In Vitro and in Vivo Interactions of Homocysteine with Human Plasma Transthyretin*

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Hyperhomocysteinemia is an independent risk factor for cardiovascular disease and an emerging risk factor for cognitive dysfunction and Alzheimer’s disease. Greater than 70% of the homocysteine in plasma is disulfide-bonded to protein cysteine residues. The identity and functional consequences of protein homocysteinylation are just now emerging. The amyloidogenic protein transthyretin (prealbumin), as we now report, undergoes homocysteinylation at its single cysteine residue (Cys10) both in vitro and in vivo. Thus, when human plasma or highly purified transthyretin was incubated with [35S]-L-homocysteine followed by SDS-PAGE and PhosphorImaging, two bands corresponding to transthyretin dimer and tetramer were observed. Treatment of the labeled samples with β-mercaptoethanol prior to SDS-PAGE removed the disulfide-bound homocysteine. Transthyretin-Cys10-S-S-homocysteine was then identified in vitro in plasma from normal donors, patients with end-stage renal disease, and homocystinurics by immunoprecipitation and high performance liquid chromatography/electrospray mass spectrometry. The ratios of transthyretin-Cys10-S-S-homocysteine and transthyretin-Cys10-S-S-sulfonate to that of unmodified transthyretin increased with increasing homocysteine plasma concentrations, whereas the ratio of transthyretin-Cys10-S-S-cysteine to that of unmodified transthyretin decreased. The hyperhomocysteinemic burden is thus reflected in the plasma levels of transthyretin-Cys10-S-S-homocysteine, which in turn may contribute to the pathological consequences of amyloid disease.

Individuals with elevated plasma total homocysteine (tHcy)1 (hyperhomocysteinemia) are at greater risk for cardiovascular disease (1), and the prognosis for patients with cardiovascular disease and other diseases in combination with the highest levels of tHcy is poor (2–4). Recent studies (5–8) suggest that hyperhomocysteinemia is also a risk factor for Alzheimer’s disease and other disorders of cognitive dysfunction. Most of the homocysteine in circulation (>70%) of tHcy is disulfide-linked to albumin and other plasma proteins (9–11). The remaining free homocysteine is found as low molecular weight disulfide forms such as homocysteine and homocysteine-cysteine mixed disulfide (12). Less than 1% of tHcy is found as free, reduced (i.e. –SH) form (13). Protein-homocysteamide (homocysteine-N-protein), the reaction product formed between a protein lysine residue and homocysteine thiolactone, is also found in circulation (14).

The functional consequences of protein homocysteinylation are beginning to emerge. For example, in vitro studies have shown that homocysteinylation of the Cys9 residue of annexin II, the endothelial cell surface docking protein for tissue plasminogen activator, inhibits the binding (15, 16). Homocysteinylation of factor Va in vitro makes it resistant to inactivation by activated protein C (17). Homocysteinylation appears to activate latent elastolytic metalloproteinase pro-MMP-2 by disulfide bond formation with the “cysteine switch” on the propeptide (18). Recently, we reported that homocysteine binds to the fibrin-binding domain of plasma fibronectin in vitro and inhibits its ability to bind fibrin (19). Taken together, these in vitro studies suggest that post-translational modification of proteins by homocysteine may have important functional consequences.

Transthyretin (prealbumin) is a 13.8-kDa protein that is synthesized predominantly in the liver and secreted into plasma. As a homotetramer transthyretin binds and transports the hormone thyroxine and the retinol-binding protein-retinal complex (20). Transthyretin has been implicated in the formation of amyloid deposits in familial transthyretin amyloidosis and senile systemic amyloidosis (21, 22). Familial transthyretin amyloidosis is an autosomal dominant disorder involving the deposition of transthyretin as amyloid fibrils in tissues and

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1 The abbreviations used are: tHcy, plasma total homocysteine; ESI MS, electrospray ionization mass spectrometry; HFLC, high performance liquid chromatography; TES, 2-[(2-hydroxyethyl)methyl]amino]ethanesulfonic acid; TTR, transthyretin; TTR-Cys10-S-S-Cys, transthyretin-Cys10-S-S-cysteine; TTR-Cys10-S-S-CysGly, transthyretin-Cys10-S-S-cysteinylglycine; TTR-Cys10-S-S-SG, transthyretin-Cys10-S-S-glutathione; TTR-Cys10-S-S-H, transthyretin-Cys10-S-S-homocysteine; TTR-Cys10-S-S-CH3, transthyretin-Cys10-S-S-homocysteine.

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Monomeric transthyretin has a single cysteine residue at position 10. In the normally folded tetrameric protein, the Cys\(^{10}\) residues are in exposed sites at the start of the helical regions. Because the Cys\(^{10}\) residue of transthyretin can conjugate with cysteine and other sulfur-containing ligands (27, 28), we hypothesized that it could be homocysteinylated and, with increasing homocysteine burden, stable transthyretin–Cys\(^{10}\)–S–S–homocysteine might become the predominant form of circulating transthyretin. In vitro as well as in vivo evidence in support of this hypothesis is now reported.

\(\beta_2\)-Microglobulin is found in amnioid deposits of patients with end-stage renal disease who are on dialysis. It too is a protein that affects about 25% of individuals over 80 years old (26). Senile systemic amyloidosis is a nonhereditary disorder that affects mainly in the heart.

EXPERIMENTAL PROCEDURES

Materials—\(\text{\^{35}}\)S-l-Homocysteine thiolactone was synthesized from \(l\)-\(\text{\^{35}}\)S]-methionine by a slight modification of the method of Mudd et al. (32) and purified as described previously (11). \(\text{\^{35}}\)S-l-Homocysteine (500 \(\mu\)mol final concentration; specific activity 50 \(\mu\)Ci/\(\mu\)mol) was prepared from \(\text{\^{35}}\)S-l-Homocysteine thiolactone (33). The thiol content of \(l\)-homocysteine was determined using Ellman’s reagent (34). All experiments were conducted with fresh preparations of \(l\)-homocysteine and \(\text{\^{35}}\)S-l-homocysteine. Purified human transthyretin was obtained from Lee Scientific (St. Louis, MO), and purified \(\beta_2\)-microglobulin was obtained from Sigma. Human plasma was obtained from healthy donors, subjects with chronic renal failure, and subjects with homocystinuria using protocols approved by the Institutional Review Boards of the Cleveland Clinic Foundation. All other reagents and solvents of analytical grade or better were obtained from Sigma.

In Vitro Binding of \(\text{\^{35}}\)S-l-Homocysteine to Human Plasma Proteins, Purified Transthyretin, and Purified \(\beta_2\)-Microglobulin—\(\text{\^{35}}\)S-l-Homocysteine (500 \(\mu\)mol final concentration) was incubated with 50% human plasma in 0.050 \(M\) TES buffer at pH 7.4, or with purified human transthyretin (1 mg/ml in 0.050 \(M\) TES buffer, pH 7.4), or with purified \(\beta_2\)-microglobulin (1 mg/ml in 0.050 \(M\) TES buffer, pH 7.4) for 5 h at 37 °C. Plasma proteins, transthyretin, and \(\beta_2\)-microglobulin were then precipitated with 1.5 \(M\) perchloric acid, washed three times with 1.5 \(M\) perchloric acid, and dissolved in nonreducing SDS-PAGE sample buffer (0.062 \(M\) Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.2% bromphenol blue). To one-half of each sample, 3 \(\mu\)l of \(\beta_2\)-mercaptoethanol was added, and the sample was heated at 100 °C for 5 min to reduce disulfide bonds. Aliquots of the \(\beta_2\)-mercaptoethanol-treated and untreated samples were applied to a 10% SDS-polyacrylamide gel, and the electrophoresis was carried out according to standard protocol (35). The gels were dried and analyzed using a PhosphorImager to identify \(\text{\^{35}}\)S-labeled proteins.

Binding of \(l\)-Homocysteine to Transthyretin—\(l\)-Homocysteine (500 \(\mu\)mol final concentration) was added to purified transthyretin (180 \(\mu\)mol final concentration) in 0.050 \(M\) TES buffer, pH 7.4, and the reaction mixture was incubated at 37 °C for 2 h in a shaking water bath. Aliquots were withdrawn after 30 min and added directly to tubes containing 0.1 ml of 1.5 \(M\) perchloric acid to precipitate the transthyretin. The tubes were vortexed, incubated on ice for 10 min, and centrifuged at 12,000 rpm for 10 min. The protein pellet was washed 3 times with perchloric acid and was solubilized in Tris-HCl (0.50 \(M\), pH 8.5). The concentrations of S-cysteinylated and S-homocysteinylated transthyretin were determined as described previously (11).

RESULTS

The objectives of this study were to determine whether homocysteine forms disulfide conjugates with the amyloid proteins transthyretin and \(\beta_2\)-microglobulin under in vitro conditions and, more importantly, in vivo. Human plasma from healthy donors and purified transthyretin were incubated with \(\text{\^{35}}\)S-l-homocysteine for 5 h at 37 °C. The samples, before and after
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Fig. 2. Binding of L-homocysteine to transthyretin (in vitro). Human plasma from a healthy donor was treated with L-homocysteine (0–500 μM) for 5 h at 37 °C. The transthyretin was then immunoprecipitated, purified by reversed-phase HPLC, and mass analyzed by ESI MS. This figure depicts the transthyretin molecular mass region in the deconvoluted ESI mass spectra of transthyretin from the plasma of a healthy individual without (A) and with (B) the addition of 500 μM homocysteine. For assignments of peaks, please see text.

treatment with β-mercaptoethanol, were subjected to SDS-PAGE and analyzed by PhosphoImaging. As shown in Fig. 1, lane 1, incubation of normal human plasma with 35S-L-homocysteine produced faint bands corresponding to the transthyretin dimer (27.5 kDa) and tetramer (55 kDa). The heavily labeled band in lane 1 is albumin, which forms a disulfide bond with homocysteine (11). When purified human transthyretin was incubated with 35S-L-homocysteine (Fig. 1, lane 2), labeling was found primarily on the dimeric form of transthyretin. Treatment of the plasma or purified transthyretin sample with β-mercaptoethanol prior to SDS-PAGE analysis resulted in the removal of homocysteine from transthyretin (Fig. 1, lanes 3 and 4), suggesting that the homocysteine was indeed disulfide-linked to the protein. These in vitro experiments provide strong evidence that L-homocysteine readily reacts with Cys10, the only cysteine residue found in transthyretin. In contrast, when human plasma from healthy donors and purified β2-microglobulin were incubated with 35S-L-homocysteine for 5 h at 37 °C, subjected to SDS-PAGE, and then analyzed by PhosphoImaging, no bands corresponding to homocysteinylated β2-microglobulin were visualized, suggesting that homocysteine does not react with the single disulfide bond of the molecule (data not shown). Our attention was then focused on transthyretin alone.

Human plasma from a healthy donor was treated with increasing concentrations of L-homocysteine (0–500 μM) for 5 h at 37 °C. Plasma transthyretin was then immunoprecipitated and purified by reversed-phase HPLC. The masses of the transthyretin-related components were determined using ESI MS. The deconvoluted ESI mass spectra of immunoprecipitated transthyretin and purified by HPLC from the plasma of a healthy individual without and with the addition of 500 μM homocysteine is shown in Fig. 2, A and B, respectively. The ESI mass spectrum of the transthyretin sample in the absence of homocysteine (Fig. 2A) showed peaks corresponding to the unmodified transthyretin molecule (mass = 13,761 Da) and the Cys10 homocysteine adducts for S-sulfonate (TTR-Cys10-S-SO3H, mass = 13,841 Da), S-cysteine (TTR-Cys10-S-S-Cys, mass = 13,880 Da) S-cysteinylglycine (TTR-Cys10-S-S-CysGly, mass = 13,937 Da), and S-glutathione (TTR-Cys10-S-SG, mass = 14,067 Da). S-Homocysteine of transthyretin (TTR-Cys10-S-S-Hcy, mass = 13,894) was detected as a minor component. In contrast, the ESI mass spectrum of the transthyretin sample in the presence of 500 μM homocysteine (Fig. 2B) displayed a major peak corresponding to TTR-Cys10-S-S-Hcy, in addition to peaks corresponding to the transthyretin-related components seen in Fig. 2A.

These ESI mass spectra were analyzed to determine the relative abundance of the TTR-Cys10-S-S-Cys and TTR-Cys10-S-S-Hcy adducts to that of the unmodified transthyretin molecule. These ratios were plotted as a function of L-homocysteine concentration in the in vitro dose-response study (Fig. 3). In Fig. 3A, the ratio of the relative abundance of TTR-Cys10-S-S-Cys to that of unmodified transthyretin decreased from 1.71 in normal plasma (0 μM exogenous homocysteine) to 1.09 in the presence of 50 μM exogenous homocysteine. The ratio then increased up to 1.21 when 250 μM homocysteine was added. The ratio then substantially increased when the transthyretin was incubated with 500 μM homocysteine (Fig. 3A). This substantial increase is attributed to the increase in the concentration of free cysteine in plasma due to the interaction of homocysteine with albumin-Cys34-S-S-Cys (S-cys-
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Fig. 3. Relative abundance of the S-cysteinylated and S-homocysteinylated adducts to that of the unmodified transthyretin molecule as a function of L-homocysteine concentration. The ratios of the relative abundance of the S-cysteine transthyretin (A) and S-homocysteine TTR (B) to that of the unmodified transthyretin were plotted as a function of homocysteine concentration.

Fig. 4. Reaction of transthyretin with L-homocysteine. L-Homocysteine (500 μM) was incubated with 180 μM purified transthyretin. After 30 min, aliquots were withdrawn; the transthyretin was precipitated using perchloric acid, and the concentrations of TTR-Cys10-S-S-Cys and TTR-Cys10-S-S-Hcy were determined using HPLC with fluorescence detection as described previously (38).

teine albumin), wherein albumin-Cys34-S-S-Hcy is formed releasing free cysteine in a two-step process (11). In contrast, the ratio of TTR-Cys10-S-S-Hcy to that of the unmodified transthyretin remained essentially unaltered up to 50 μM added homocysteine (Fig. 3B). The ratio then increased, albeit not substantially, up to 250 μM homocysteine. Like TTR-Cys10-S-S-Cys (Fig. 3A), the ratio of the TTR-Cys10-S-S-Hcy to that of the unmodified transthyretin substantially increased at 500 μM homocysteine (Fig. 3B). However, the ratios of the TTR-Cys10-S-SO2H, TTR-Cys10-S-S-CysGly, and TTR-Cys10-S-S-G to that of the unmodified TTR remained relatively constant over the entire concentration range of L-homocysteine (data not shown).

We had reported earlier (11) that during the reaction of homocysteine with human serum albumin, homocysteine strips the cysteine attached to Cys34 of albumin (albumin-Cys34-S-S-Cys), resulting in the formation of albumin thiolate anion and homocysteine-cysteine mixed disulfide in the first step of the reaction. To determine whether a similar reaction is involved here, we incubated 500 μM homocysteine with 180 μM purified transthyretin and found that after 30 min, homocysteine had stripped almost all of the cysteine from TTR-Cys10-S-S-Cys (Fig. 4). During the same time period, only 10 μM of TTR-Cys10-S-S-Hcy was formed, indicating that homocysteine attacks the S-cysteine sulfur on TTR-Cys10-S-Cys, resulting in the formation of TTR-Cys10-S-thiolate anion and homocysteine-cysteine mixed disulfide. These observations are similar to those reported by us previously (11) for the reaction of homocysteine with albumin.

All the results clearly suggest that homocysteine readily forms a disulfide conjugate with transthyretin in vitro. Next, we wanted to determine whether transthyretin is a carrier of homocysteine in vivo. Transthyretin from plasma of healthy individuals and patients with hyperhomocysteinemia due to chronic renal failure or homocystinuria was immunoprecipitated, HPLC-purified, and subjected to the same analysis as those used in the in vitro studies. Representative ESI deconvoluted mass spectra of the transthyretin isolated from the plasma of a patient with end-stage renal disease (tHcy = 20.7 μM) and a homocystinuric patient (tHcy = 434 μM) are shown in Fig. 5, A and B, respectively. In the plasma from the patient with end-stage renal disease, the transthyretin existed predominantly as in S-cysteine form (Fig. 5A). Only a small amount of the S-homocysteine transthyretin was detected (Fig. 5A). In contrast, the majority of the transthyretin isolated from the plasma of the homocystinuric patient was S-sulfonate (Fig. 5B). Additionally, the S-homocysteine transthyretin was now a major adduct, whereas the amount of the S-cysteine transthyretin was greatly reduced (Fig. 5B).

Like the results obtained for the in vitro studies, the ratios of the relative abundance of the S-sulfonate, S-cysteine, and S-homocysteine adducts of the transthyretin to that of the unmodified transthyretin molecule were plotted as function of L-homocysteine concentration (Fig. 6). The ratio of the relative abundance of the S-sulfonate transthyretin to that of the unmodified transthyretin increased with increasing concentrations of plasma homocysteine and plateaued at about 200 μM homocysteine (Fig. 6A). In contrast, the ratio of the S-cysteine transthyretin to that of the unmodified transthyretin from the hyperhomocysteinemic patients decreased up to ~200 μM plasma homocysteine and then increased (Fig. 6B). However, the ratio of the S-homocysteine transthyretin to that of the unmod-
ified transthyretin increased initially to a small extent and then there was a substantial increase after the 200 μM plasma homocysteine concentration (Fig. 6C). Overall, the ratio of Cys10-conjugated transthyretin to Cys10-free transthyretin increased linearly as the concentration of tHcy increased (Fig. 6D).

**DISCUSSION**

These studies show that L-homocysteine reacts with transthyretin in human plasma to form a stable covalent adduct both in vitro and in vivo. Transthyretin is the third plasma protein, after albumin (9–11) and fibronectin (19), to be identified as a carrier of homocysteine in vivo. Because transthyretin contains only one cysteine residue (Cys10), the homocysteine adduct must be TTR-Cys10-S-S-Hcy. Earlier reports (27, 28, 36, 39) have identified other transthyretin-Cys10 adducts as S-sulfonate, S-cysteine, S-cysteinylglycine, and S-glutathione. However, to our knowledge, this is the first report demonstrating the presence of S-homocysteine transthyretin in normal human serum. The relatively low abundance of S-homocysteine transthyretin in normal human serum probably explains why it was not detected in earlier studies. It should be noted that while this work was under review, Sass et al. (40) also identified TTR-Cys10-S-S-Hcy in the plasma and serum from hyperhomocysteinemic individuals.

Our in vitro studies show that homocysteine displaces cysteine from TTR-Cys10-S-S-cysteine (Fig. 4) to form homocysteine-cysteine mixed disulfide (Hcy–S–S–Cys) and transthyretin thiolate anion (TTR-Cys10-S−) (Reaction 1),

\[
\text{Hcy}^- + \text{TTR-Cys}^{10-}-\text{S-S-Cys} \rightarrow \text{TTR-Cys}^{10}-\text{S}^- + \text{Hcy-S-S-Cys}
\]

which is consistent with our studies on the interaction of homocysteine with albumin-Cys34-S-S-cysteine (11, 41). (About one-third of the albumin molecules in normal plasma are cysteinylated at Cys34 (42).)

When normal human plasma was treated with increasing concentrations of L-homocysteine, the ratio of the relative abundance of S-homocysteine transthyretin to that of the unmodified transthyretin remained relatively constant up to 50 μM added L-homocysteine. This was followed by a small increase in the ratio up to about 200 μM added L-homocysteine. However, at higher concentrations of added homocysteine (>250 μM), the formation of S-homocysteine transthyretin increased substantially (Fig. 3B).

This phenomenon can be explained if we consider that, in plasma, albumin is the most abundant protein, accounting for ~50–60% of total plasma proteins. We propose that transthyretin thiolate anion and the albumin thiolate anion competitively attack the low molecular weight disulfides homocysteine-cysteine or homocysteine. Because the concentration of albumin is about 100 times greater than that of transthyretin in plasma, homocysteinylation of albumin would be the predominant reaction. However, the in vitro binding capacity of plasma albumin for homocysteine is ~150–200 μM (43, 44). We determined that the in vitro binding capacity of plasma albumin for homocysteine was similar (10, 11). Therefore, when the binding capacity of albumin for homocysteine is reached, homocysteine will then react with transthyretin. This explains the substantial increase in the formation of S-homocysteine transthyretin when exogenously added homocysteine exceeds 200 μM.

The in vivo study results are similar. The in vivo ratio of the
relative abundance of S-homocysteine transthyretin to that of unmodified transthyretin increased to a small extent with increasing concentrations of \( \text{Hcy} \) (Fig. 6C). However, at homocysteine concentrations \( >200 \mu\text{M} \), the ratio increased dramatically. In contrast, the ratio of the relative abundance of the S-cysteine transthyretin to that of the unmodified transthyretin decreased initially and then increased at higher homocysteine concentrations (Fig. 6B). Based on these results, we propose that homocysteine, upon entering circulation, preferentially strips cysteine from both albumin-Cys\(^{34} – \text{S–S–Cys} \) and transthyretin-Cys\(^{10} – \text{S–S–Cys} \) forming the respective protein thiolate anions and homocysteine-cysteine mixed disulfide. The protein thiolate anions then react with the mixed disulfide to form the respective S-homocysteine protein adducts and cysteine thiolate anion (Reaction 2).

**REACTION 2**

Because the \( pK_a \) of cysteine (\( \sim 8.3 \)) is at least an order of magnitude lower than the \( pK_a \) of \( \text{Hcy} \) (\( \sim 9.5 \)) (45), the cysteine thiolate anion would be much more stable and the preferred leaving group at neutral pH as we found for the albumin reaction (11).

The \( \textit{in vivo} \) ratio of the relative abundance of the S-sulfonate transthyretin (TTR-Cys\(^{10} – \text{S–SO}_3\text{H} \)) to that of the unmodified transthyretin also increased with increasing homocysteine concentrations until it plateaued at about 200 \( \mu\text{M} \) added homocysteine (Fig. 6A). The \( \textit{in vitro} \) ratio of the relative abundance of the S-sulfonate transthyretin to that of the unmodified transthyretin remained essentially unchanged on addition of exogenous homocysteine (data not shown). Why the concentration of the S-sulfonate transthyretin increases in hyperhomocysteinemic patients is unknown. It is possible that the flux of cysteine through the catabolic pathway leading to cysteine sulfinic acid and then sulfite may increase in homocystinurics. Enhanced cysteine flux may be due to the reaction between homocysteine and S-cysteine albumin followed by the reaction of albumin thiolate anion with homocysteine-cysteine mixed disulfide (Reaction 2). The sulfite formed as a result of enhanced cysteine catabolism in homocystinurics could then react with transthyretin and/or its S-conjugated forms to produce S-sulfonate transthyretin. The formation of S-sulfonate albumin and S-sulfonate fibronectin has been reported previously (46) in...
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rabbit plasma. Irrespective of the underlying mechanism, the formation of S-sulfonate transthyretin may have pathophysiological consequences.

Transthyretin forms amyloid fibrils under weakly acidic conditions (pH 4.0 to 4.5) (23). Kishikawa et al. (39) reported that S-sulfonation enhanced the amyloidogenicity of transthyretin. The formation of transthyretin fibrils was studied with three different preparations: unmodified transthyretin (with thiol compounds attached to Cys10 and containing ~20% S-sulfonated transthyretin), dithiobisulfite-treated transthyretin (with a free sulphydryl group at Cys10), and transthyretin conjugated with sulfite (S-sulfonate transthyretin). At pH 4.0 there was a 3-fold enhancement of fibril formation with S-sulfonate transthyretin compared with unmodified transthyretin, whereas reduction of transthyretin had very low capacity to form fibrils. These results show that sulfonation of Cys10 of transthyretin might increase the fibril forming capacity of transthyretin, which could lead to a more rapid progression of familial transthyretin amyloidosis or senile systemic amyloidosis. Interestingly, a higher percentage of S-conjugated transthyretin to the unmodified transthyretin has been reported in patients with symptomatic amyloid disease (47).

Our studies show that the amyloid protein transthyretin can undergo homocysteinylation in human plasma. In contrast, the amyloid protein β-2-microglobulin, although it has a single disulfide bond that could be targeted by homocysteine thiolate anion, is not homocysteinylated under the same in vitro reaction conditions used to homocysteinylate transthyretin. The reason that β-2-microglobulin is resistant to homocysteinylatation is probably due to the buried nature of the disulfide bond in the native protein (48). The ratio of the relative abundance of the S-homocysteinyl transthyretin to that of the unmodified transthyretin increases with increasing tHcy concentrations. Thus, homocysteinylated transthyretin is a novel indicator of plasma homocysteine burden in hyperhomocysteinemia and homocystinuria. It remains to be determined whether post-translational modification of transthyretin by homocysteine plays a role in its pathogenicity in amyloidosis and other diseases.

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