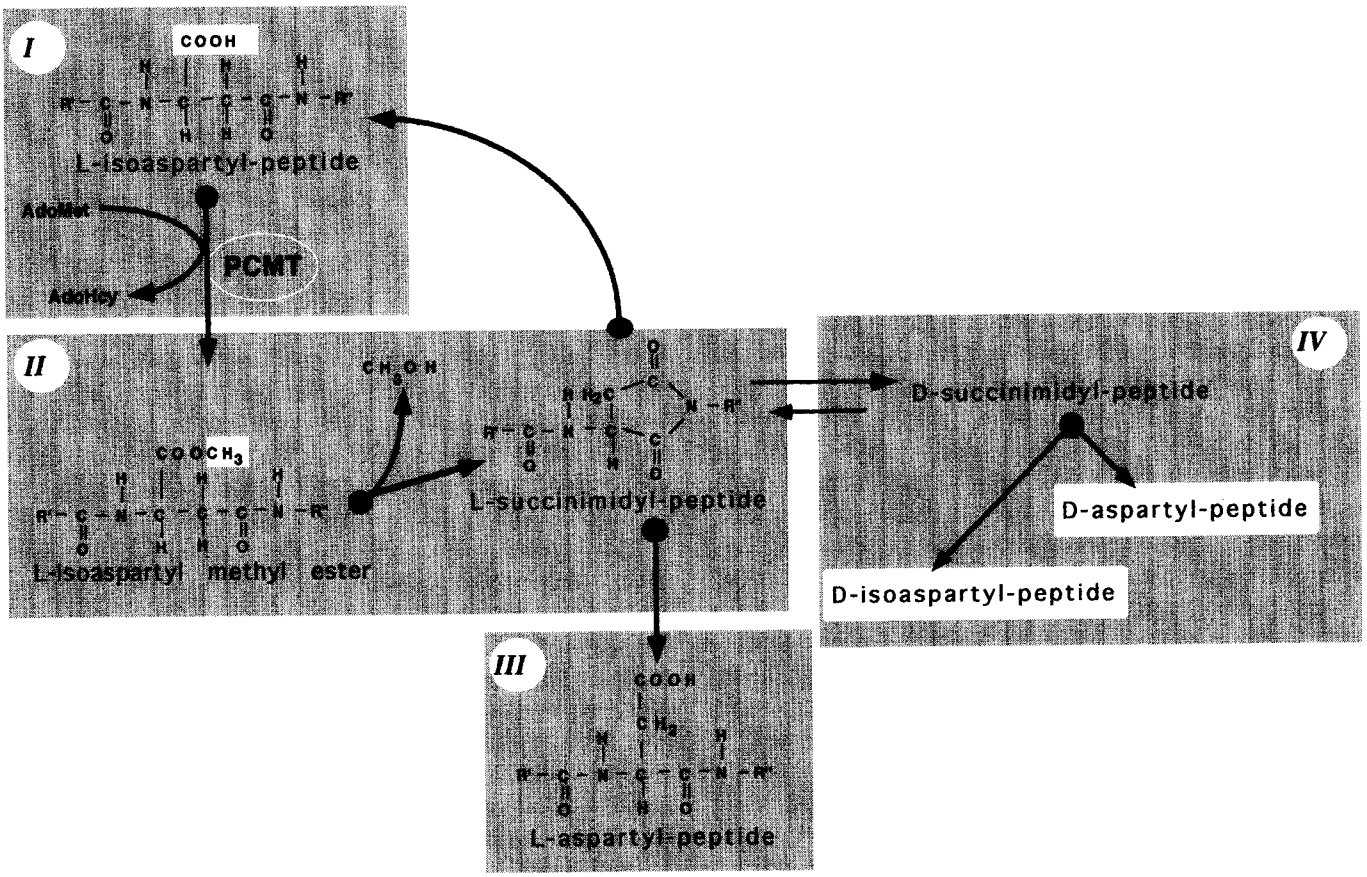

Figure 1. Peptides and proteins containing L-isoaspartyl residues are substrates of PCMT enzyme (panel I). Methyl esters are hydrolyzed through the formation of the succinimidyl derivative of the peptide (panel II). The spontaneous opening of the succinimide ring yields the repaired L-Asp residue (panel III), as well as substantial amounts of L-isoaspartyl residues. Thus, the complete conversion of the L-isoAsp residues into normal L-Asp can only occur through repeated cycles of the methylation-demethylation steps. D-isoAsp residues are the side product of the repair reaction through the slow and spontaneous racemization of L-succinimide to D-succinimide (panel IV) (1, 2).

synthetic peptide (6), the calculated fraction of racemized residues in 60-day-old cells (0.79) closely matches the experimental value (0.82) (see Figure 2). Furthermore, when only 11% of the normal PCMT activity is used in the model, the fraction of racemized residues in 60-day-old cells (0.29) is very similar to the experimental value (0.25) from uremic cells (Figure 2). Therefore, mathematical modeling demonstrates that inhibition of PCMT can account for the decrease in D-Asx observed in uremic patients.

A consequence of the inhibition of PCMT is the accumulation of L-isoAsp residues. In the mathematical model described above, only 0.0046% of the Asx residues in 60-day-old control cells are in the L-isoAsp configuration (Figure 3). Under conditions where 89% of the PCMT activity is inhibited, however, 2.88% of the residues are in the L-isoAsp form, a >600-fold increase (Figure 3). This model predicts that PCMT in control cells can almost eliminate L-isoAsp residues but is not active enough in uremic cells to prevent the continuous accumulation of these damaged residues.

The calculations described above assume that *S*-adenosylhomocysteine acts simply as a competitive inhibitor of *S*-adenosylmethionine binding. Johnson and Aswad (36) report that *S*-adenosylhomocysteine can also act as a mixed-type inhibitor of L-isoAsp residue binding with bovine brain PCMT *in vitro*, indicating that the degree of PCMT inhibition will depend on the concentration of L-isoAsp. To investigate the effect of this type of inhibition, the equations denoting PCMT activity (37) were modified, using the equation and kinetic constants of Johnson and Aswad (36) to describe the continuous change in PCMT activity with the variation of substrate concentration (Figure 2, inset). The output of this model most closely represents the observed percentage of D-Asx (% D-Asx) when the rate of succinimide racemization is slightly decreased ($k = 0.00022\ min^{-1}$; $t_{1/2} = 26.3$ h). Whereas the % D-Asx values calculated by this model (55% decrease) are not as close to the measured values as were those of the simpler model (Figure 2, inset), they are still well within the SD of the experimental values.

Discussion

In this study, we have found that RBC membrane proteins from patients with ESRD contain only about 31% of the D-Asx residues of control individuals. The fact that this difference in racemization appears to be specific for Asp residues suggested that it might be linked to a recently proposed pathway that limits the accumulation of damaged Asp residues arising spontaneously with age from L-Asn and L-Asp residues in RBC and

membrane proteins from uremic patients does not result from these cells being younger than are the control cells.

Another process that could yield the results observed here would be the selective loss of damaged proteins (*i.e.*, those enriched in racemized residues) from the RBC membranes in uremic patients. Examination of the amino acid composition of these proteins, however, suggests that this type of loss is not occurring. As shown in Table 3, no significant difference was found in the relative abundance (nmol/mg) of Asp, Glu, Ser, His, Gly + Thr, Arg, Ala, Tyr, Val, Met, Ile, Phe, Leu, and Lys in membrane proteins from the uremic and control groups. The total non-D-Asx D-amino acid content was also measured, and by an enzymatic procedure using D-amino acid oxidase (see Methods), was found to be 1.37 ± 0.21 nmol/mg in control membrane proteins ($N = 6$) and 1.12 ± 0.13 nmol/mg in membrane proteins from uremic RBC ($N = 6$). Because these values are not statistically different, there is no evidence that proteins containing more racemized residues are selectively eliminated from uremic RBC or that the overall membrane protein composition of these cells differs from that of control RBC. In addition, the ratio between total D-amino acids and total D+L-amino acids is not different between the two groups (control, O.O21%; uremic, 0.019%). Thus, ESRD affects only the D-Asx content of these proteins.

Mathematical Simulation of the Degradation and Repair Reactions Affecting Aspartyl Residues in Uremic and Control Erythrocytes

We have previously described how the spontaneous degradation and enzymatic methylation reactions outlined in Figure 1 can be mathematically modeled to simulate what occurs in intact red cells (24). Briefly, we estimated that a newly matured RBC contains 165 mM L-Asp and 111 mM L-Asn in proteins and that 1% of these residues are labile for succinimide formation ($t_{1/2}$ = 50 days), 25% degrade at an intermediate rate ($t_{1/2}$ = 400 days), and 74% are resistant to succinimide formation ($t_{1/2}$ = 2000 days) (24). The values for succinimide hydrolysis ($t_{1/2}$ = 2.9 h for isoAsp formation, $t_{1/2}$ = 10.7 h for Asp formation) and for succinimide racemization ($t_{1/2}$ = 19.5 h) are derived from studies with synthetic peptides performed *in vitro* under physiologic conditions (24). The rates of deesterification of methylated residues ($t_{1/2}$ = 1.5 h for 57% of the esters; $t_{1/2}$ = 25 h for 43% of the esters) come from *in vitro* measurements with *in situ*-methylated RBC membrane proteins. The V_{max} value used for the methyltransferase-catalyzed steps in "control cells"(0.66 μM/min) was calculated from measurements with erythrocyte cytosol to reflect the activity in a single cell (24). Finally, the K_m values used for L-isoAsp (5 μM) and D-Asp residues (5 mM) are based on the analyses of a variety of peptides and proteins *in vitro* (24).

To model the effect of the buildup of *S*-adenosylhomocysteine, which is the product and the competitive inhibitor of the methylation reaction (34,35), on the accumulation of damaged Asx residues, we first calculated the change in PCMT activity using the Michaelis-Menten equation. In control cells, *S*-adenosylmethionine (the methyl donor) concentration is 2.56 μM, and the *S*-adenosylhomocysteine concentration is 0.77 μM, whereas in RBC from patients undergoing dialysis, the *S*-adenosylmethionine concentration is 2.10 μM, and the *S*-adenosylhomocysteine concentration is 6.87 μM (20). Assuming that *S*-adenosylhomocysteine is a competitive inhibitor of *S*-adenosylmethionine binding and that the K_m of *S*-adenosylmethionine is 2.0 μM and the K_i of *S*-adenosylhomocysteine is 0.076 μM (24), PCMT in uremic cells has only 11% of the activity of control cells. When the rate of succinimide racemization used in the modeling ($k = 0.000225\ min^{-1}$; $t_{1/2}$ = 25.7 h) is slightly lower than that measured *in vitro* with a

Table 3. Amino acid content (both D and L residues) of erythrocyte membrane proteins from control subjects and from uremic patients[a]

	Controls		Uremics	
	Protein (nmol/mg)	Average abundance relative to Asx	Protein (nmol/mg)	Average abundance relative to Asx
Asx	395.0 ± 63.0	1.0	368.0 ± 48.0	1.0
Glx	776.0 ± 187.0	2.0	670.0 ± 91.5	1.8
Ser	671.0 ± 178.9	1.7	566.8 ± 98.4	1.5
Hys	241.0 ± 159.0	0.6	252.5 ± 184.7	0.7
Gly+Thr	485.8 ± 110.3	1.2	432.3 ± 88.4	1.2
Arg	375.0 ± 27.9	0.9	356.0 ± 28.6	1.0
Ala	720.0 ± 192.0	1.8	689.6 ± 183.6	1.9
Tyr	227.8 ± 64.0	0.6	205.0 ± 15.7	0.6
Val	540.0 ± 95.6	1.4	416.0 ± 39.0	1.1
Met	352.8 ± 162.7	0.9	285.0 ± 189.8	0.8
Ile	399.0 ± 55.1	1.0	355.0 ± 38.1	1.0
Phe	250.0 ± 186.0	0.6	195.0 ± 113.3	0.5
Leu	748.0 ± 230.5	1.9	820.0 ± 320.5	2.2
Lys	376.4 ± 152.0	1.0	323.8 ± 118.4	0.9

[a] Results are given as the mean ± SD (N=8). Average abundance relative to Asx is also reported. Asx, asparagine, aspartate, and isoaspartate.

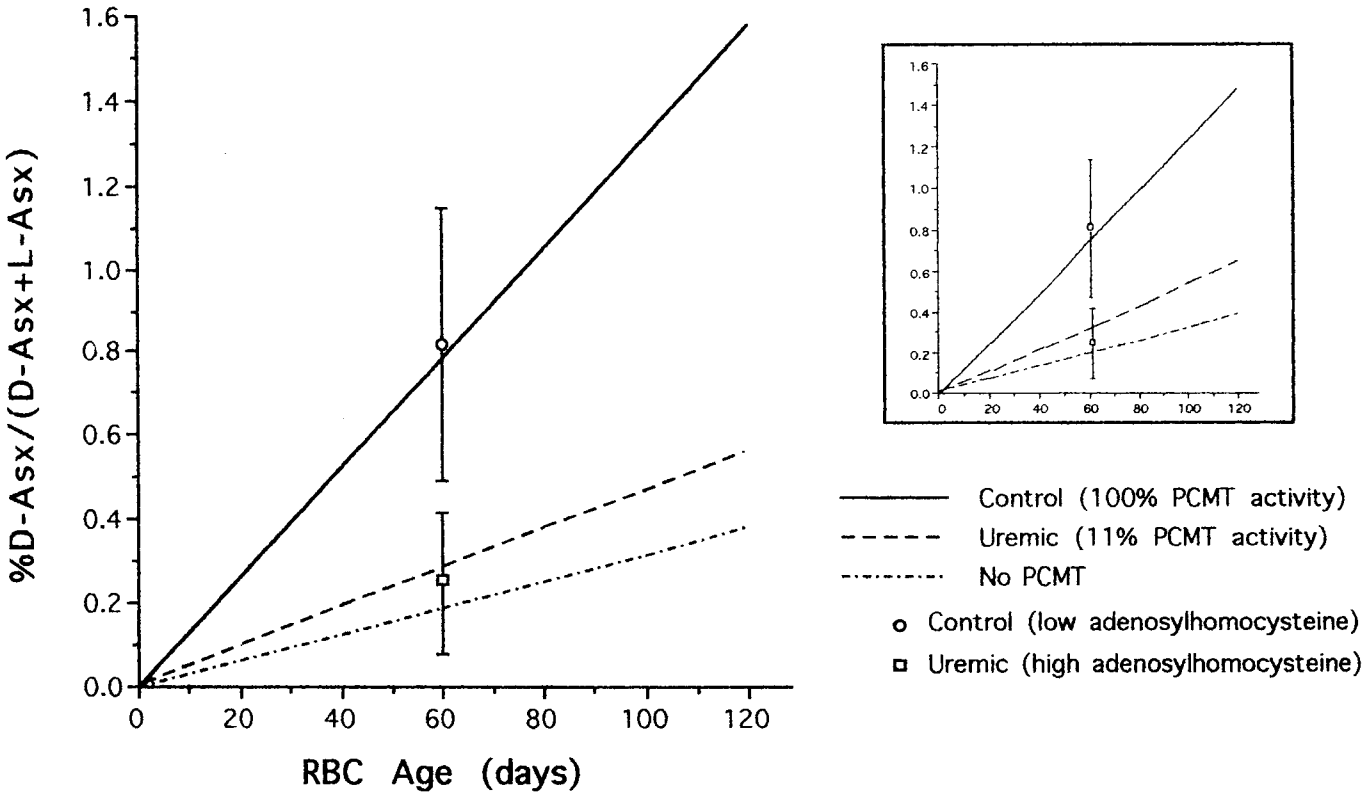

Figure 2. Measured and modeled percentages of D-Asx/D-Asx+L-Asx content of erythrocyte membrane proteins in normal and uremic subjects. The kinetic constants used in the modeling are the best estimates from a variety of previous studies, and the half-life of succinimide racemization used here ($t_{1/2}$ = 25.7 to 26.3 h) differs by only about 25% from the value measured with a synthetic peptide *in vitro* ($t_{1/2}$ = 19.5 h; 6). In the simpler model (full size graph) it has been assumed that *S*-adenosylhomocysteine acts only as a competitive inhibitor. Mathematical modeling of the relevant reactions, thought to affect Asx residues in membrane proteins, indicates that at 60 days of RBC age, a 63% reduction in the relative abundance of D-Asx will be present, matching the 69% decrease observed experimentally (see Table 1). When *S*-adenosylhomocysteine is allowed to function as both a competitive and mixed-type inhibitor, the model predicts that the relative abundance of D-Asx will decrease by 55% in the uremic patients (inset). This value still falls well within the range of the SD for the experimental data (inset).

other tissues (1,2). Central to this pathway is the PCMT enzyme, which selectively methyl esterifies L-isoAsp (and with lower affinity D-Asp) residues in proteins but does not recognize normal L-Asp residues. In combination with several nonenzymatic reactions, this methyltransferase initiates the conversion of L-isoAsp residues to L-Asp residues via a succinimidyl intermediate (Figure 1). Because L-succinimidyl residues are prone to racemization and D-Asp residues are relatively poor substrates for the methyltransferase, Figure 1 predicts a slight but progressive accumulation of racemized residues, particularly D-isoAsp, as side products of the repair of L-isoAsp residues. This price can be advantageous to pay in exchange for maintaining low levels of L-isoAsp residues because racemized residues are calculated to be, eventually, one to two orders of magnitude less abundant than the L-isoAsp residues (24); also, the position of the D-isoAsp side chain carboxyl group in space is similar to that of normal L-Asp residues (38), thus suggesting that D-isoAsp is less detrimental than L-isoAsp to protein structure and function (24).

Three types of evidence support the above hypotheses. First, incubation of an L-isoAsp-containing form of the *Escherichia coli* HPr phosphocarrier protein with purified methyltransferase *in vitro* converts the L-isoAsp residues to L-Asp with the generation of a small but significant amount of D-isoAsp (39). D-isoAsp buildup has also been shown to occur as a side effect of the repair of other L-isoaspartyl-containing peptides *in vitro* (7,15). Second, human α A-crystallin purified from the eye lens of elderly individuals (mean age 80 yr) contains primarily D-isoAsp and L-Asp residues at positions 58 and 151, suggesting that the active PCMT in this tissue had converted the L-isoAsp residues *in vivo* (40). Finally, mathematical modeling of the spontaneous aging and enzymatic methylation reactions thought to occur in RBC demonstrates that PCMT could easily be responsible for a several-fold increase in the number of D-Asx residues over the life span of the cell (24).

How might uremia be linked to this repair pathway? We have recently reported that patients with ESRD treated with hemodialysis have an elevated level of homocysteine, which causes an up-to-ninefold increase in the concentration of s-adenosylhomocysteine (41), a potent inhibitor of *S*-adenosylmethionine-dependent methyltransferases (34,35). Because PCMT is the most active methyltransferase in the RBC, accounting for >90% of *S*-adenosylmethionine consumption (1,2), inhibition of this enzyme is presumably the major consequence of *S*-adenosylhomocysteine elevation.

Can inhibition of PCMT account for the decrease in D-Asx

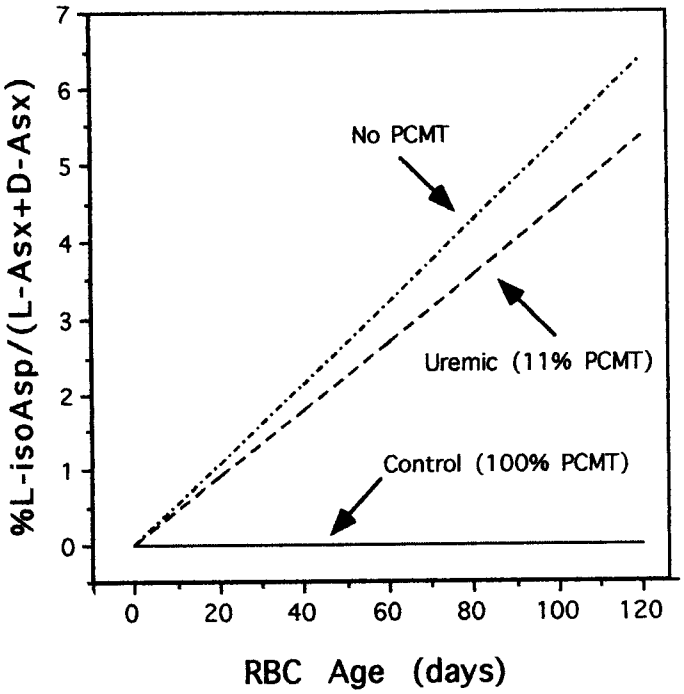

Figure 3. Accumulation of L-isoAsp residues in erythrocyte membrane proteins in normal and uremic subjects predicted by modeling. Mathematical modeling shows that the fraction of L-isoAsp residues in 60-day-old cells, when only 11% of the activity of PCMT is available, will be increased 600-fold, predicting that PCMT in control cells can greatly reduce L-isoAsp residues, but in uremia cannot prevent the continuous accumulation of damaged residues.

observed under uremic conditions? The concentration of S-adenosylhomocysteine present in uremic RBC, when considered as a competitive inhibitor of *S*-adenosylmethionine, should reduce PCMT activity by 89%. Mathematical modeling of the reactions thought to affect Asx residues in intact RBC indicates that this inhibition could cause a 63% decrease in the relative abundance of D-Asx [% D/(D+L)] in 60-day-old RBC, which closely matches the 69% decrease observed experimentally upon comparison of control and uremic RBC of average age (Table 1, Figure 2). If *S*-adenosylhomocysteine is allowed to function as both a competitive and mixed-type inhibitor, the model predicts that the relative abundance of D-Asx will decrease in the uremic patients by 55%, still well within the range of the SD for the experimental data (Table 1, Figure 2, inset). The similarity of the calculated and experimental values supports the accuracy of the pathway shown in Figure 1.

Based on the information presented above, we propose that uremia is linked to chronic inhibition of PCMT in erythrocytes and that this substantially limits the repair of L-isoAsp residues, with an associated decrease in D-Asx residues. Accumulation of D-Asx residues has previously been considered merely as a marker of protein aging in both living tissues and in biological remains; in fact, D-Asx residue accumulation has been used to estimate the time since death of some biological samples (42). Our results now demonstrate for the first time that the D-Asx content of living organisms can be influenced by enzymatic activity and altered significantly by diseases that affect this activity. Because damaged Asp residues have been observed to modify the structure and activity of a variety of proteins and peptides, it would seem that the two- to threefold decrease of D-Asx residues in membrane proteins may be advantageous to the cell. Unfortunately, this decrease is associated with a predicted 12- to 600-fold increase (depending on the model used) in L-isoAsp residues, greatly overshadowing the small decrease in D-Asx resulting from PCMT inhibition.

Regarding the relationship between abnormal aspartyl residues in membrane proteins and RBC pathophysiology, an increase of membrane protein methylation has been shown to parallel erythrocyte aging, which suggests that the PCMT-linked repair pathway is unable to keep up with the rate of accumulation of the protein fatigue damage in older erythrocytes (1,2).

In addition, we have previously demonstrated a significant increase of protein methylation in spherocytosis, a chronic hereditary anemia characterized by critical structural defects in the red cell membrane (43,44). In contrast to uremia, this condition causes no inhibition of PCMT; thus, methylation levels can be taken as a marker of the underlying membrane protein alteration (43), which appears to be triggered by RBC passage through spleen microcirculation (44). We were also able to show, in spherocytosis, a direct correlation between the degree of spectrin deficiency, a marker of both membrane structural disarray and clinical derangement, and the increase of methylation levels (43).

Uremia is the first disease in which a chronic inhibition of protein methylation has been clearly established, with the damage deriving not only from intrinsic "protein fatigue" (1) but also from inadequate repair. We have previously shown that reduction of methylation in uremia is particularly dramatic for ankyrin, a cytoskeletal protein involved in the maintenance of membrane stability and integrity (20). From a pathophysiologic standpoint, however, the role of aspartyl damage in uremia is difficult to interpret because of the complexity contributed by several other types of functional alterations that arise concurrently in these erythrocytes (18,19,32,45–47). For example, membrane oxidation in uremic erythrocytes leads to a reduction in membrane fluidity (48). Therefore, although different types of damage may affect proteins and other cell components in uremic cells, we must now consider the effects of abnormal aspartyl residues, as well.

Acknowledgments

This work was partially supported by the Ministry of University and Scientific and Technological Research (MURST) (40%), MURST (60%), Targeted Project Prevention and Control of Disease Factors (FATMA), and National Research Council (CNR) contracts to the Chair of Nephrology; by grant AI93.00316.14 of the Italian National Research Council to Dr. Ingrosso; and by a grant from the American Health Assistance Foundation to Dr. Clarke.

References

1. Galletti P, Ingrosso D, Manna C, Clemente G, Zappia V: Protein damage and methylation mediated repair in the erythrocyte. *Biochem J* 306: 313–325, 1995
2. Ota IM, Clarke S: The function and enzymology of protein

D-aspartyl/L-isoaspartyl methyltrasferases in eukaryotic and prokaryotic cells. In:. *Protein Methylation*, edited by Paik WK, Kim S, Boca Raton, FL, CRC Press, 1990, pp 179–194
3. Brunauer LS, Clarke S: Age-dependent accumulation of protein residues that can be hydrolyzed to D-aspartic acid in human erythrocytes. *J Biol Chem* 261: 12538–12543, 1986
4. Stephenson RC, Clarke S: Succinimide formation from aspartyl and asparaginyl peptides as a model for the spontaneous degradation of proteins. *J Biol Chem* 264: 6164–6170, 1989
5. Clarke S: Propensity for spontaneous succinimide formation from aspartyl and asparaginyl residues in cellular proteins. *Int J Peptide Protein Res* 30: 808–821, 1987
6. Geiger T, Clarke S: Deamidation, isomerization, and racemization at asparaginyl and aspartyl residues in peptides: Succinimide-linked reactions that contribute to protein degradation. *J Biol Chem* 262: 785–794, 1987
7. McFadden PN, Clarke S: Chemical conversion of aspartyl peptides to isoaspartyl peptides: A method for generating new methyl-accepting substrates for the erythrocyte D-aspartyl/L-isoaspartyl protein methyltransferase. *J Biol Chem* 261: 11503–11511, 1986
8. Roher AE, Lowenson JD, Clarke S, Wolkow C, Wang R, Cotter RJ, Reardon IM, Zürcher-Neely HA, Heinrikson RL, Ball MJ, Greenberg BD: Structural alterations in the peptide backbone of β-amyloid core protein may account for its deposition and stability in Alzheimer's disease. *J Biol Chem* 268: 3072–3083, 1993
9. Fabian H, Szendrei GI, Mantsch HH, Greenberg BD, Ötvös L: Synthetic post-translationally modified human Aβ peptide exhibits a markedly increased tendency to form β-pleated sheets in vitro. *Eur J Biochem* 221: 959–964, 1994
10. Roher AE, Lowenson JD, Clarke S, Woods AS, Cotter RJ, Gowing E, Ball MJ: β-amyloid-(1–42) is a major component of cerebrovascular amyloid deposits: Implications for the pathology of Alzheimer disease. *Proc Natl Acad Sci USA* 90: 10836–10840, 1993
11. Tomiyama T, Asano S, Furiya Y, Shirasawa T, Endo N, Mori H: Racemization of Asp^{23} residue affects the aggregation properties of Alzheimer amyloid β protein analogues. *J Biol Chem* 269: 10205–10208, 1994
12. Hefman PM, Bada JL: Aspartic acid racemization in dentine as a measure of aging. *Nature (Lond)* 262: 279–281, 1976
13. Masters PM, Bada JL, Zigler JS: Aspartic acid racemization in heavy molecular weight crystallins and water-insoluble protein from normal human lenses and cataracts. *Proc Natl Acad Sci USA* 75: 1204–1208, 1978
14. Cohen CM: New trends in understanding the regulation of red cell membrane skeleton organization. *Adv Cell Biol* 2: 1–24, 1988
15. Johnson BA, Murray ED, Clarke S, Glass DB, Aswad DA: Protein carboxyl methyltransferase facilitates the conversion of atypical L-isoaspartyl peptides to normal L-aspartyl peptides. *J Biol Chem* 262: 5622–5629, 1987
16. Galletti P, Ciardiello A, Ingrosso D, Di Donato A, D'Alessio G: Repair of isopeptide bonds by protein carboxyl *O*-methyltransferase: Seminal ribonuclease as a model system. *Biochemistry* 27: 1752–1757, 1988
17. McFadden PN, Clarke S: Conversion of isoaspartyl peptides to normal peptides: Implications for the cellular repair of damaged proteins. *Proc Natl Acad Sci USA* 84: 2595–2599, 1987
18. Inauen W, Stäubli M, Descoeudres C, Galeazzi RL, Straub PW: Erythrocyte deformability in dialysed and nondialysed uraemic patients. *Eur J Clin Invest* 12: 173–176, 1982
19. Maduell F, Fernandez J, Diez J: Alterations of the $Cl^-/NaCO_3^-$ anion exchanger in erythrocytes of uraemic patients. *Nephrol Dial Transplant* 5: 1018–1022, 1990
20. Perna AF, Ingrosso D, Galletti P, Zappia V, De Santo NG: Enzymatic methyl esterification of erythrocyte membrane proteins is impaired in chronic renal failure: Evidence for high levels of the natural inhibitor *S*-adenosylhomocysteine. *J Clin Invest* 91: 2497–2503, 1993
21. D'Aniello A, Giuditta A: Identification of D-aspartic acid in the brain of *Octopus vulgaris* Lam. *J Neurochem* 29: 1053–1057, 1977
22. D'Aniello A, D'Onofrio G, Pischetola M, D'Aniello G, Vetere A, Petrucelli L, Fisher GH: Biological role of D-amino acid oxidase and D-aspartate oxidase. Effects of D-amino acids. *J Biol Chem* 268: 26941–26949, 1993
23. D'Aniello A, Strazzullo L: Peptidyl-D-amino acid hydrolase from *Loligo vulgaris* Lam: Purification and characterization. *J Biol Chem* 259: 4237–4243, 1984
24. Lowenson JD, Clarke S: Recognition of D-aspartyl residues in polypeptides by the erythrocyte L-isoaspartyl/D-aspartyl protein methyltransferase. Implications for the repair hypothesis. *J Biol Chem* 267: 5985–5995, 1992
25. Bradford MM: A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. *Anal Biochem* 72: 248–254, 1976
26. D'Aniello A, Petrucelli L, Gardner C, Fisher G: Improved method for hydrolyzing proteins and peptides without inducing racemization and for determining their true D-amino acid content. *Anal Biochem* 213: 290–295, 1993
27. Doi E, Shibata D, Matoba T: Modified colorimetric ninhydrin methods for peptidase assay. *Anal Biochem* 118: 173–184, 1981
28. Godel H, Graser T, Földi P, Pfaender P, Fürst P: Measurement of free amino acids in human biological fluids by high-performance liquid chromatography. *J Chromatogr* 297: 49–61, 1984
29. D'Aniello A, Vetere A, Petrucelli L: Further study on the specificity of D-amino acid oxidase and of D-aspartate oxidase and time course for complete oxidation of D-amino acids. *Comp Biochem Physiol* 105B: 731–734, 1993
30. Aswad DW: Determination of D- and L-aspartate in amino acid mixtures by high performance liquid chromatography after derivatization with a chiral adduct of O-phthaldialdehyde. *Anal Biochem* 137: 405–407, 1984
31. Kaplan NO, Colowick SP, Barnes CC: Effect of alkali on diphosphopyridine nucleotide. *J Biol Chem* 191: 461–471, 1951
32. Akmal M, Telfer N, Ansari AN, Massry SG: Erythrocyte survival in chronic renal failure. Role of secondary hyperparathyroidism. *J Clin Invest* 76: 1695–1698, 1985
33. Haram S, Carriero D, Seaman C, Piomelli S: The mechanism of decline of age-dependent enzymes in the red blood cell. *Enzyme* 45: 47–53, 1991
34. Zappia V, Zydek-Cwick CR, Schlenk F: The specificity of s-adenosylmethionine derivatives in methyl transfer reactions. *J Biol Chem* 244: 4499–4505, 1969
35. Cantoni GL, Chiang PK: The role of *S*-adenosylhomocysteine and *S*-adenosylhomocysteine hydrolase in the control of biologic methylations. In: *Natural Sulfur Compounds: Novel Biochemical and Structural Aspects*, edited by Cavallini D, Gaull GE, Zappia V, New York, Plenum Press, 1980, pp 67–89
36. Johnson BA, Aswad DW: Kinetic properties of bovine brain protein L-isoaspartyl methyltransferase determined using a synthetic isoaspartyl peptide substrate. *Neurochem Res* 18: 87–94, 1993
37. Lowenson JD, Clarke S: Spontaneous degradation and enzymatic repair of aspartyl and asparaginyl residues in aging red cell

proteins analyzed by computer simulation. *Gerontology* 37: 128–151, 1991

38. Murray ED, Clarke S: Synthetic peptide substrates for the erythrocyte protein carboxyl methyltransferase. *J Biol Chem* 259: 10722–10732, 1984
39. Brennan TV, Anderson JW, Jia Z, Waygood EB, Clarke S: Repair of spontaneously deamidated HPr phosphocarrier protein catalyzed by the L-isoaspartate-(D-Aspartate)-*O*-methyltransferase. *J Biol Chem* 269: 24586–24595, 1994
40. Fujii N, Satoh K, Harada K, Ishibashi Y: Simultaneous stereoinversion and isomerization at specific aspartic acid residues in alpha A-crystallin from human lens. *J Biochem* 116: 663–669, 1994
41. Perna AF, Ingrosso D, De Santo N G, Galletti P, Zappia V: Mechanism of erythrocyte accumulation of methylation inhibitor *S*-adenosylhomocysteine in uremia. *Kidney Int* 47: 247–253, 1995
42. Lubec G, Weniger M, Anderson SR: Racemization and oxidation studies of hair protein in the Homo tirolensis. *FASEB J* 8: 1166–1169, 1994
43. Ingrosso D, D'Angelo S, Perna AF, Miraglia del Giudice E, Iolascon A, Perrotta S, Zappia V, Galletti P: Increased membrane protein methylation in hereditary spherocytosis: A marker of cytoskeletal disarray. *Eur J Biochem* 228: 894–898, 1995
44. Ingrosso D, D'Angelo S, Perrotta S, D'Urzo G, Iolascon A, Perna AF, Galletti P, Zappia V, Miraglia del Giudice E: Cytoskeletal behaviour in spectrin and in band 3 deficient spherocytic red cells: Evidence for a differentiated splenic conditioning role. *Br J Haematol* 93: 38–41, 1996
45. Kaji D, Kahn T: Na^+-K^+ pump in chronic renal failure. *Am J Physiol* 252: F785–F793, 1987
46. Kelly RA, Canessa ML, Steinman TI, Mitch WE: Hemodialysis and red cell cation transport in uremia: Role of membrane free fatty acids. *Kidney Int* 35: 595–603, 1989
47. Corry DB, Lee DBM, Tuek ML: A kinetic study of cation transport in erythrocytes from uremic patients. *Kidney Int* 32: 256–260, 1987
48. McGrath LT, Douglas AF, McClean E, Brown JH, Doherty CC, Johnston GD, Pooler G, Archbold R: Oxidative stress and erythrocyte membrane fluidity in patients undergoing regular dialysis. *Clin Chim Acta* 235: 179–188, 1995