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DIALYSIS – TRANSPLANTATION

# Plasma proteins containing damaged L-isoaspartyl residues are increased in uremia: Implications for mechanism

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**Plasma proteins containing damaged L-isoaspartyl residues are increased in uremia: Implications for mechanism.**

*Background.* Several alterations of protein structure and function have been reported in uremia. Impairment of a transmethylation-dependent protein repair mechanism possibly related to a derangement in homocysteine metabolism is also present in this condition, causing erythrocyte membrane protein damage. Homocysteine may affect proteins via the accumulation of its parent compound S-adenosylhomocysteine (AdoHcy), a powerful in vivo methyltransferase inhibitor. However, since plasma homocysteine is mostly protein bound, a direct influence on protein structures cannot be ruled out. We measured the levels of L-isoaspartyl residues in plasma proteins of uremic patients on hemodialysis. These damaged residues are markers of molecular age, which accumulate when transmethylation-dependent protein repair is inhibited and/or protein instability is increased.

*Methods.* L-isoaspartyl residues in plasma proteins were quantitated using human recombinant protein carboxyl methyl transferase (PCMT). Plasma concentrations of homocysteine metabolites were also measured under different experimental conditions in hemodialysis patients.

*Results.* The concentration of damaged plasma proteins was increased almost twofold compared to control (controls 147.83 ± 17.75, uremics 282.80 ± 26.40 pmol of incorporated methyl groups/mg protein, $P < 0.003$). The major protein involved comigrated with serum albumin. Although hyperhomocysteinemia caused a redistribution of thiols bound to plasma proteins, this mechanism did not significantly contribute to the increase in isoaspartyl residues. The S-adenosylmethionine (AdoMet)/AdoHcy concentration ratio, an indicator of the flux of methyl group transfer, was altered. This ratio was partially corrected by folate treatment (0.385 ± 0.046 vs. 0.682 ± 0.115, $P < 0.01$), but protein L-isoaspartate content was not.

*Conclusions.* Plasma protein damage, as determined by protein L-isoaspartyl content, is increased in uremia. This alteration is to be ascribed to an increased protein structural instability, rather than the effect of hyperhomocysteinemia.

**Key words:** recombinant PCMT, transmethylations, homocysteine, S-adenosylhomocysteine, hemodialysis.

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Deamidation of asparaginyl residues and/or isomerization of aspartyl residues in proteins is a spontaneous, postbiosynthetic type of damage (also termed protein "fatigue" damage), which manifests itself by the appearance of abnormal L-isoaspartyl residues [1].

L-isoaspartyl sites are able to destroy local conformation of proteins and destabilize their structure, thus affecting physicochemical properties, such as solubility, and interfering with biological activity [2–6]. The formation of L-isoaspartyls is increased during cell aging and in various cell stress models [7], as well as in some pathological conditions, such as anemias caused by intrinsic erythrocyte defects [8, 9]. L-isoaspartyl residues have been also found in diseases such as Alzheimer's disease and prion encephalopathies in which the accumulation of altered proteins plays a pathogenetic role [10–13].

The enzyme protein L-isoaspartate (D-aspartate) *O*-methyltransferase (PCMT; EC 2.1.1.77) selectively recognizes and modifies such deamidated/isomerized residues, thus promoting the repair of abnormal isopeptide bonds [1]. PCMT, as well as all other transmethylation enzymes, is powerfully inhibited by S-adenosylhomocysteine (AdoHcy), the homocysteine precursor [1]. In this respect, it has been recently demonstrated that in healthy women, plasma homocysteine, through an increase of plasma and intracellular AdoHcy, leads to DNA hypomethylation in lymphocytes [14]. In fact, when homocysteine accumulates, hydrolysis of AdoHcy slows down, leading to AdoHcy elevation [1]. PCMT also has been utilized as a highly specific enzymic tool for the identification and quantitation of isoaspartyl residues in proteins [15].

Protein carboxyl methyl transferase-dependent membrane protein repair of L-isoaspartyl residues is altered in erythrocytes of uremic patients on hemodialysis, and this has been regarded as an effect of hyperhomocysteinemia via the accumulation of AdoHcy [16–20].

L-isoaspartyl protein damage was measured using human recombinant PCMT in plasma samples of uremic patients on hemodialysis, in an attempt to verify whether

the hyperhomocysteinemia, which is characteristic of these patients, through AdoHcy, has repercussions in the plasma compartment as well as intracellularly. Partial characterization of the affected proteins was carried out.

The possibility that direct formation of homocysteinylated protein adducts could affect protein composition was tested.

Protein damage and the S-adenosylmethionine (AdoMet)/AdoHcy concentration ratio, a reliable indicator of the flux of methyl group transfer, was also measured after a two-month course of folate therapy to verify whether homocysteine reduction is able to modify these parameters.

## METHODS

### Patients and treatment

Controls were volunteer healthy subjects recruited among hospital staff. End-stage renal disease patients were selected, provided that they were not affected by systemic diseases such as lupus erythematosus, arterial hypertension antecedent to renal disease, diabetes mellitus, and cardiac disease. Patients were under regular bicarbonate hemodialysis treatment, three times weekly, utilizing non-reusable hollow-fiber dialyzers. Kt/V was routinely checked at monthly intervals, following the NKF-DOQI recommendations, and it was always above 1.4 during the study and in the previous six months. Patients did not consume any B complex vitamins or phosphodiesterase inhibitors for a washout period of two months [21]. Methyltetrahydrofolate (MTHF) was administered for two months at the dose of 15 mg/day per mouth. Compliance with treatment was assessed by pill count. Informed consent was obtained from each patient. Patients did not mention any unusual symptoms during the study period.

Blood was drawn by venipuncture immediately before the dialysis session in hemodialysis patients when in a fasting state using ethylenediaminetetraacetic acid (EDTA; 1 mg/mL of blood). Whole blood was immediately centrifuged to separate plasma from cells. Plasma aliquots were stored at −80°C before analysis.

Routine blood biochemical tests (BM/Hitachi 911, F. Hoffmann-La Roche Ltd., Basel, Switzerland) including urea, creatinine, glucose, total protein, albumin, and electrolyte concentrations, were performed before and after treatment.

### Materials

S-adenosyl-L-[methyl-$^{3}$H]methionine [specific activity (sp. act) 500 mCi/mmol] and S-adenosyl-L-[methyl-$^{14}$C] methionine (sp. act 50 mCi/mmol) were purchased from Amersham International (Little Chalfont, Buckinghamshire, UK). All standards and reagents were from Sigma Chemical Co. (St. Louis, MO, USA) and were of the purest grade available.

### Human recombinant PCMT

A cDNA clone encoding for the more acidic isoenzyme II of PCMT, from a human brain library, was a generous gift of Dr. Steven Clarke (Department of Chemistry and Biochemistry and the Molecular Biology Institute, University of California Los Angeles, Los Angeles, CA, USA). For enzyme overexpression into *Escherichia coli* and purification, the method described by MacLaren and Clarke was followed, with minor modifications [15]. Briefly, *E. coli* strain DH5a was used for cloning and propagation of plasmid constructs. For PCMT overexpression, *E. coli* strain BL21(DE3) was transformed with pDM2x expression plasmid and grown in Luria-Bertani medium, in the presence of 100 μg/mL ampicillin. PCMT was purified from transformed bacteria basically as described by MacLaren and Clarke [15], except that the original DEAE-cellulose chromatography final step, under nonequilibrium conditions, was replaced by Q-Sepharose HP chromatography, using a Hiload 26/10 column (Pharmacia, Uppsala, Sweden). Column was equilibrated with buffer A (20 mmol/L Tris-HCl, 0.2 mmol/L EDTA disodium salt, 10% wt/vol glycerol, 15 mmol/L β-mercaptoethanol, 25 μmol/L phenylmethylsulfonyl fluoride, 0.1 mol/L NaCl, pH 8.0). After sample loading (10 mL, 6 mg/mL protein concentration), the column was washed with 10 volumes of buffer A (at 1 mL/min flow rate), followed by a linear gradient from 0.1 to 0.7 mol/L NaCl over 210 minutes. PCMT activity was detected by means of the radiochemical assay described by Macfarlane, using ovalbumin as a standard methyl-accepting substrate [21]. One enzyme unit is defined as 1 pmol methyl groups transferred $\times$ min$^{-1}$.

### In vitro transmethylation assay of plasma proteins

L-isoaspartyl residues in proteins can be directly detected by an in vitro assay using PCMT as an enzymic probe with selective substrate specificity toward such damaged aspartyl residues [15]. Damaged residues were specifically recognized and methyl esterified by PCMT, using radiolabeled AdoMet as the methyl donor, under conditions designed to insure complete labeling, on a 1:1 molar ratio, of accessible damaged residues in proteins. This method has proven highly sensitive, specific, reproducible, and particularly suitable when analysis of deamidated protein mixtures is to be carried out [22].

Methylation of plasma proteins was performed in vitro in a 90 μL final assay volume containing approximately 1 mg plasma proteins (15 μL), 80 units of PCMT enzyme (5 μL), 47.07 μmol/L of [methyl-$^{14}$C]AdoMet (10 μL), and 60 μL of 0.4 mol/L sodium citrate buffer, pH 6.0. The mixture was incubated at 37°C for 90 minutes. Under these conditions, extensive methyl esterification of plasma proteins was achieved. The reaction was stopped by adding 90 μL of quench solution [0.2 mol/L NaOH, 1%

sodium dodecyl sulfate (SDS)]. One hundred thirty-five microliters were spotted on filter paper for the vapor diffusion assay and were read with a scintillation counter after two hours, as previously described [16]. Results are expressed as pmol incorporated methyl groups/mg protein. For this purpose, the specific activity of [methyl-$^{14}$C]AdoMet was corrected for the dilution due to endogenous AdoMet concentration in individual plasma samples, if not previously diafiltrated.

In preparation for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, plasma proteins were methyl esterified as described previously in this article, except that the vapor diffusion step was omitted, and protein samples were loaded onto the gels after treatment with sample buffer, as detailed later in this article. [methyl-$^{14}$C] AdoMet was replaced by [methyl-$^{3}$H]AdoMet (4 μL added to a 44.44 μmol/L final concentration into the assay) for methylation of samples to be analyzed by SDS-PAGE, followed by either fluorography or gel cut, extraction, and protein methyl esters quantitation by scintillation counting [16].

### Electrophoresis analysis of methylated plasma proteins

*SDS-PAGE.* SDS-PAGE of methylated plasma proteins was performed according to the general procedure of Fairbanks, Steck, and Wallach [23], with some modifications; 1.5 mm thick gels, containing acrylamide 5.6% (wt/vol) and bisacrylamide 0.2% (wt/vol), in the presence of 1% SDS, pH 7.4, were used. Samples containing approximately 70 μg of proteins were diluted 1:4 with SDS reducing buffer (62.5 mmol/L Tris-HCl, pH 6.8, 10% vol/vol glycerol, 2% wt/vol SDS, 0.71 mol/L β-mercaptoethanol, 0.01% wt/vol Bromophenol blue) and incubated at 37°C for 15 minutes. Samples were run under constant voltage mode (120 V), using a vertical slab gel unit, which was purchased from Hoefer Scientific Instruments (San Francisco, CA, USA).

*Nondenaturing PAGE.* Continuous PAGE under nondenaturing conditions was also employed, after in vitro methyl esterification with recombinant PCMT. This system reproduced the general protocol of Fairbanks and Avruch, in the presence of 50 mmol/L final $(H_3/Na_2H_2)PO_4$ buffer at pH 2.4 [24]. The procedure was carried out in the absence of SDS and dithioerythritol and without protein denaturation prior to sample loading. Horizontal resolving gels were poured, containing 10% total acrylamide (acrylamide:bisacrylamide 40:1.5 ratio), and photopolymerized. Protein samples were run at 150 V under constant voltage conditions. This system allowed recovery of nondenatured proteins from gel slices, which could be analyzed on cellulose acetate electrophoresis, as described later in this article. Native plasma proteins therefore could be identified on an acetate cellulose system without any interference caused by SDS traces.

For further standard electrophoresis separation of plasma proteins, a commercial system commonly available for clinical diagnostic purposes was employed (Helena Laboratories Ltd., Beaumont, TX, USA), using a Barbital/Tris buffer system at pH 8.8 on a cellulose acetate support.

*Protein band detection.* Gels were then cut into halves and each was loaded with parallel samples. One half was stained with Coomassie brilliant blue and subjected to band densitometry using a Pharmacia/LKB Ultro Scan XL laser densitometer (Pharmacia). The second half was sliced into 2 mm fractions, and radioactivity was determined after protein elution using 0.4 mL of 50 mmol/L sodium acetate buffer, 0.25% Triton X-100, pH 5.2, and incubated overnight at room temperature under constant shaking [16]. Samples were then counted for radioactivity. Data were expressed as pmol of incorporated methyl groups/band area. In some experiments, protein methyl esters were evaluated, after gel drying under vacuum, by means of either fluorography (Amplify; Amersham International) or gel imaging by electronic autoradiography (Instant Imager-Electronic Autoradiography; Packard Inc., Camberra, Australia).

### Determination of homocysteine concentration

Homocysteine concentration was determined according to the method of Ubbink, Vermaak, and Bissbort [25], as modified by Perna et al [17]. Homocysteine represented the total protein-bound and non-protein–bound homocysteine. Briefly, the procedure involves a preliminary step of reduction and release from plasma proteins, using tri-*n*-butyl-phosphine in dimethylformamide, followed by precolumn derivatization with ammonium 7-fluorobenzo-2-oxa-1, 3-diazole-4-sulfonate (SBD-F).

Separation was accomplished on a C18, 5 μm, 250 × 4.6 mm, reverse phase column ("Luna"; Phenomenex, Torrance, CA, USA). The mobile phase was 0.1 mol/L $KH_2PO_4$, pH 2.1, containing 4% acetonitrile, with a flow rate of 1.0 mL/min. Micromolar concentrations of homocysteine are referred to 1 L of plasma. Detection conditions were optimized for homocysteine. Fluorescence intensities were measured with excitation at 385 nm and emission at 515 nm, using a Shimadzu RF-535 fluorescence detector (Shimadzu Co., Kyoto, Japan), equipped with a Shimadzu Chromatopac C-R6A data processor.

### Effect of homocysteine binding on plasma proteins

Homocysteinylation of plasma proteins can theoretically affect protein structure directly, thus bypassing AdoHcy accumulation and transmethylation inhibition or other postulated mechanisms of homocysteine toxicity. To test this possibility, 0.8 mL of plasma from control healthy individuals (mean homocysteine plasma concentration = 5 μmol/L) were incubated with or without 100 μmol/L of D-L-homocysteine for the indicated time periods at 37°C. The assay mixture was then diafiltrated

with Centricon YM-10 filters, 10,000 molecular weight cut-off (Amicon, Millipore, Bedford, MA, USA), with a Beckman Model J2-21 centrifuge, 5000 × *g* for 30 minutes. Protein-containing retentate was then washed twice with buffered saline solution. After diafiltration, the first diafiltrate and the retentate were analyzed for their content in homocysteine, cysteine (Cys), and the cysteinylglycine (CysGly) dipeptide. Results were corrected for the final volumes of retentate and diafiltrate. Plasma proteins were also subject to the PCMT-catalyzed in vitro methylation and vapor diffusion assay, as described previously in this article. Methylation results are the mean ± SE of triplicate determinations.

### High-performance liquid chromatography determination of plasma AdoHcy and AdoMet

Since plasma concentrations of AdoMet and AdoHcy were expected to be in the 0.2 to 0.6 μmol range [26], to obtain the etheno derivatives of both nucleotides, precolumn derivatization was performed according to the general procedure of Wagner et al [27], with some modifications. Ten percent of 0.625 mL of perchloric acid was added to 1 mL of thawed plasma and vortexed. After centrifugation at 12,000 × *g* for 10 minutes, the supernatant was filtered through a Millipore membrane (0.22 μm). Two hundred microliters of filtered samples were treated with 50 μL of chloracetaldehyde, and pH was adjusted to 3.5-4.0 by the addition of 50 μL of 3 mol/L sodium acetate. Samples were then derivatized by incubation at 39°C for eight hours, and the reaction was stopped by ice cooling.

A System Gold Solvent Module High-performance liquid chromatography system (model 126; Beckman Instruments, Inc., Palo Alto, CA, USA) controlled by a System Gold software package, running on an IBM PS2 personal computer, was used. Fluorescence of etheno derivatives was measured with excitation at 270 nm and emission at 410 nm, using a Shimadzu RF-535 fluorescence detector (Shimadzu Co.) equipped with a Shimadzu Chromatopac C-R6A data processor. $1,N^6$-etheno-AdoMet and $1,N^6$-etheno-AdoHcy concentrations were measured separately under different conditions by a combination of the methods reported by Loehrer et al [26] and Della Ragione et al [28], respectively, with some modifications, according to two experimental conditions.

*Condition A.* Etheno-AdoMet separation [26] was obtained on a 4.0 × 200 mm Hypersil RP-18 (particle size 3 μm) column with a guard column (4.0 × 20 mm) filled with the same packing material (MZ analyzentechnik, Mainz, Germany). The column was equilibrated with a 0.1 mol/L sodium acetate buffer containing 0.5 mmol/L heptanesulfonic acid and 5.9% vol/vol acetonitrile. After 40 minutes of isocratic elution (0.7 mL/min flow rate), the column was flushed with 100% acetonitrile for 10 minutes, followed by re-equilibration to initial conditions within 15 minutes. Recovery, calculated by adding trace amounts of S-adenosyl-[methyl-$^3$H]-methionine to plasma samples, processed in parallel, was complete. Retention times of the compounds of interest was 10 minutes for etheno-AdoMet and 2 minutes for etheno-AdoHcy.

*Condition B.* To optimize conditions for etheno-AdoHcy analysis, the chromatographic system was reversed by using an Ultrasil CX column (particle size 10 μm, 25 × 4.6 mm; Beckman Inc.), with a guard column (4.5 × 3.2 mm) filled with the same packing material. The column was equilibrated with a 0.25 mol/L ammonium formiate buffer, adjusted to pH 4.0 with formic acid, at a 1.5 mL/min flow rate [28]. Etheno-AdoHcy was eluted with a 7.8-minute retention time.

Peak assignment was accomplished by three methods in combination: (*1*) comparison with etheno-derived external standards of AdoMet and AdoHcy; (*2*) addition of an internal standard of AdoMet and AdoHcy etheno derivatives; (*3*) for AdoMet, plasma was treated with cathecol *O*-methyltransferase, EC 2.1.1.6 (10 U of cathecol *O*-methyl transferase was added to 1 mL of plasma in the presence of saturating concentrations of the methyl accepting substrate, protocatechuic acid 0.1 mmol/L, and 0.05 mmol/L $MgCl_2$, and the mixture was incubated at 37°C for 1 hour), and processed as described previously in this article.

### Statistical analysis

Statistical analysis was performed employing the Student unpaired or paired *t* test, as appropriate. Linear regression analysis was done to assess the independent effects of the different variables [29]. All calculations were performed using the software package SPSS® (SPSS Inc., Chicago, IL, USA), running on an IBM-compatible personal computer. All results are presented as the mean ± SE.

## RESULTS

### Molecular "fatigue" damage in plasma proteins of uremic patients on hemodialysis

Levels of L-isoaspartyl residues were measured in plasma samples from hemodialysis patients and from healthy controls. Figure 1 shows a significant increase of damaged aspartyl residues in hemodialysis patients ($N$ = 15) compared with controls ($N$ = 9). The mean value in uremic patients was almost double the value in controls.

### Major damaged plasma protein in hemodialysis patients comigrates with albumin

Plasma proteins from hemodialysis patients, as well as from normal controls, were methyl esterified in vitro by PCMT and analyzed by means of SDS-PAGE or nondenaturing PAGE, as described in the **Methods** section.

Methyl esters incorporated into proteins were quanti-

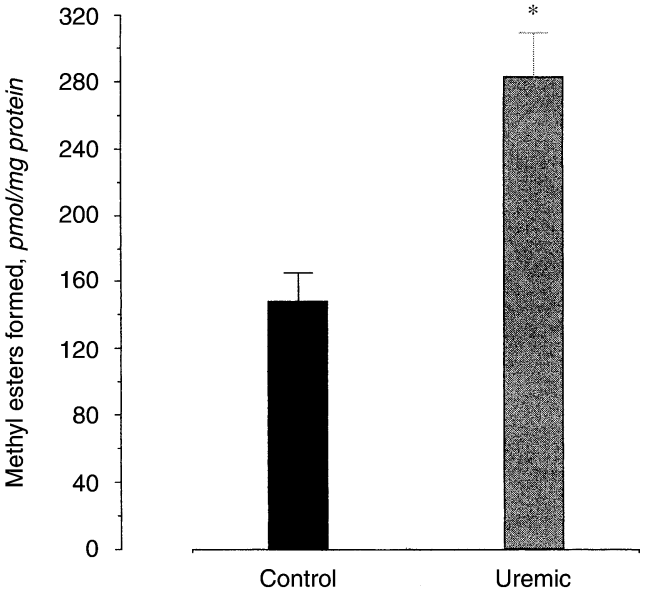


**Fig. 1. Protein damage measured with human recombinant protein L-isoaspartate (D-aspartate) *O*-methyltransferase (PCMT) assay.** Plasma protein content of L-isoaspartyl residues was quantitated by means of specific radioenzymatic assay employing human recombinant PCMT (**Methods** section) in control and hemodialysis patients. Control $N = 9$, uremic $N = 15$. $^*P < 0.003$ (unpaired $t$ test).

tated by gel cut, overnight protein elution, and scintillation counting; an example is given in Figure 2A. The most heavily affected protein comigrated with serum albumin (Fig. 2A). The values of albumin methyl esters obtained in this set of experiments were normalized by protein band area (Fig. 2B). The results in Figure 2 demonstrate that the radioactive methyl incorporation in proteins was higher in the uremic plasma samples, confirming that most radioactivity incorporated as protein methyl esters comigrated with albumin.

Figure 2B therefore depicts the pmol of methyl groups relative to the protein comigrating with albumin, normalized per area of the relevant protein band obtained from the stained electrophoretic profile. In uremia, the increase of isoaspartyl content of the protein comigrating with albumin with respect to control was greater when compared with the average increase in total plasma proteins. On the other hand, it is worth noting that radioactivity associated with the other peaks shows little difference between uremic and control (Fig. 2A). This indicates that (*1*) not all proteins in plasma are subject to the same increase in isoaspartyl content in uremia, and (*2*) proteins comigrating with albumin in uremia are selective targets for isoaspartyl formation compared with other proteins.

Analysis of methyl esterified proteins was also accomplished by a nondenaturing gel electrophoresis system and radioactive band detection by electronic autoradiography (Fig. 3A). Re-analysis by cellulose acetate electrophoresis further confirmed that the major methyl esterified protein in uremia comigrated with serum albumin (Fig. 3B). Fluorography also allowed the identification of methyl-esterified serum albumin (comigrating with the molecular weight standard of 66.00 kD) and five other minor radioactive protein bands with a molecular mass of 134, 53, 50, 34, 28 kD, respectively (Fig. 3C).

A quantitative estimate of the methylation stoichiometry of albumin could be made from data accessible from individual analyses as in Figure 2, taking into account the patients' albumin concentration (available from routine blood testing) and the percentage of incorporated methyl esters comigrating with albumin compared with the total. This estimate provided a stoichiometry of $0.078 \pm 0.005$ mol of methyl esters incorporated/mol of albumin in the uremic versus $0.017 \pm 0.003$ mol/mol in the normal.

### Effects of homocysteine on in vitro protein methylation levels

Plasma homocysteine concentration in hemodialysis patients was significantly elevated compared with controls (control, $11.06 \pm 0.75$ μmol/L, $N = 8$; uremic patients, $69.44 \pm 16.46$ μmol/L, $N = 13$; unpaired $t$ test, $P < 0.01$). We verified the hypothesis that direct exposure of proteins to elevated homocysteine levels may induce an increase of aspartyl-damaged sites. Plasma from normal individuals was incubated in the presence of homocysteine at concentrations comparable to those detected in uremic patients. Evaluation of L-isoaspartyl content was accomplished by an in vitro PCMT methylation assay of plasma protein samples preincubated without or with homocysteine for different time periods, and freed from unbound thiols by diafiltration prior to the PCMT assay.

No increase in the normal plasma protein methyl-accepting capability, which was specifically induced by homocysteine in the medium, could be detected (Fig. 4A). An increase in protein methylation was observed in both treated and untreated samples after several hours of incubation, likely caused by in vitro deamidation/isomerization of plasma proteins. This increase in vitro never approached the levels observed in uremic plasma, indicating that normally plasma proteins in vivo are effectively protected against deamidation.

A methionine load significantly increases homocysteine plasma levels in healthy subjects. This situation is slightly closer to physiological conditions compared with the in vitro homocysteine addition to normal plasma. The effect of a methionine load on plasma protein deamidation was checked, and the results showed no specific acute effect of homocysteine on plasma protein damage (Fig. 4B).

After two hours of incubation in the presence of homocysteine, protein-bound thiols were concomitantly evaluated by means of high-performance liquid chromatography. Chromatographic analysis allowed an evaluation of protein-bound thiols (namely Cys, CysGly, and homocys-

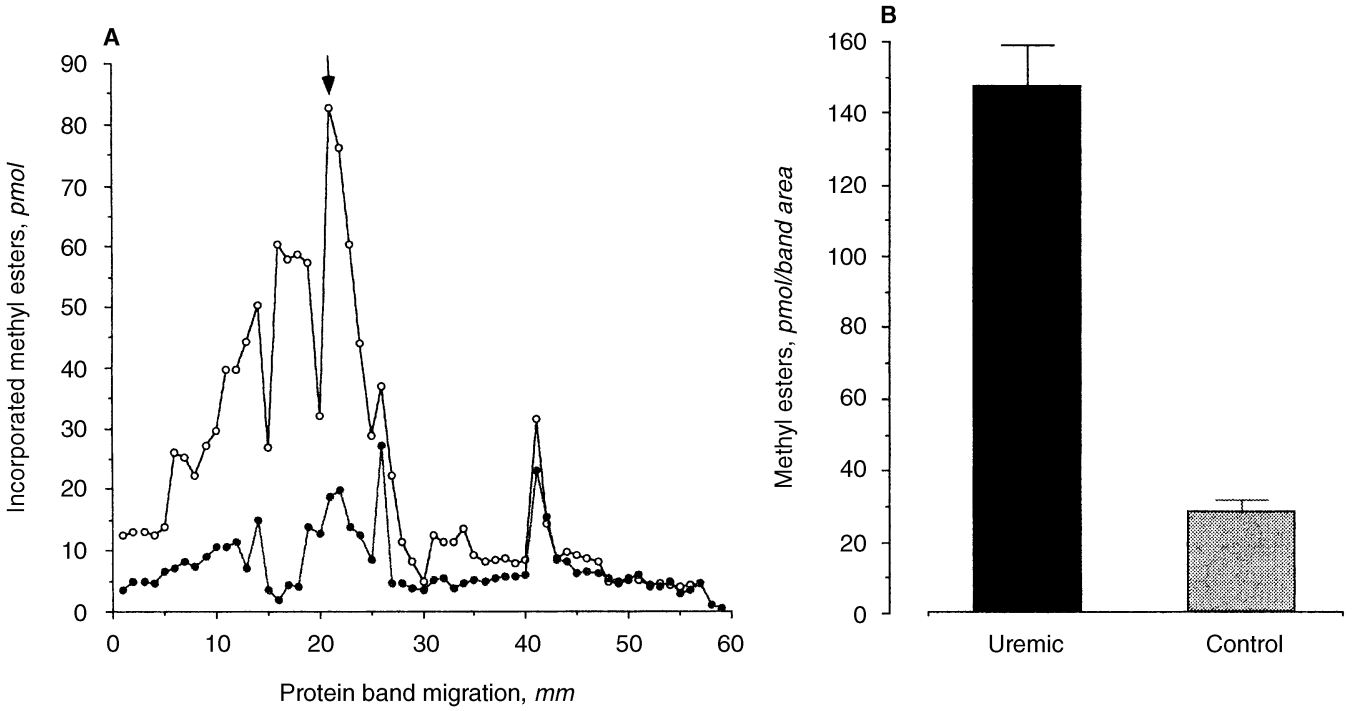


**Fig. 2. Identification of methyl-labeled plasma proteins in uremics (○) and controls (●).** Plasma proteins from both normal controls and hemodialysis patients were methylated in vitro by human recombinant PCMT, as a specific marker reaction for L-isoaspartyl residues, using [$^3$H-methyl]AdoMet. Duplicate samples of methyl-labeled plasma proteins were then separated by SDS-PAGE. Gel halves were processed in parallel. One half was Coomassie blue stained for protein molecular mass determination. In the other gel half, individual lanes were dissected into 2 mm slices; proteins were extracted and samples were counted for radioactivity. (*A*) The arrow points to the position of standard serum albumin. (*B*) Values of albumin methyl esters were normalized by protein area, measured by means of laser densitometry of Coomassie blue-stained profile of electrophoresis separated protein bands.

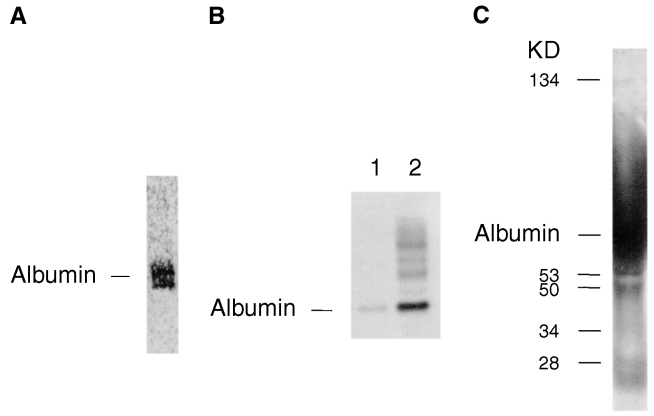


**Fig. 3. Characterization of methyl-labeled, isoaspartyl-containing, damaged plasma proteins in uremia.** Plasma proteins from hemodialysis patients were methylated in vitro using PCMT and [$^{14}$C-methyl]AdoMet. Radiolabeled proteins were then separated by PAGE under nondenaturing conditions and visualized by electronic autoradiography (*A*). The radioactive protein band was excised, extracted from the gel slice, and re-analyzed on cellulose acetate electrophoresis system (*B*), where it co-migrated with albumin, when compared with a profile of total plasma proteins (lane 1 and 2, respectively). Results were further confirmed by methylating a parallel plasma protein sample with PCMT, in the presence of [$^3$H-methyl] AdoMet, followed by SDS-PAGE analysis and detection of radioactive protein bands by fluorography (*C*; arrows indicate the molecular weight based on the position of protein standards).

teine) in plasma incubated for two hours at 37°C without and with homocysteine. Analysis of protein-bound and -unbound thiols in samples incubated without homocysteine showed that almost all plasma homocysteine bound to proteins. In the sample incubated with homocysteine, the proportion of free homocysteine increases. After a correction for the final volumes of retentate (~800 μL) and diafiltrate (~1300 μL), protein-bound homocysteine represented approximately 99% and 68% of the total in samples incubated without and with homocysteine, respectively. Protein-bound Cys and the dipeptide CysGly decreased in the sample incubated with homocysteine compared with the sample in the absence of homocysteine. After correction for the final volume, protein-bound Cys and CysGly were 49% (Cys) and 44% (CysGly) in the high homocysteine sample and 86% (Cys) and 81% (CysGly) in the low homocysteine samples.

## The AdoMet/AdoHcy ratio is an in vivo marker of homocysteine derangement, but not of plasma protein damage

The AdoMet/AdoHcy ratio is altered in uremia both in the intracellular compartment [16] and in plasma, where it is thought to reflect mainly the metabolism of these two compounds in the liver [26].

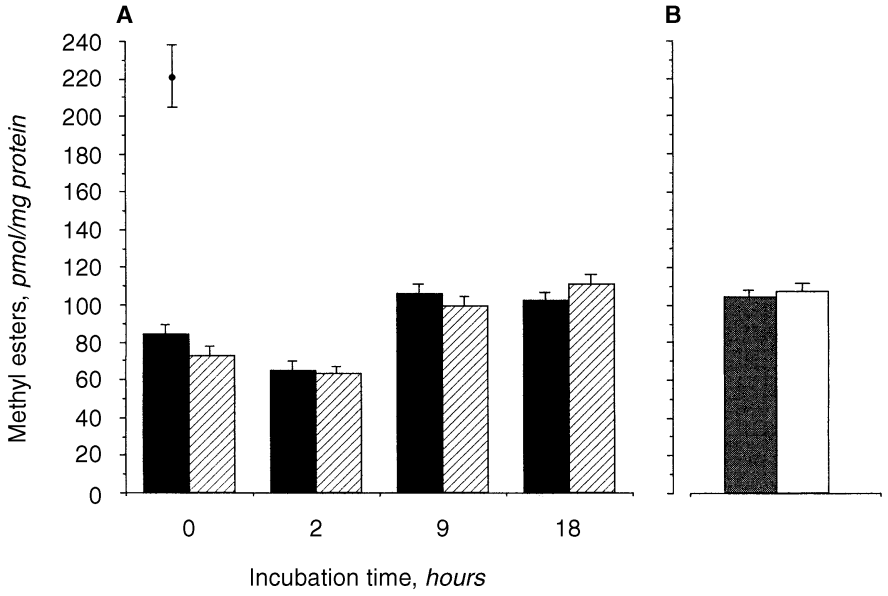


**Fig. 4. In vitro and in vivo acute homocysteine effects on plasma protein methylation.** (*A*) Plasma from normal volunteers was incubated with DL-homocysteine (100 μmol/L; ▨) and without (■), at 37°C for the indicated times. At the end of incubation, plasma proteins were diafiltrated, and the recombinant PCMT assay was performed. Closed circle symbol points to the mean methylation value of uremic diafiltrated plasma proteins (homocysteine concentration: 44.05 ± 0.94). (*B*) The effects of acute hyperhomocysteinemia on plasma protein deamidation and isomerization were evaluated by PCMT assay after a methionine load performed in three normal subjects (homocysteine concentration before load 10.9 ± 0.47 and after load 32.26 ± 0.79). Symbols are: (▨) fasting; (□) post-methionine loading. The oral methionine load was performed by administering 100 mg of methionine/kg diluted in fruit juice. Blood samples were drawn before and after four hours, and samples were subjected to homocysteine analysis and the PCMT-catalyzed assay. Plasma samples to be assayed for PCMT were diafiltrated as in (A).

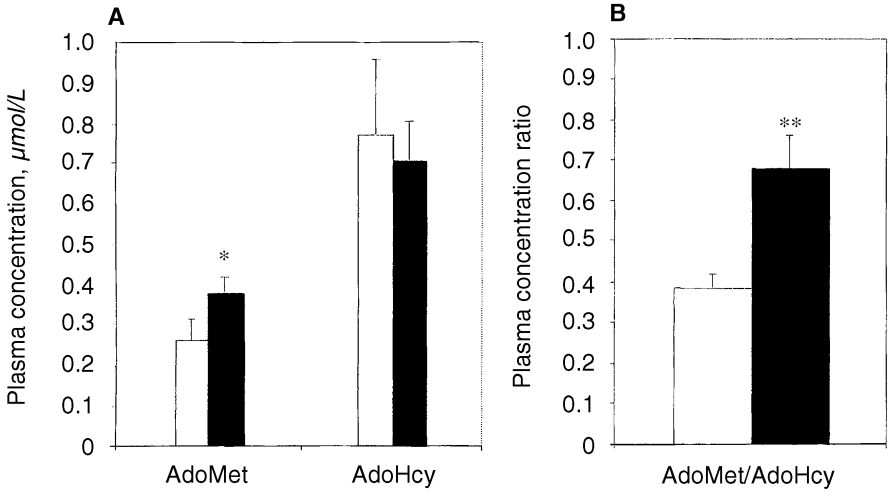


**Fig. 5. Plasma AdoMet, AdoHcy, and AdoMet/AdoHcy ratio before (□) and after (■) folate administration to uremic patients.** Plasma concentrations of AdoMet and AdoHcy were measured in uremic hemodialysis patients before and after folate treatment. Results are expressed as concentrations of both nucleosides (*A*), as well as the AdoMet/AdoHcy ratio (*B*; $N = 13$, $^*P < 0.04$; $^{**}P < 0.01$).

Folates have clear-cut lowering effects on plasma homocysteine levels in hemodialysis patients and significantly ameliorate the AdoMet/AdoHcy ratio in erythrocytes [20]. In the present study, the homocysteine concentration in uremic patients was 69.44 ± 16.46 μmol/L and after folate intake was 17.47 ± 2.61 μmol/L ($N = 13$, $P < 0.005$, paired $t$ test).

The plasma AdoMet concentration before and after therapy with folates was measured (Fig. 5). Mean values before therapy were 0.268 ± 0.058 μmol/L and after folates were 0.381 ± 0.052 μmol/L, $P < 0.04$.

The AdoHcy concentration before and after folate administration was measured, and data are shown in Figure 5. Mean values before therapy were 0.776 ± 0.218 μmol/L and after were 0.709 ± 0.111 μmol/L ($P$ = NS).

AdoMet/AdoHcy values also are included in Figure 5. The mean before therapy was 0.385 ± 0.045 and after was 0.682 ± 0.115 ($P < 0.01$). Results indicate that upon folate administration (*1*) homocysteine is effectively removed by remethylation to methionine, and (*2*) metabolic conditions sustain the flow of transmethylations through an increased AdoMet concentration and AdoMet/AdoHcy ratio.

## Protein damage after folate therapy

Levels of plasma protein damage were also measured by the in vitro protein methylation assay after folate administration in uremic patients. Results show that methyl esterification of plasma proteins was not significantly modified after folate administration (before folate 289.20 ±

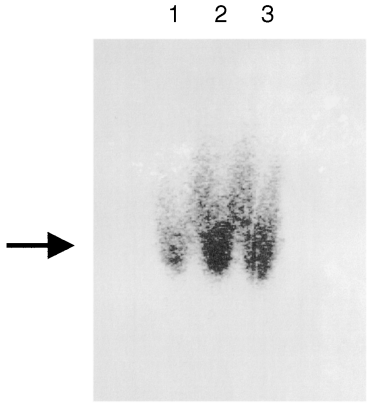


**Fig. 6. Effect of folate administration on in vitro methylation of plasma proteins in uremic patients.** Plasma proteins, enzymatically methyl labeled by means of PCMT in the presence of [$^{14}$C-methyl]AdoMet, were analyzed by means of SDS-PAGE and were subjected to gel imaging by electronic autoradiography. Lane 1, normal healthy control; lane 2, uremic patient before folate treatment; and lane 3, uremic patient after folate treatment.

30.08, after folate 266.84 ± 21.90 pmol/mg protein; paired *t* test, $N = 13$, $P$ = NS). This observation was further validated by the SDS-PAGE analysis of in vitro methylated plasma proteins (Fig. 6), confirming that (*1*) major isoaspartyl-containing plasma proteins comigrated with albumin both in normal and uremic patients, and (*2*) the isoaspartyl content of this plasma protein component is evidently increased in uremia (Fig. 6, lane 2 vs. lane 1), with a slight tendency to decrease after folate treatment (Fig. 6, lane 3 vs. lane 2).

## DISCUSSION

The key finding of the present study is that damaged plasma proteins are significantly increased in hemodialysis patients. The protein that is most affected comigrates with albumin under different analytical conditions. Direct binding of homocysteine to proteins is not responsible for this type of alteration. Although folate therapy is able to reduce homocysteine levels and favorably affect the AdoMet/AdoHcy ratio, it did not significantly modify the levels of damaged plasma proteins, at least under the conditions employed in this study. This indicates that protein damage is not substantially mediated by the methylation inhibition caused by AdoHcy.

The physiological role of PCMT in vivo is linked to the repair/disposal of damaged proteins [1]. In uremic patients, methylation of erythrocyte membrane proteins is reduced in intact cells. This is due to the inhibition exerted by increased AdoHcy concentration, caused by homocysteine elevation [17]. An abnormal accumulation of L-isoaspartyl residues in red cell membrane proteins was evaluated in uremia by quantitating side products of the repair process, complemented by data fitting in computer-aided mathematical models [18].

Protein carboxyl methyl transferase under normal conditions is an intracellular enzyme, even if the elegant work by Weber and McFadden showed that after endothelial injury, altered aspartyl residues of type I and type III collagen become methylated by PCMT, which is released into the extracellular space [30, 31]. However, circulating proteins are not selectively hypermethylated in vitro in PCMT knockout mice [32]. This indicates that during their passage in circulation, plasma proteins are not accessible to this modification unless some type of cell or tissue stress takes place.

The aim of our study was to determine the isoaspartyl content of extracellular proteins in uremia, which could be more prone to this kind of damage than intracellular proteins.

In this respect, other types of postbiosynthetic protein damage have been described in uremia [33, 34]. In addition, a self-limited cell breakage, not measurable with the usual markers of endothelial damage, cannot be ruled out in uremia. Furthermore, protein deamidation can occur before proteins enter the bloodstream or be the result of reduced protein stability, rather than impaired repair. Several conditions may be simultaneously operative.

As to the causes underlying this kind of damage of plasma proteins in uremia, the inhibition of protein repair does not seem to be involved because the plasma AdoMet/AdoHcy ratio was ameliorated by folate therapy, but protein L-isoaspartyl content was not. This finding can be interpreted that AdoHcy does not play a role in the isoaspartyl levels in plasma proteins, but it acts prevalently in cell proteins, as shown in our previous work [16–20]. Direct binding of homocysteine to proteins does not appear to be involved either.

A number of uremic toxins are present in plasma and in the intracellular compartment at concentrations, which may modify protein stability, and the intracellular and the extracellular environment may be affected by the physicochemical stresses appropriate of uremia (acidosis, hemodialysis, etc.). In addition, the elevation of plasma protein isoaspartyl content in uremia may reflect the presence of more long-lived, aged proteins in circulation. However, we think this possibility is rather unlikely because protein catabolism is frequently increased in uremia [35], although the isopeptide bond is resistant to proteolytic cleavage [22].

Therefore, plasma proteins in uremia may be "prematurely" aged, either because of reduced repair (before secretion) or because of increased instability caused by the microenvironment. Data in the present manuscript to some extent support the second hypothesis. In light of these findings, a new hypothesis is proposed: Damaged residues are present in plasma proteins probably because the uremic milieu causes premature protein aging.

Molecular alterations in the form of isomerized aspartyl residues, generated through deamidation of asparaginyl residues or isomerization of aspartyl residues, may accumulate in proteins in vivo [18]. These modifications may play a pathogenetic role in diseases in which altered proteins accumulate. This is the case of Alzheimer's disease, in which the isoaspartyl content of β-amyloid has been related to the aging of brain lesions [36]. Isomerized and/or racemized aspartyl residues lead to significant changes in the chemical-physical properties (aggregation, solubility) of β-amyloid [10, 11, 37]. Isomerized aspartyl residues also have been detected in prion proteins, generating altered forms of these macromolecules [12, 13]. Proteins in central nervous tissue or connective tissue contain isoaspartyl sites [38]. In addition, isoaspartyl residues have been recently reported to increase the immunogenic properties of peptides [39]. The effects of deamidation on biological functions have been carefully assessed: Deamidation indeed reduces the activity of several proteins, such as calmodulin, CD4, and human growth hormone [6].

The availability of natural or recombinant PCMT represents an advancement in the identification of isoaspartyl residues, since this enzyme selectively recognizes and methyl esterifies the free α-carboxyl group of isoaspartyl residues, thus allowing quantitation of such altered sites in proteins [1, 6, 15, 40–43]. Other aspartyl residues are poorly or not recognized at all (that is, the L-Asp and the D-isoAsp), so PCMT is a reliable enzymatic tool for in vitro characterization of L-isoAsp–containing proteins [1, 22].

A human recombinant PCMT assay allowed us to demonstrate that proteins containing L-isoAsp residues were significantly increased in uremic plasma. The major in vitro substrate for PCMT comigrated with albumin, but at least five other methylated species were detected.

In the albumin sequence, six possible spots for protein deamidation/isomerization lie within regions endowed with important functions, including immunologic and binding properties [44–46].

Homocysteine, a cardiovascular risk factor that is elevated in uremia, may exert its toxic action at least in part by modifying protein structure and function [reviewed in 47]. Moreover, alterations of postbiosynthetic processing of proteins are common findings in both uremic [33, 34] and homocystinuric patients [48]. Among the possible biochemical mechanisms of toxicity, homocysteine can: (*1*) act as a transmethylation inhibitor through its precursor AdoHcy, (*2*) induce oxidative modifications, or (*3*) induce protein acylation. Formation of homocysteinylated protein adducts is possible, in consideration of the reactivity of homocysteine sulfhydryl and carbonyl group (the latter for homocysteine thiolactone [47]. In the set of experiments presented in this article, in vitro or after a methionine load, the L-isoaspartyl content of plasma proteins is not influenced by the direct binding of homocysteine to plasma proteins. Our data confirm that homocysteine binding to plasma proteins induces a significant redistribution of bound thiols, but it is not sufficient to increase content of altered aspartyl residues per se.

The L-isoaspartyl content of plasma proteins is not significantly modified by folate treatment, under conditions in which other homocysteine metabolic parameters—that is, homocysteinemia, and [AdoMet]/[AdoHcy]—are remarkably affected. This indicates that, in line with previous results on animal models, plasma proteins are not significantly recognized and repaired in vivo by endogenous PCMT, at least from the moment of their secretion into the bloodstream [32].

Damage accumulation in the form of isoaspartyl residues in plasma proteins therefore can be ascribed to their increased structural instability in uremia, rather than the effect of hyperhomocysteinemia, due to either reduced repair or direct binding of this amino acid to macromolecules.

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