

Modification of Fibrinogen by Homocysteine Thiolactone Increases Resistance to Fibrinolysis: A Potential Mechanism of the Thrombotic Tendency in Hyperhomocysteinemia[†]

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ABSTRACT: We have previously shown functional differences in fibrinogen from hyperhomocysteinemic rabbits compared to that in control rabbits. This acquired dysfibrinogenemia is characterized by fibrin clots that are composed of abnormally thin, tightly packed fibers with increased resistance to fibrinolysis. Homocysteine thiolactone is a metabolite of homocysteine (Hcys) that can react with primary amines. Recent evidence suggests that Hcys thiolactone-lysine adducts form in vivo. We now demonstrate that the reaction of Hcys thiolactone with purified fibrinogen in vitro produces fibrinogen (Hcys fibrinogen) with functional properties that are strikingly similar to those we have observed in homocysteinemic rabbits. Fibrinogen purified from homocysteinemic rabbits and Hcys fibrinogen are similar in that (1) they both form clots composed of thinner, more tightly packed fibers than their respective control rabbit and human fibrinogens; (2) the clot structure could be made to be more like the control fibrinogens by increased calcium; and (3) they both form clots that are more resistant to fibrinolysis than those formed by the control fibrinogens. Further characterization of human fibrinogens showed that Hcys fibrin had similar plasminogen binding to that of the control and an increased capacity for binding tPA. However, tPA activation of plasminogen on Hcys fibrin was slower than that of the control. Mass spectrometric analysis of Hcys fibrinogen revealed twelve lysines that were homocysteinylated. Several of these are close to tPA and plasminogen binding sites. Lysines are major binding sites for fibrinolytic enzymes and are also sites of plasmin cleavage. Thus, modification of lysines in fibrinogen could plausibly lead to impaired fibrinolysis. We hypothesize that the modification of lysine by Hcys thiolactone might occur in vivo, lead to abnormal resistance of clots to lysis, and thereby contribute to the prothrombotic state associated with homocysteinemia.

Homocysteine (Hcys) is a sulfur-containing amino acid that is produced metabolically from methionine and is a critical intermediate in several metabolic cycles. There has been a recent explosion of interest in Hcys as a risk factor for the development of cardiovascular disease. An association between premature atherosclerosis and a rare genetic disorder leading to a massive elevation of Hcys was first reported by McCully in 1969 (1). It is now recognized that even a mild elevation of plasma Hcys, present in 5–7% of the general population (2), is an independent risk factor for cardiovascular disease, including atherosclerosis, myocardial infarction, stroke, and venous thrombosis (3, 4). Although the morbidity and mortality of vascular diseases ultimately result from inappropriate blood clotting, most studies have focused

on the effects of homocysteine on vascular cells, and few have examined the effects of Hcys on proteins involved in blood clot formation and lysis.

Fibrinogen is a key protein in blood coagulation because it polymerizes to form the structure of the fibrin clot. It is a 340 kDa glycoprotein composed of two symmetrical halves. Each half molecule contains three nonidentical peptide chains: $\alpha\alpha$, $B\beta$, and γ . The fibrinogen molecule has a generally rigid trinodular structure, stabilized by 29 disulfide bonds (5). A central E domain is separated from two distal D domains by collagenlike coiled–coil regions. The amino termini of all six constituent polypeptide chains are linked by disulfide bonds in the E domain. The $B\beta$ and γ chains have their carboxy termini in the D domains, whereas the longer $\alpha\alpha$ chain terminates in a flexible αC domain that is noncovalently associated with the E domain (6). Thrombin cleaves fibrinopeptides A and B from the amino termini of the $\alpha\alpha$ and $B\beta$ chains to initiate fibrin polymerization. Fibrin monomers associate in a half-staggered, double-stranded array. Lateral association thickens the fibers and leads to branching and the formation of a complex fibrin network (7).

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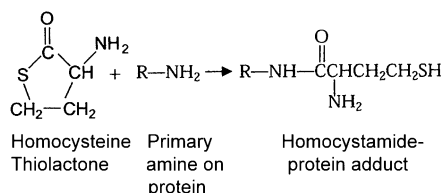


FIGURE 1: Reaction of homocysteine thiolactone with a primary amine. The most favored of such reactions under physiologic conditions is with the ϵ -amino group of lysine. This reaction introduces a new free sulfhydryl group into the protein structure.

Fibrinolysis is the process by which a fibrin clot is dissolved. It is initiated when tissue plasminogen activator (tPA) and plasminogen bind to the fibrin clot, where tPA cleaves plasminogen to form plasmin. Plasmin cleaves all three chains at multiple sites in the coiled-coil regions between the E and D domains as well as in the α -chain at the amino terminal boundary of the α C domain. Lysine residues are sites where fibrinolytic enzymes bind to fibrin and where plasmin cleavage occurs during fibrinolysis. Binding sites for tPA and plasminogen have been localized to A α 392–610 in the α C domain (8) and γ 312–324 and A α 148–160 in the D domain (9).

We have recently shown that rabbits that are made hyperhomocysteinemic by chronic injections of homocysteine or by dietary manipulation develop an acquired dysfibrinogenemia (10, 11). Clots formed from the fibrinogen or the plasma from homocysteinemic rabbits are composed of thinner, more tightly packed fibers than clots from the control fibrinogen or plasma. The most striking manifestation of altered fibrinogen function in hyperhomocysteinemic rabbits is that whole blood, plasma, or purified fibrinogen forms clots that are abnormally resistant to fibrinolysis (10, 11). Thus, some modification of the fibrinogen protein occurs in vivo during hyperhomocysteinemia that produces a defect in fibrin structure and fibrinolysis. Fibrinolysis is essential for the removal of the hemostatic clot during wound healing as well as for removing intravascular clots that might otherwise be manifest as thrombosis. Intravascular deposition of fibrin is associated with the development of atherosclerosis. Therefore, an effective fibrinolytic system protects against the chronic process of atherosclerotic vascular disease as well as the acute process of thrombosis. Because defects in fibrinolysis have been associated with atherosclerosis and thrombosis (12, 13), we hypothesized that the alteration of fibrinogen function observed in homocysteinemic rabbits could contribute to the vascular pathology associated with homocysteine.

We hypothesized that Hcys or a metabolite reacts with fibrinogen in vivo to modify its function. There are several mechanisms by which Hcys (which has a free sulfhydryl group) or its metabolites modify plasma proteins, including (1) the formation of mixed disulfides with cysteine residues in the protein (14); (2) the promotion of the oxidation of proteins, potentially leading to fragmentation and rearrangement of amino acid side chains (15); and (3) the metabolism to its cyclic thioester, Hcys thiolactone (16), which can react with primary amines, such as those in lysine, asparagine, arginine, and glutamine (17). The latter reaction is shown in Figure 1. Although homocysteine thiolactone can react with several of the amino acids, the amino groups in asparagine, arginine, and glutamine are not as reactive as

the primary amine in lysine because their bonding electrons are delocalized across adjacent carbon–oxygen double bonds (asparagine and glutamine) or carbon–nitrogen double bonds (arginine). Thus, under physiologic conditions the reaction with the ϵ -amino group of lysine predominates (18).

Many plasma proteins, including fibrinogen, can be covalently modified by Hcys thiolactone in vitro (19). Thus, lysine homocysteinylolation is a plausible mechanism for protein modification in vivo. Note that homocysteinylolation introduces a new free sulfhydryl group into the protein and alters the size of the modified amino acid. Although the production of Hcys thiolactone in vivo has been a matter of controversy, Hcys thiolactone has recently been detected in human plasma by gas chromatography/mass spectrometry (20). Another recent publication provided evidence that Hcys thiolactone-lysine adducts are formed in human subjects and that an immune response to them is associated with the risk of stroke (21). Thus, protein homocysteinylolation is likely an important pathogenic mechanism. These reports along with the knowledge that lysines in fibrinogen are critical to the binding and activity of fibrinolytic enzymes prompted us to specifically examine the effect of Hcys thiolactone on fibrinogen and fibrinolysis.

We hypothesized that modification of lysine residues in fibrinogen by Hcys thiolactone alters the structure of the resulting clots and impairs the ability of fibrinolytic enzymes to bind to and cleave the modified fibrin. To test this hypothesis we examined the ability of Hcys thiolactone to react with fibrinogen in vitro and modify its clotting function and resistance to fibrinolysis.

MATERIALS AND METHODS

Modification of Human Fibrinogen (Homocysteinylolation). Purified plasminogen-free human fibrinogen (CalBiochem, 4.12 mg/mL) was incubated with 0 or 300 μ M or 30 mM L-Hcys thiolactone (Sigma-Aldrich Co., St. Louis, MO) at 37 °C for 18 h. The control and Hcys thiolactone-treated fibrinogen samples were gel filtered on a Sephadex G-25 column to remove unreacted Hcys thiolactone. The fibrinogen concentrations were adjusted to 1 mg/mL and assayed for fibrin clot structure and lysis time as described below. The incorporation of Hcys thiolactone into the fibrinogen protein was assessed by assaying for free sulfhydryls using Ellman's reagent (Sigma-Aldrich) (22). Incubation with 300 μ M Hcys thiolactone did not impair fibrin clot formation, whereas 30 mM Hcys thiolactone reduced the clottability of the fibrinogen. Thus, incubation with 300 μ M Hcys thiolactone was used for further functional studies, whereas 30 mM was used to produce more extensive modification of fibrinogen for MS analysis.

Measurement of Fibrin Fiber Structure (Mass/Length Ratio (μ)). The relative μ of fibers in fibrin clots was determined using a microplate-reading spectrophotometer as previously described (23). Samples of the control and Hcys fibrinogen were clotted with 2.5 nM thrombin in the presence of CaCl₂ to final concentrations of 0, 2.5, 5.0, and 7.5 mM. The absorbance was monitored for 1 h in a microplate spectrophotometer to verify that clotting was complete as evidenced by the stabilization of the clot turbidity. The optical density was then scanned from 400 to 800 nm. This data was used to calculate the relative μ values.

Fibrinolysis Assay. Lysis of fibrin clots was measured with a Hemodyne Hemostasis Analysis System (Richmond, VA). This instrument is an elastometer that has been adapted for semi-automated clinical use and continuously measures the elastic modulus (rigidity) of the clot. Aliquots of citrated fibrinogen-deficient plasma (HRF Inc., Raleigh, NC), to which either the control or Hcys fibrinogen had been added to a final concentration of 1 mg/mL, were recalcified with 10 mM CaCl_2 and clotted with 10 nM thrombin in the presence of 0.45 $\mu\text{g/mL}$ of tissue plasminogen activator (tPA, American Diagnostica, Stamford, CT). During these assays, the fibrin clot forms and then is lysed as plasminogen from the plasma is activated by tPA on the fibrin template. The half-lysis time is defined as the time for the elastic modulus to decline to 50% of its peak value.

Mass Spectrometric Analysis. Purified samples of the control and Hcys fibrinogen were reduced in 10 mM dithiothreitol (DTT), with 8 M urea in 0.1 M Tris, pH 7.4 overnight at 37 °C. Sulfhydryl groups were acetylated by incubation with 10 mM iodoacetamide for 30 min before overnight incubation with sequencing-grade modified trypsin (Promega) at an enzyme–substrate ratio of 1:100 (wt/wt). The resulting tryptic fragments of the control and Hcys fibrinogen were analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). Briefly, an aliquot of each sample was mixed with an equal volume of MALDI-matrix (saturated α -cyano-4-hydroxycinnamic acid, 50% MeCN, 0.1% formic acid) and then analyzed by a MALDI-TOF-TOF instrument (Applied Biosystems, Framingham, MA) operating in the positive-ion reflectron mode. The detected peptide masses were queried by MASCOT against the NCBI protein database for protein identification and potential Hcys modification of lysine, which is evident as an increase in peptide mass due to the modification. Potentially modified peptides were subjected to sequence analysis by MS/MS to confirm the amino acid sequence and modification status.

Tryptic digests of Hcys fibrinogen were further analyzed by LC/MS/MS on a Q-TOF API US mass spectrometer and capLC system from Waters/Micromass (Millford, MA). Ten microliters of digest was injected into a Dionex trapping column and then separated on a 75 μm Dionex Pep-map analytical column connected online to the electrospray source of the mass spectrometer. The data was acquired as a continuous cycle of MS and MS/MS acquisitions of the five most intense peaks. As with the MALDI experiment, potential Hcys modifications of lysine were found by submitting the LC-MS/MS data via MASCOT to the NCBI database.

The MASCOT search engine used for these analyses is an in-house version at the UNC Proteomics Core Facility and can accommodate custom modifications not offered on the publicly available worldwide web version. Prior to database searching, the masses of homocysteine-modified lysine (245.1190 Da monoisotopic mass) and carbamidomethylated homocysteine-modified lysine (302.1413 Da monoisotopic mass) were added to a list of potential modifications.

tPA Binding Assay. We measured the solid-phase binding of tPA to the control and Hcys fibrinogen and fibrin as previously described (24). The control and Hcys fibrinogens (0.1 mg/mL) in the presence of 1 mM CaCl_2 were added to the wells of a microtiter plate. To initiate fibrin formation,

we added thrombin (10 nM final concentration) to some of the wells. In other wells, the fibrinogen was immobilized directly to the plastic in the absence of thrombin. One row of wells contained only a buffer as a control for nonspecific binding. The plate was incubated for 4 h at 37 °C and then placed in the refrigerator overnight. The wells were washed, blocked with 1% BSA for 2 h at 37 °C, and then washed again. A series of 1:2 dilutions of tPA, starting at 2000 nM, was prepared. Each dilution of tPA was added to a fibrin well, a fibrinogen well, and a control well and allowed to bind for 2 h at 37 °C. At the end of the binding period the wells were washed, and 100 μL of 0.5 mM Pefachrome tPA (Pentapharm, Switzerland; diluted in Tris-imidazole buffer, pH 8.3) was added to each well. The reaction was monitored for 1 h at 405 nm on a ThermoMax plate reader. The amount of tPA activity bound to the fibrinogen, fibrin, and the background was determined by comparison to a standard curve.

The V_{max} values (mOD/minute) were recorded for each absorbance curve, and the background V_{max} values (due to nonspecific binding) were subtracted. The data were analyzed by nonlinear regression analysis using Prism (GraphPad software, Inc.) using the equation $y = (B_{\text{max}} * x) / (K_{\text{app}} + x)$, where x is the concentration of total tPA (nM), y is the concentration of bound tPA, B_{max} is the amount of tPA bound at saturation, and K_{app} is the apparent dissociation constant.

Plasminogen Binding Assay. Solid-phase binding of plasminogen to the control and Hcys fibrinogen and fibrin was assayed in a manner similar to that described for tPA binding (24). The control or Hcys fibrinogens at a final concentration of 0.1 mg/mL in the presence of 1 mM CaCl_2 were added to the microtiter wells. The fibrinogens were either clotted with 10 nM thrombin or immobilized directly to the microtiter plate in the absence of thrombin as described above. One row of wells contained only buffer as a control for nonspecific binding. After incubation, the wells were washed, blocked with 1% BSA, and then washed again. A series of 1:2 dilutions of human glu-plasminogen (Haematologic Technologies, Essex Junction, VT) was prepared and the different concentrations of plasminogen added to fibrinogen, fibrin, and background control wells. After a 2 h binding period at 37 °C, the wells were washed thoroughly, then incubated for 1 h at room temperature with a 1:10 000 dilution of antihuman plasminogen peroxidase conjugated antibody (Enzyme Research Laboratories, South Bend, IN). The wells were washed, and 100 μL of peroxidase substrate was added (SureBlue TMB Microwell Peroxidase, Kirkegaard & Perry Labs, Gaithersburg, MD). To stop the reaction, 100 μL of 1 M phosphoric acid was added, and the absorbance values were read at 450 nm on a ThermoMax plate reader. Plasminogen binding data were analyzed as for tPA binding data.

Plasminogen Activation Assay. We assayed the activation of plasminogen by tPA on the immobilized control and Hcys fibrinogen and fibrin as described (24). The control and Hcys fibrinogens (0.1 mg/mL with 1 mM CaCl_2) were added to microtiter wells. The fibrinogen was either clotted with 10 nM thrombin or immobilized directly to the microtiter wells as described above. After immobilization, washing, and blocking, 100 μL of a mixture of 1 nM tPA, 5 $\mu\text{g/mL}$ of glu-plasminogen, and 0.1 mM Spectrozyme PCa (American Diagnostica) was added to all of the wells. The kinetics of

plasmin activation was monitored for 1 h at 405 nm, at room temperature, on a ThermoMax plate reader. Two parameters of the plasmin generation curves can be derived from these data: lag time and V_{\max} . The V_{\max} value is determined by drawing a line through the steepest slope of the absorbance curve (the slope is defined as V_{\max}). Extending the line until it crosses the x -axis yields the lag time (defined as the x intercept). The values for modified fibrin(ogen) were compared to the control using an unpaired t -test.

ELISA Assay to Monitor the Immobilization of Fibrin(ogen) to Microtiter Plates. In each series of experiments in which fibrin or fibrinogen was immobilized in microtiter wells, the efficiency of binding of the control and Hcys fibrin(ogens) was assessed with an ELISA assay. After allowing the fibrinogen to bind to the wells, blocking, and washing, the wells were incubated for 1 h at room temperature with a 1:50 000 dilution of goat antihuman fibrinogen peroxidase conjugated antibody (Enzyme Research Laboratories). The wells were washed, and 100 μ L of TMB peroxidase substrate was added. The reactions were stopped with phosphoric acid, and the absorbance values were read at 450 nm on the ThermoMax plate reader. We consistently found that the control and modified fibrinogens bound equally well to the microtiter wells. Thus, any differences in the solid-phase assays were not due to the differences in fibrin(ogen) available for interaction with the fibrinolytic enzymes.

RESULTS

Incubation with Homocysteine Thiolactone Leads to the Chemical Modification of Fibrinogen and the Modification of Fibrin Structure. Fibrinogen was incubated with different concentrations of homocysteine thiolactone for different periods of time until conditions were found under which clotability was retained, but the resulting fibrin clot structure was altered. We found that incubation of 300 μ M homocysteine thiolactone with 12.5 μ M fibrinogen (3.28 mg/mL) for 18 h resulted in Hcys fibrinogen that could be clotted completely and appeared to be indistinguishable from the control fibrinogen on reduced and unreduced polyacrylamide gel electrophoresis. Under these conditions, we assayed an average of 52.4 ± 3.6 sulfhydryl groups/control fibrinogen molecule and 57.7 ± 0.5 sulfhydryls/Hcys fibrinogen molecule in three different preparations. Maximal sulfhydryl incorporation was obtained at 30 mM homocysteine thiolactone, which yielded 62.0 ± 11.0 sulfhydryl groups/fibrinogen molecule.

The control fibrinogen and fibrinogen that had been reacted with 300 μ M Hcys thiolactone (Hcys fibrinogen) were clotted with thrombin in the presence of the concentrations of calcium indicated in Figure 2. The relative thickness of the fibrin fibers (μ) was calculated from optical measurements. As we have previously seen with fibrinogen from hyperhomocysteinemic rabbits, the Hcys fibrinogen formed clots with significantly thinner fibers than those formed by the control fibrinogen. This difference was reduced by increasing calcium levels, as observed for homocysteinemic rabbit fibrinogen.

Hcys Fibrinogen Forms Clots that are Resistant to Fibrinolysis. The control ($n = 7$) and Hcys-fibrinogen samples ($n = 10$) were added to fibrinogen-deficient plasma at 1 mg/mL, recalcified, and clotted with thrombin in the

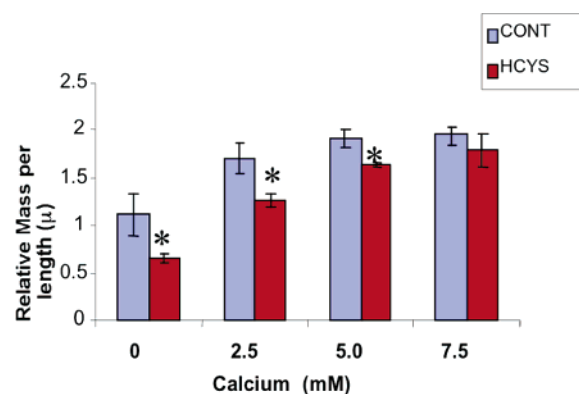


FIGURE 2: Relative mass/unit length of clots formed from the control and Hcys fibrinogen. Samples of fibrinogen ($n = 4$ each) were adjusted to the indicated concentration of calcium, clotted by the addition of thrombin, and the relative μ of the fibers in the resulting clots determined as detailed in Materials and Methods. * $p < 0.05$ vs control.

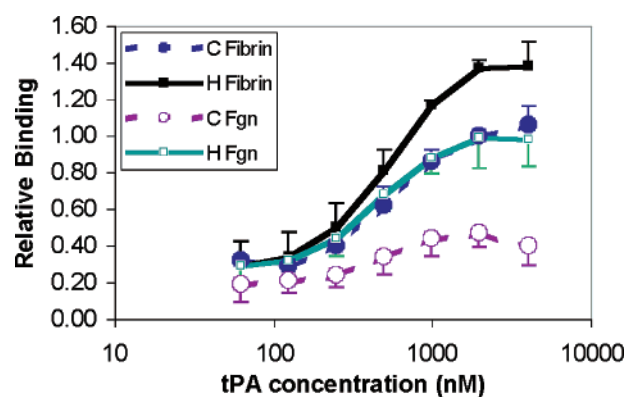


FIGURE 3: tPA binding to fibrin(ogen). The control and Hcys fibrinogen were clotted or directly plated in wells as described in Materials and Methods. tPA was allowed to bind to fibrinogen, fibrin, and control wells. Binding was detected by the cleavage of a chromogenic substrate. The tPA bound to control fibrin at 2 μ M tPA was set equal 1 in each experiment. The mean \pm sd from four experiments are shown.

presence of tPA in the hemodyne hemostasis analyzer. The formation and lysis of the resulting clots were monitored, and the half-lysis time was determined as described in Materials and Methods. The half-lysis time was significantly ($p < 0.01$) increased for the clots from Hcys fibrinogen (15.5 ± 3.8 min) compared to the clots from the control fibrinogen (11.7 ± 2.1 min).

tPA Binding to Hcys Fibrinogen and Hcys Fibrin Is Increased. The binding of tPA to immobilized fibrinogen and fibrin was measured as described in the Materials and Methods section. As can be seen in Figure 3, Hcys fibrinogen and Hcys fibrin bound a greater total amount of tPA than did their respective controls ($p < 0.01$). The affinity (K_{app}) was not, however, significantly changed by the Hcys modification.

The K_{app} calculated for tPA binding to the control fibrin was 381 ± 97 nM (relative maximum binding 1.17 ± 0.09), for Hcys fibrin 451 ± 83 nM (relative maximum binding 1.61 ± 0.09), for the control fibrinogen 144 ± 61 nM (relative maximum binding 0.46 ± 0.05), and for Hcys fibrinogen 287 ± 62 nM (relative maximum binding 1.09 ± 0.06). The maximal tPA binding to homocysteine-treated fibrin and fibrinogen was greater than the binding to the respective control preparations.

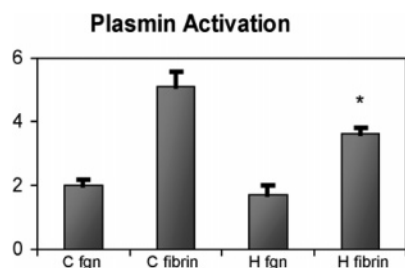


FIGURE 4: Plasminogen activation on fibrin(ogen). The control (C) and Hcys (H) fibrinogen were clotted or plated in wells. A mixture of tPA, plasminogen, and a chromogenic substrate was added to fibrinogen (fgn), fibrin, and blank wells. A405 was monitored as a measure of plasmin activity. The average maximal rate of substrate cleavage (mOD/min) \pm SD (three experiments in triplicate) is shown. * $p < 0.05$ vs control fibrin.

Plasminogen Binding to Fibrin(ogen) Is Not Significantly Altered By Reaction with Hcys Thiolactone. The binding of plasminogen to immobilized fibrinogen and fibrin was measured as described in Materials and Methods. Neither the maximum binding nor the affinity were different for Hcys fibrin ($K_{app} = 1.4 \pm 0.4 \mu\text{M}$) and the control fibrin ($K_{app} = 1.2 \pm 0.3 \mu\text{M}$). Likewise, the capacity and affinity of plasminogen binding to Hcys fibrinogen ($K_{app} = 5.2 \pm 1.3$

μM) were not significantly different from the control fibrinogen ($K_{app} = 4.1 \pm 1.0 \mu\text{M}$).

Plasminogen Activation Is Impaired on Hcys Fibrin. Although previous experiments did not show any defects in tPA or plasminogen binding to Hcys fibrin or Hcys fibrinogen, the maximal rate of plasmin cleavage of the chromogenic substrate was decreased in the presence of Hcys fibrin compared to that in the presence of the control fibrin, as shown in Figure 4. The difference was statistically significant ($p < 0.05$).

Homocysteinylation of Lysine Residues Are Present in Hcys Fibrinogen. Tryptic digests of the control and Hcys fibrinogen were analyzed by mass spectrometry (MS) as described in Materials and Methods. Initial analysis by MALDI-MS revealed that three lysines (K413, K418, and K448) were modified in the A α chain. No modifications were detected in the B β and γ chains. Digests were further analyzed by LC-MS/MS, which resulted in more extensive sequence coverage (up to 65%) and detection of additional modification sites. A total of 12 homocysteinylation sites were found, including seven in the A α chain, two in the B β chain and three in the γ chain. The modifications were detected on the basis of the mass of the peptide and confirmed by sequence information from tandem MS. As an example, the

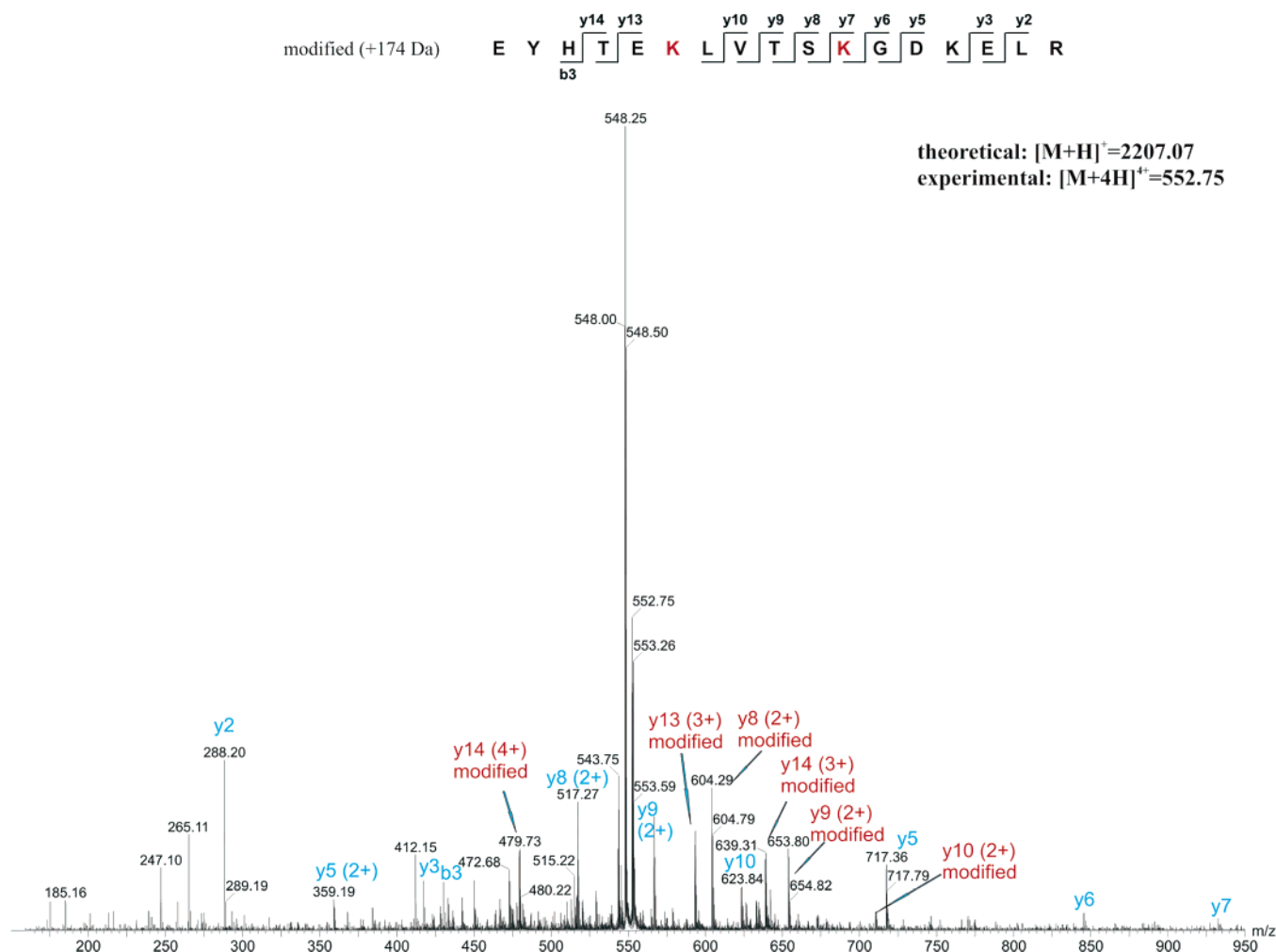


FIGURE 5: MS/MS spectrum of one modified tryptic peptide from the A α chain of fibrinogen. The mass of these peptide-containing residues E408–R424 was 174 Da greater than the theoretical mass based on the amino acid sequence. The mass shift corresponds to the modification by a single carbamidomethylated Hcys thiolactone. Predominantly y-ions were produced in the MS/MS spectrum, which confirmed the bulk of the peptide sequence. Analysis of the MS/MS data reveals two isoforms of this peptide with modification occurring at either Lys413 or Lys418.

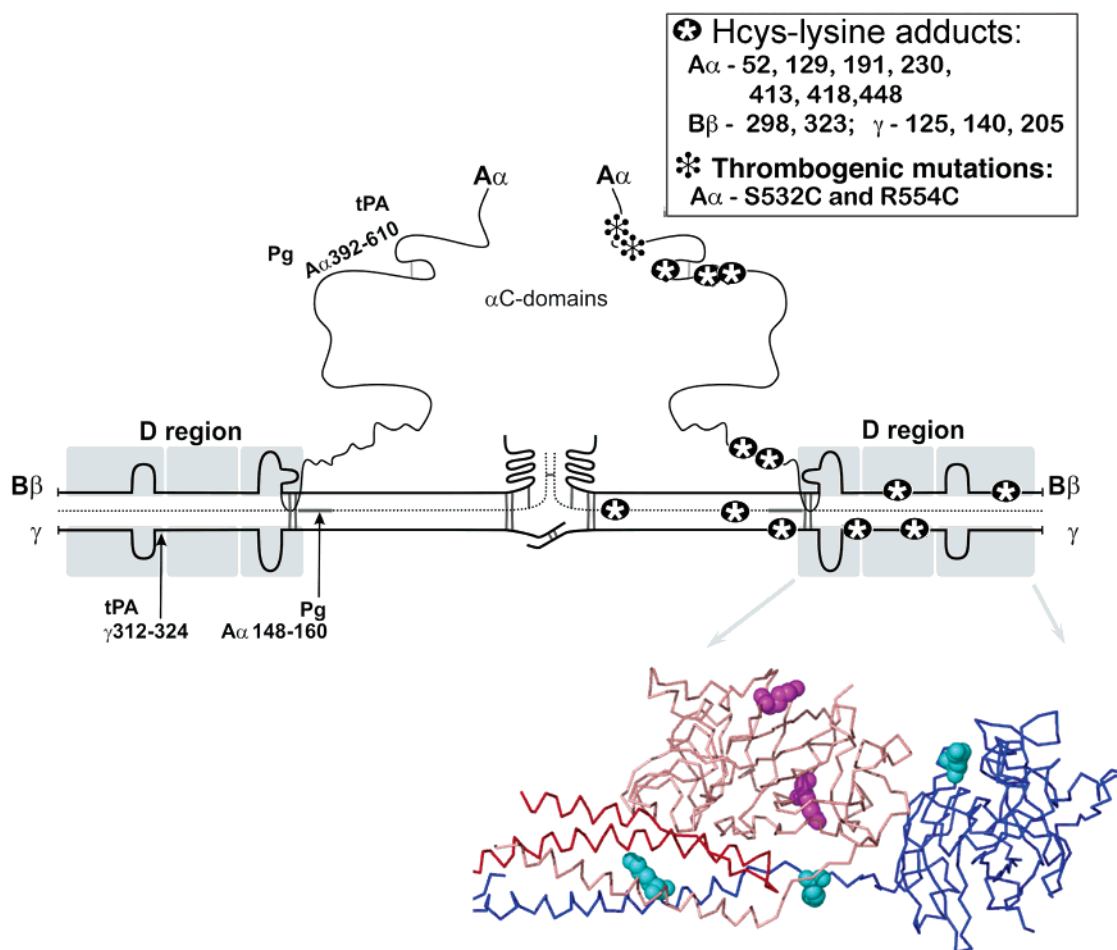


FIGURE 6: Hcys lysines in fibrinogen. Hcys lysines are shown on the right side of the molecule, and sites of tPA and plasminogen (Pg) binding are on the left side. The inset shows the 3D structure of the D region with the B β chain in pink, γ in blue, and A α in red. Hcys lysines are indicated as spheres.

MS/MS spectrum of the tryptic peptide containing A α lysines 413 and 418 is shown in Figure 5.

DISCUSSION

We have shown that the modification of human fibrinogen by reaction with Hcys thiolactone *in vitro* leads to functional alterations that mimic the dysfibrinogenemia seen in hyperhomocysteinemic rabbits. The changes include the formation of fibrin clots composed of thinner fibers, altered responsiveness to calcium concentration, and resistance of the resulting clots to tPA/plasmin lysis. Although we used much higher levels of homocysteine than are reported to circulate in plasma, we feel that this is still a reasonable model for modifications that might occur *in vivo*. We were only able to react isolated fibrinogen with homocysteine thiolactone for a few hours before some degradation began to occur. However, although homocysteine thiolactone levels are lower, fibrinogen circulates in plasma with a half life of 5–6 days. The physiological relevance of our findings remains to be determined; however, the similarities between the alterations in fibrinogen function observed *in vivo* in a rabbit model and the alterations observed upon reaction of purified human fibrinogen with homocysteine thiolactone are striking.

MS analysis identified a limited number of lysine residues that are homocysteinylated *in vitro*. More sites could probably be modified, but we only wanted to modify as many sites as were consistent with maintaining fibrinogen clotting

function. These sites are indicated by a white star in a black circle on the right side of the schematic of fibrinogen in Figure 6. These include seven sites in the A α chain with three in the α C domain, two in the B β chain, and three in the γ chain within the D region. The inset in Figure 6 shows the details of the D region on the basis of its crystal structure (25). The Hcys lysines in the B β and γ chains are shown as spheres. None of the modifications are directly at the sites of tPA and plasminogen binding, probably because those sites are not accessible in fibrinogen but are unveiled during fibrin polymerization (26). Structural details of the α C region are not available because it is highly mobile and not visualized in the fibrinogen crystal structure. All of the sites of modification seem reasonable because they are on solvent-exposed parts of the molecule.

The regions of plasminogen (Pg) and tPA binding are indicated on the left-hand side of the fibrinogen molecule in Figure 6. Three sites of Hcys modification in the A α chain are in the region of the α C domain identified as being involved in tPA and plasminogen binding (A α 392–610) (6). They are also close to the sites of two well-characterized mutations of Arg-554 (fibrinogen_{Dusart}) or Ser-532 (fibrinogen_{CaracasV}) to Cys, which are associated with thrombosis (27, 28). The sites of these prothrombotic mutations are indicated by asterisks. Similar to what is seen in homocysteinylated fibrinogen, these mutations lead to clots composed of thin, tightly packed fibers with abnormal resistance to fibrinolysis.

The new Cys residues introduced in the prothrombotic mutations mediate disulfide bond formation between the mutant fibrinogen and albumin in human plasma. Linking a protein to the α C domain is theorized to disrupt polymerization because the α C domain is involved in the lateral association of fibrin protofibrils (29). It seems likely that the introduction of one or more free sulfhydryls by homocysteinylation could have the same effect in vivo. Thus, we feel that homocysteinylation in the α C domain may be functionally significant because it could potentially explain the abnormalities noted in our structural and functional studies and may produce an acquired dysfibrinogenemia that is analogous to thrombotic congenital dysfibrinogenemias.

We found that the abnormality in fiber thickness induced by homocysteinylation could be counteracted by the addition of calcium. This suggests that Hcys thiolactone modification of fibrinogen might impair calcium binding. Therefore, we compared the sites of calcium binding with sites of homocysteinylation. The fibrinogen molecule has 3 high-affinity calcium binding sites (μ M) and between 11 and 20 low-affinity binding sites (mM). Two high-affinity sites are located in the C-terminus of the γ -chain in the D domain and the third site has been tentatively localized in the central E domain (30). However, sites of lysine modification by Hcys thiolactone do not overlap in any obvious way with the calcium binding sites. Perhaps this should not be surprising because the positively charged lysine residues would not be expected to directly play a role in the binding of the positively charged calcium ions. At this point, we speculate that lysine modification may cause some changes in the conformation of the β or γ domains that reduce the affinity of calcium binding.

In summary, homocysteinylation of lysine residues may be linked to three important functional consequences. First, modification in the α C domain could alter the lateral association of fibers and thereby alter clot structure. Second, the alteration of the protein conformation could interfere with calcium binding, which could contribute to alterations in fibrin clot structure. Third, modification of lysine sites that are directly involved in fibrinolytic enzyme binding and activity could lead to increased resistance to fibrinolysis. Fibrinogen modification by Hcys thiolactone in vitro mimics the effects of elevated Hcys in vivo. We hypothesize that the modification of lysines in fibrinogen by Hcys thiolactone might occur in vivo, leading to abnormal resistance of the resulting fibrin to lysis and thereby contributing to the prothrombotic state associated with homocysteinemia.

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