Plasma Protein Aspartyl Damage Is Increased in Hemodialysis Patients: Studies on Causes and Consequences

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Abstract. Plasma proteins in hemodialysis patients display a significant increase in deamidated/isomerized Asx (asparagine and aspartic acid) content, a marker of protein fatigue damage. This has been linked to the toxic effects of hyperhomocysteinemia in uremic erythrocytes; however, treatment aimed at abating homocysteine levels did not lead to significant reductions in plasma protein damage. The hypothesis that lack of reduction in protein damage could be due to protein increased intrinsic instability, as result of interference with the uremic milieu rather than to hyperhomocysteinemia, was put forward. The deamidated/isomerized Asx content of normal plasma incubated with several uremic toxins for 24 h, 72 h, and 7 d was measured, identifying a group of toxins that were able to elicit this kind of damage. Uremic toxins were also incubated with purified human albumin, and dose-response experiments with the two most toxic agents in terms of protein damage (guanidine and guanidinopropionic acid) were carried out. The effect of the hemodialysis procedure on protein damage was evaluated. For investigating also the consequences of these alterations, human albumin was treated in vitro to produce an increase in deamidated/isomerized Asx residues, and the effects of albumin deamidation on protein binding were evaluated. Among the uremic toxins that are able to elicit protein damage, guanidine produced a dose-dependent increase in protein damage. No difference was found after a hemodialysis session. Deamidated albumin shows normal binding capacity to warfarin, salicylic acid, or diazepam but reduced binding to homocysteine. In conclusion, uremic toxins, especially guanidine, display an ability to induce significant protein damage, which can in turn have functional consequences.

Plasma proteins are a group of very heterogeneous molecules, in terms of structure complexity and function, that can deteriorate in response to stresses of various nature (1). Among these, the milieu offered by the uremic plasma is certainly one of the most representative of a stressful environment (2). A particular kind of protein damage is represented by the accumulation of deamidated/isomerized Asx residues, originating from asparaginyl deamidation, or aspartyl isomerization through a succinimide intermediate, either during protein aging or in response to oxidation, mechanical stress, etc. (3). The presence of damaged residues interferes with normal protein structure and function. We previously demonstrated that in chronic renal failure patients who undergo hemodialysis therapy, these residues significantly accumulate in plasma and that the most affected protein is albumin (4). Under normal conditions, these residues are repaired by a mechanism that involves an enzymatic step catalyzed by a methyltransferase (protein isoaspartyl carboxyl-O-methyltransferase [PCMT]; EC 2.1.1.77). In this reaction, PCMT transfers a methyl group from the methyl donor S-adenosylmethionine (AdoMet) to the various methyl acceptors (in this case, the deamidated/isomerized Asx residue). AdoMet demethylated product S-adenosylhomocysteine (AdoHcy) is able to competitively inhibit PCMT, as well as potentially all AdoMet-dependent methyltransferases, when its concentration increases. In uremia, AdoHcy accumulates as a result of the elevation of homocysteine, thus reaching inhibitory concentrations toward methyltransferase activity in general and PCMT in particular (5–8). Although this is an important mechanism of homocysteine toxicity in erythrocytes, in the plasma compartment, the picture seems to be somewhat different. In uremic patients, folate therapy is able to reduce plasma homocysteine levels to restore DNA methylation back to normal levels and correct the altered gene expression patterns (9). However, in plasma, 2 mo of folate therapy, which significantly reduces homocysteine levels, does not lead to a significant reduction in deamidated/isomerized Asx residues (4). The hypothesis was put forward that the uremic toxins that are present in plasma could directly induce this form of protein damage. We therefore tested this hypothesis in various in vitro experiments using several known uremic toxins. We also examined patients before and after dialysis to check whether dialysis itself produced some damage. In addition, an in vitro deamidated albumin was obtained and
functionally studied in terms of its binding capacity to different substances, such as warfarin, salicylic acid, diazepam, and homocysteine.

Materials and Methods
S-adenosyl-L-[methyl-14C]methionine (sp act 50 mCi/mmol) was purchased from Amersham International (Buckinghamshire, UK). Urea, guanidine HCl, methyguanidine HCl, guanidinoacetic acid, guanidinopropionic acid, creatinine, creatine, p-cresol, 3-indoxylsulphate K, putrescine, spermidine, phenol, parathyroid hormone, asymmetric dimethyl arginine, and homocysteine, were from Sigma Chemical Co. (St. Louis, MO), as all other standards and reagents, and of the purest grade available.

In Vitro Transmethylation Assay of Plasma Proteins
Deamidated/isomerized Asx residues in proteins were detected by an in vitro assay using PCMT as an enzyme probe with selective substrate specificity toward such damaged aspartyl residues (10). Methylation of plasma proteins was performed in vitro as described previously (4). Results are expressed as picomoles of incorporated substrate specificity toward such damaged aspartyl residues (10).

Incubation Experiments
Experiments with Plasma. Selected uremic toxins were representative of several studied groups of uremic toxins (guanidino compounds, small molecules, proteins), and choice was by no means intended to be comprehensive or exhaustive of the entire scope of uremic toxins. Ten microliters of uremic toxins diluted with normal saline was incubated with 90 μl of plasma from a normal healthy subject at 37°C for 24, 72, and 168 in a shaking water bath, under sterile conditions. Final concentration of each uremic toxin was that present on the average, in uremic serum, as derived from the literature. Final concentrations were as follows: urea, 33 mM; guanidine HCl, 3 μM (12); methyguanidine HCl, 10 μM (13,14); guanidinoacetic acid, 5 μM (13,14); guanidinopropionic acid, 5 μM (13,14); creatinine, 884 μM; creatine, 80 μM (12); p-cresol, 92.5 μM (15); 3-indoxylsulphate K, 79.6 μM (16); putrescine, 1 μM (17); spermidine, 1 μM (17); phenol, 42.5 μM (18); parathyroid hormone, 1000 pg/ml (106 pM); and asymmetric dimethyl arginine, 1 μM (19). Control sets were represented by plasma incubated with saline, and normal saline incubated with each uremic toxin.

Experiments with Albumin. Human albumin in its powder form was dissolved in saline to a concentration similar to that of normal plasma (5 g/dl) and incubated, under the same conditions described for plasma, with the same uremic toxins used in the experiments with plasma.

Dose-Response Experiments. Dose-response experiments with guanidine and guanidinopropionic acid were performed incubating 90 μl of albumin solution with guanidine (31.4, 15.7, 9.45, 6.3, and 3.14 μM final concentrations) and guanidinopropionic acid (50, 25, 15, 10, and 5 μM final concentrations) for 7 d at 37°C. All incubation experiments were carried out under sterile conditions to avoid any bacterial contamination.

Experiments before and after a Hemodialysis Session in Uremic Patients
To verify whether hemodialysis membranes could interfere with proteins and induce the formation of L-isoaspartyl residues during a hemodialysis session, were selected ESRD patients, provided that they had been on dialysis for >6 mo and were not affected by systemic diseases, such as lupus erythematosus, diabetes, and cardiac disease. Patients were under regular bicarbonate hemodialysis treatment, thrice weekly, using hollow-fiber no-reuse dialyzers (either polysulfone or polyethyleneimine carboxylate [PMMA]). Dialysis was carried out with a blood flow at or above 300 ml/min and a dialysate flow rate of 500 ml/min. Patients were dialyzed with “pure” dialysis fluid, according to the European Best Practice Guidelines (20). Kt/V was checked routinely at monthly intervals, following the European Best Practice Guidelines recommendations, and was >1.4 during the study and in the previous 6 mo. For a washout period of 2 mo, patients did not take any group B vitamins or phosphodiesterase inhibitors (21).

Blood was drawn immediately before the hemodialysis session, in the fasting state, by venipuncture, using EDTA (1 mg/ml blood). After the dialysis session, usually after 4 h, blood was drawn according to the 2-min slow-flow technique. Whole blood was immediately centrifuged to separate plasma from cells. Plasma aliquots were stored at −80°C before analysis.

Preparation of Deamidated Albumin
Deamidated albumin was prepared according to the method described previously (22), with modifications. Human purified albumin was incubated with a 0.15 M potassium bicarbonate buffer at two different pH, pH 7.3 or 9.1 (200 mg of albumin diluted in 2.0 ml of buffer), under sterile conditions. Vials were flushed with nitrogen and sealed. Aliquots of the pH 7.3 solution (preparation A) and pH 9.1 solution (preparation B) were frozen as controls, and two other aliquots, one from the pH 7.3 solution (preparation C) and from the pH 9.1 solution (preparation D), were incubated for 48 h at 37°C. Samples were then dialysed extensively with water using YM-10 Centricon filters, 10,000 MW cut-off (Amicon, Millipore, Bedford, MA), to get rid of the bicarbonate buffer and then resuspended in distilled water, 2.0 ml final volume. Subsequently, samples were assayed for their deamidated/isomerized Asx content, as described above.

Binding Experiments with Deamidated Albumin
Quenching of the intrinsic tryptophan fluorescence of albumin induced by binding with warfarin, diazepam, and salicylic acid was monitored by spectrofluorimetry and used to study protein function, as described by Parikh et al. (23). Warfarin, diazepam, and salicylic acid were used to study three different binding sites of albumin. The four preparations of albumin (preparations A, B, C, and D) diluted with PBS to reach a 2.02 μM albumin concentration were mixed with a stock solution of warfarin, diazepam, and salicylic acid to reach various final concentrations (warfarin: 166.5, 133.2, 106.6, 85.2, 68.2, 54.6, 43.6, 34.92, 17.44, 8.73, 4.36, and 2.18 μM; diazepam: 1000, 800, 640, 320, 256, 163.8, 104.9, 67.1, 43, 27.5, 14.1, 9.0, 4.61, and 2.36 μM; and salicylic acid: 10,000, 1000, 500, 100, 50, 10, 1.5, 0.1, and 0.05 μM). After 30 min, fluorescence was measured (excitation, 280 nm; emission, 340 nm; slit width, 2.5 to 4.0 nm). Additional binding experiments with homocysteine, which covalently binds to the cysteine residue in position 34 of albumin, through a disulfide bond, were carried out. For these experiments, preparations A, B, C, and D of albumin were incubated with 100 μM final concentration of homocysteine for 2.5 h at 37°C. After this, samples were dialyzed with YM-30 Centricon to get rid of unbound homocysteine. The retentate was then washed again with PBS, and the homocysteine concentration was measured with the HPLC method of Ubink et al. (24), as modified by Perna et al. (6). Briefly, the procedure involves a preliminary step of reduction and release from albumin, using tri-n-butyl-phosphate in dimethylformamide, followed by precolumn
derivatization with ammonium 7-fluorobenzo-2-oxa-1, 3-diazo-4-sulfonate. Separation was accomplished on a C18, 5-µm, 250 × 4.6-mm, reverse-phase column (“Luna”; Phenomenex, Torrance, CA). The mobile phase was 0.1 M KH2PO4, pH 2.1, containing 4% acetonitrile, with a flow rate of 1.0 ml/min. Micromolar concentrations of homocysteine are referred to 1 L. Detection conditions were optimized for homocysteine. Fluorescence intensities were measured with excitation at 385 nm and emission at 515 nm. A Shimadzu HPLC (Shimadzu Co., Kyoto, Japan), model LX10AVP was used.

Statistical Analyses
Statistical analysis was performed using unpaired or paired t test, as appropriate. All calculations were performed using the software package SPSS, (SPSS, Chicago, IL), running on an IBM-compatible personal computer. All results, unless otherwise stated, are presented as the mean (SD) of triplicate experiments, performed independently on different occasions; and for each set of experiments, samples were assayed two times.

Results
Normal Plasma Incubated with Uremic Toxins for Various Time Periods
Results relative to the incubation experiments of normal plasma with uremic toxins are depicted in Table 1. Almost all tested uremic toxins affected protein deamidated/isomerized Asx content, and the effect was more pronounced after 168 h.

Table 1. Effects of incubation of several different uremic toxins with normal plasma for 24, 72, 168 h on protein methyl ester concentration (pmol/mg protein) a

<table>
<thead>
<tr>
<th></th>
<th>24</th>
<th>72</th>
<th>168</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>276 (9)</td>
<td>440 (14)</td>
<td>481 (10)</td>
</tr>
<tr>
<td>Urea</td>
<td>343 (7)</td>
<td>475 (11)</td>
<td>710 (8)</td>
</tr>
<tr>
<td>Guanidino propionic acid</td>
<td>272 (5)</td>
<td>467 (13)</td>
<td>878 (6)</td>
</tr>
<tr>
<td>Creatinine</td>
<td>283 (9)</td>
<td>558 (14)</td>
<td>950 (16)</td>
</tr>
<tr>
<td>Guanidine</td>
<td>314 (3)</td>
<td>529 (9)</td>
<td>641 (12)</td>
</tr>
<tr>
<td>Guanidino acetic acid</td>
<td>312 (7)</td>
<td>615 (11)</td>
<td>749 (12)</td>
</tr>
<tr>
<td>PTH</td>
<td>355 (6)</td>
<td>435 (8)</td>
<td>697 (11)</td>
</tr>
<tr>
<td>Putrescine</td>
<td>318 (5)</td>
<td>595 (9)</td>
<td>661 (10)</td>
</tr>
<tr>
<td>Methylguanidine</td>
<td>322 (4)</td>
<td>541 (10)</td>
<td>532 (8)</td>
</tr>
<tr>
<td>p-cresol</td>
<td>293 (6)</td>
<td>622 (7)</td>
<td>671 (11)</td>
</tr>
<tr>
<td>Creatine</td>
<td>314 (8)</td>
<td>621 (13)</td>
<td>727 (14)</td>
</tr>
<tr>
<td>Indoxylsulphate</td>
<td>346 (6)</td>
<td>595 (10)</td>
<td>661 (12)</td>
</tr>
<tr>
<td>Spermidine</td>
<td>328 (9)</td>
<td>432 (12)</td>
<td>537 (14)</td>
</tr>
<tr>
<td>Phenol</td>
<td>345 (8)</td>
<td>594 (8)</td>
<td>541 (13)</td>
</tr>
<tr>
<td>ADMA</td>
<td>319 (7)</td>
<td>489 (11)</td>
<td>744 (10)</td>
</tr>
</tbody>
</table>

a SD is shown in brackets. At time 0, the concentration of protein methyl esters was 256 (11). PTH, parathyroid hormone; ADMA, asymmetric dimethylarginine.

Albumin Incubated with Uremic Toxins for Various Time Periods, and Dose-Response Experiments
All uremic toxins used for the experiments with plasma were used in experiments with human albumin and incubated for 24, 72, and 168. Results are presented in Table 2. Guanidine and guanidinopropionic acid were the compounds that affected albumin the most. For this reason, a dose-response curve for guanidine and guanidinopropionic acid was carried out, selecting 168 h as the time after which the maximum response could be observed. Results can be observed in Figure 1, and a clear dose-response could be obtained for guanidine.

Protein Damage in Plasma of Hemodialysis Patients before and after a Dialysis Session
To verify whether dialysis membranes could per se damage plasma proteins, we studied a group of patients before and after a standard 4-h hemodialysis session. Selected membranes, polysulfone or PMMA, are highly biocompatible and specifically are not thought to activate the complement cascade. There was no significant difference between predialysis and postdialysis values (predialysis, 381.00 ± 23.3 pmol/mg protein; postdialysis, 360.57 ± 26.1 pmol/mg protein [n = 9]; NS).

Albumin Deamidation
To verify whether the in vitro deamidation protocol successfully induced albumin damage, we tested the four albumin preparations for their deamidated/isomerized Asx content. Results are depicted in Table 3 and represent the mean (SD) of four different sets of preparations. Preparation A, the control

<table>
<thead>
<tr>
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<th>24</th>
<th>72</th>
<th>168</th>
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<tr>
<td>Control</td>
<td>119 (12)</td>
<td>170 (11)</td>
<td>246 (13)</td>
</tr>
<tr>
<td>Urea</td>
<td>104 (6)</td>
<td>226 (6)</td>
<td>168 (7)</td>
</tr>
<tr>
<td>Guanidino propionic acid</td>
<td>169 (5)</td>
<td>220 (8)</td>
<td>533 (9)</td>
</tr>
<tr>
<td>Creatinine</td>
<td>129 (7)</td>
<td>176 (12)</td>
<td>308 (14)</td>
</tr>
<tr>
<td>Guanidine</td>
<td>270 (4)</td>
<td>266 (7)</td>
<td>593 (6)</td>
</tr>
<tr>
<td>Guanidino acetic acid</td>
<td>270 (11)</td>
<td>387 (9)</td>
<td>263 (13)</td>
</tr>
<tr>
<td>PTH</td>
<td>303 (10)</td>
<td>221 (9)</td>
<td>316 (11)</td>
</tr>
<tr>
<td>Putrescine</td>
<td>278 (10)</td>
<td>242 (13)</td>
<td>276 (12)</td>
</tr>
<tr>
<td>Methylguanidine</td>
<td>113 (11)</td>
<td>155 (7)</td>
<td>156 (14)</td>
</tr>
<tr>
<td>p-cresol</td>
<td>105 (10)</td>
<td>143 (12)</td>
<td>184 (9)</td>
</tr>
<tr>
<td>Creatine</td>
<td>178 (14)</td>
<td>184 (8)</td>
<td>142 (11)</td>
</tr>
<tr>
<td>Indoxylsulphate</td>
<td>110 (15)</td>
<td>148 (11)</td>
<td>166 (14)</td>
</tr>
<tr>
<td>Spermidine</td>
<td>155 (10)</td>
<td>165 (7)</td>
<td>140 (12)</td>
</tr>
<tr>
<td>Phenol</td>
<td>164 (9)</td>
<td>175 (11)</td>
<td>171 (13)</td>
</tr>
<tr>
<td>ADMA</td>
<td>165 (11)</td>
<td>151 (8)</td>
<td>155 (9)</td>
</tr>
</tbody>
</table>

a SD is shown in brackets. At time 0, the concentration of protein methyl esters was 110 (9).
sample that was not incubated and stored in a pH 7.3 solution, represents the basal deamidated/isomerized Asx content of albumin. The deamidated/isomerized Asx content gradually increases in preparations B and C, and the highest level is reached by preparation D. In this preparation, levels were significantly different from preparation A ($P < 0.02$).

**Table 3.** Protein aspartyl damage levels of albumin subject to different treatments

<table>
<thead>
<tr>
<th>Protein Methylesters (pmol/mg)</th>
<th>Preparation A</th>
<th>Preparation B</th>
<th>Preparation C</th>
<th>Preparation D</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>726.5 (74.0)</td>
<td>779.3 (61.7)</td>
<td>817.6 (55.0)</td>
<td>1226.0 (134.6)$^b$</td>
</tr>
</tbody>
</table>

$^a$ SD is shown in brackets.

$^b$ $P < 0.02$.

**Discussion**

In this article, we show that plasma protein damage, as represented by the deamidated/isomerized Asx residue content, can be elicited by uremic retention toxins, and in particular, the most effective compound in this respect is guanidine. The effect of the hemodialysis procedure itself is practically nil. In general, the binding capacity of *in vitro* deamidated albumin is intact, whereas its ability to carry homocysteine in blood is impaired.

In a previous paper, we showed that in uremic patients on hemodialysis, plasma protein damage is significantly increased, as measured by an assay using human recombinant PCMT (4). This damage involves all plasma proteins, but in particular, albumin is the most affected plasma protein. Whereas in erythrocytes, damage to membrane proteins is caused by the accumulation of homocysteine and of its precursor AdoHcy, this is only partially true in the plasma compartment. In fact, it has been shown that folate therapy with the attendant reduction in homocysteine levels can reduce plasma protein damage to a limited extent, and differences in results are not statistically significant. 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 (25,26). It therefore is possible that damage accumulation, in the form of isoaspartyl residues, is to be ascribed, in plasma proteins, to their increased structural instability in uremia rather than to the effects of hyperhomocysteinemia. A role for other uremic toxins therefore can be postulated, able to elicit this kind of protein damage.

Several uremic toxins were tested, ranging from small molecules such as urea to proteins such as parathyroid hormone. Uremic toxins were selected also considering the various classes of compounds implicated in uremic toxicity, such as guanidino compounds, polyamines, urea, middle molecules/peptides, p-cresol, indoles, etc (2). However, selection was by no means intended to be comprehensive or exhaustive. In particular, the effects of advanced glycosylation end products on protein deamidation were not tested. The same uremic toxins selected for the experiment with plasma were selected in an incubation experiment with human albumin as a model protein, which shows that guanidine and guanidinopropionic acid are the most toxic compounds. Dose-response experiments show that guanidine is able to elicit a dose-response curve.

Guanidino compounds are of major interest in uremia because of their ability to interact with various reactive species and act as metabolic toxins. Although they are removed during dialysis, levels remain high with respect to normal. A renewed interest in guanidino compounds has arisen recently; for example, neutrophil energy metabolism is affected by these compounds, accounting for the impaired defenses against bacterial...
infections in uremia (13). The present study is in line with these studies, demonstrating a toxic effect of guanidino compounds and, in particular guanidine, on protein damage, as expressed by their deamidated/isomerized Asx content.

The dialysis procedure itself could theoretically affect protein deamidated/isomerized Asx content in the 4 h during which blood comes in contact with the dialysis membranes, even if the membranes chosen are biocompatible, and therefore less likely to activate the complement system. Protein damage is elicited by stresses of various kinds, and, by all means, the dialysis procedure itself could induce protein damage, being in the nature of extracorporeal circulation systems to generate mechanical and oxidative stress. Results show that no evidence of such stress in terms of deamidated/isomerized Asx content is present in plasma proteins, at least during the 4 h of the dialysis session.

When the function of such altered proteins was examined, the ideal candidate was of course human albumin. This protein is abundant in plasma, and it is a carrier for numerous substances in circulation. In addition, it is known that in uremia, albumin binding capacity is reduced (27). The method used to study albumin function is based on the fact that binding of various ligands to albumin induces conformational changes with consequent quenching of tryptophan residue fluorescence, yielding different curves with the various concentration of the tested compound (23). Human albumin was treated in vitro to reach, in the aliquot that was incubated with the pH 9.1 solution, a deamidation level almost double that of the aliquot
that was not incubated and treated with the pH 7.3 solution. When warfarin, salicylic acid, and diazepam were used, no significant different was observed; in fact, the four albumin preparations were remarkably similar, if not identical, with respect to their binding capacity to these substances. When binding of homocysteine, which normally binds to the cysteine residue in position 34 of albumin, was considered, a different finding was detected. The albumin preparations show a reduced binding to homocysteine, with the lowest binding in the most deamidated albumin preparation.

Albumin contains 17 intrachain disulfide bonds and one additional cysteine residue at Cys, which is not involved in intrachain disulfide bonding. The disulfide bond of homocysteine to Cys of albumin forms itself through cysteine displacement and formation of an albumin thiolate anion, which then reacts with the homocysteine-cysteine mixed disulfide or free homocysteine in blood to form albumin-bound homocysteine. In addition, human albumin mediates the conversion of homocysteine to its low molecular weight disulfide forms by thiol/disulfide exchange reactions (28–32).

Deamidation probably modifies the quaternary arrangement of the protein and, therefore, its binding properties. The consequence of this alteration would be an increase in the percentage of plasma homocysteine, which is in the non–protein-bound form. If deamidated albumin increases in chronic renal failure and in uremia and it has been shown that this is the case, then an increased availability of non–protein-bound homocysteine is possible. In uremia, both the protein-bound and the non–protein-bound forms of homocysteine are increased (33). Because the deamidated form of albumin is increased as well, this could contribute in part to the elevated homocysteine levels in uremia, especially in its non–protein-bound form. Free homocysteine is in dynamic equilibrium with other forms, such as AdoHcy, and homocysteine thiolactone, not considering the capability of free homocysteine to elicit oxidative stress. We have previously shown that AdoHcy is able, through transmethylation inhibition, to induce erythrocyte membrane protein damage (5,6) and is at least partly involved in plasma protein damage (4) and in alterations of the allelic expression of imprinted genes in uremia (9). The formation of homocysteine thiolactone, a highly reactive homocysteine derivative, is another plausible mechanism of homocysteine toxicity, which depends on the cyclization of free homocysteine, as a result of methionyl tRNA synthetase proofreading activity, a process that is able to prevent homocysteine misincorporation into proteins (34). Oxidative stress production has also been envisioned as a mechanism of homocysteine toxicity (35). Recently, Antony et al. (36) showed that free homocysteine (or homocysteine thiolactone) plays a key role in the regulation of folate receptor synthesis, directly modulating RNA-protein binding to influence the translational efficiency of folate re-
in the sequence or in the tridimensional structure, from Cys34. Many different Asn residues, located at various distances, either and Cys34, the higher the probability that deamidation may involve the single Cys34, whereas deamidation may involve protein structure, to a point at which deamidation is accelerated. Nevertheless, even the most stable conditions cannot block deamidation from taking place. In this respect, we previously reported that even proteins that are kept at low temperature for various periods of time may undergo deamidation to a detectable extent, which is significantly modified when ligands are added (38). Therefore, the observation that in Table 3, even preparation A of albumin shows some degree of deamidation, can be due to small but significant conformational changes of macromolecules occurring during freezing and/or thawing. In addition, small alterations of local protein conformation at the solvent–protein interface may play a role over time, so the most correct way of interpreting this phenomenon is that proteins tend toward deamidation slowly but definitely even under the mildest conditions as a result of their intrinsic instability. When we modify the microenvironment by adding uremic toxins, which may critically alter protein conformation even locally (i.e., particularly where asparagine residues are exposed at the solvent–protein interface), this results in protein destabilization and increased susceptibility to deamidation to an extent that may vary significantly from toxin to toxin.

The increase in deamidation levels of albumin preparations and reduction in homocysteine binding seem to follow a different pattern, in response to the conditions described in Table 3 and Figure 3. In particular, the highest increase in methyl-accepting capability occurs when the proteins were treated under conditions that maximize Asn deamidation. Conversely, the effect on homocysteine binding seems to reflect an almost linear decrease pattern. In our opinion, this difference may be explained by the consideration that homocysteine binding involves the single Cys34, whereas deamidation may involve many different Asn residues, located at various distance, either in the sequence or in the tridimensional structure, from Cys34. Therefore, we may expect that the closer the two residues (Asn and Cys34), the higher the probability that deamidation may influence homocysteine binding through conformational alterations induced at the homocysteine binding site. On the contrary, when an asparagine residue is distant from homocysteine binding site, its deamidation would poorly affect homocysteine binding. However, susceptibility to deamidation is, in general, not the same for all Asn residues, but it depends on various structural determinants (e.g., local sequence, neighboring residues, conformational flexibility). Therefore, it is possible to explain the results presented in Table 3 and Figure 3 if we hypothesize that a small number of the most rapidly deamidated residues are also the few that are closer to the homocysteine binding site, whereas the remaining deamidation-susceptible Asn sites (the most abundant) are distant. The latter residues therefore are relatively more stable, and their deamidation poorly affects homocysteine binding.

In conclusion, damaged plasma proteins in uremia are increased as a consequence of the toxic action of uremic retention solutes, and guanidine seems to be the most toxic compound, because it is able to elicit a dose-response effect. The hemodialysis procedure does not affect plasma protein deamidated/isomerized Asx content. The binding properties of damaged albumin to warfarin, diazepam, and salicylic acid are not altered; however, homocysteine binding is significantly reduced.

Acknowledgments

This work was supported by M.U.R.S.T., Programs of Relevant National Interest 2001, 2002, 2003; and the Italian Space Agency (A.S.I.).


We gratefully acknowledge Dr. Steven Clarke (Professor of Biochemistry, Department of Chemistry and Biochemistry and the Molecular Biology Institute, University of California Los Angeles, Los Angeles, CA) for the generous gift of PCMT enzyme. We also acknowledge the dedicated work of Dr. Davide Stellato and Dr. Lucia Spitali, who were involved in patient care.

References