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Modification by Homocysteine Thiolactone Affects Redox Status of Cytochrome *c*

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ABSTRACT: Homocysteine (Hcy)-thiolactone mediates a post-translational incorporation of Hcy into protein in humans. Protein N-homocysteinylation is detrimental to protein structure and function and is linked to pathophysiology of hyperhomocysteinemia observed in humans and experimental animals. The modification by Hcy-thiolactone can be detrimental directly by affecting the function of an essential lysine residue or indirectly by interfering with the function of other essential residues or cofactors. Previous work has shown that cytochrome *c* is very sensitive to Hcy-thiolactone, which causes formation of *N*-Hcy-cytochrome *c* multimers. However, it was unclear what sites in cytochrome *c* were prone to Hcy attachment and whether N-linked Hcy can affect the structure and redox function of cytochrome *c*. Here we show that 4 lysine residues (Lys8 or -13, Lys86 or -87, Lys99, and Lys100) of cytochrome *c* are susceptible to N-homocysteinylation. We also show that N-homocysteinylation of 1 mol of lysine/mol of protein affects the redox state of the heme ligand of cytochrome *c* by rendering it reduced. The modification causes subtle structural changes, manifested as increased resistance of the *N*-Hcy-cytochrome *c* to proteolysis by trypsin, chymotrypsin, and Pronase. However, no major secondary structure perturbations were observed as shown by circular dichroism spectroscopy. Our data illustrate how N-homocysteinylation can interfere with the function of heme-containing proteins.

Homocysteine (Hcy)[1] is a sulfur non-protein amino acid, involved in conversions of methionine and cysteine. Severe hyperhomocysteinemia due to genetic disorders in methionine metabolism leads to neurologic and vascular complications and premature death of affected individuals (*1*). Mild hyperhomocysteinemia is prevalent in a general population, and it is associated with an increased risk of vascular events and predicts mortality in heart disease patients (*2*, *3*). One of the proposed mechanisms of Hcy toxicity is the modification of proteins by a metabolite of Hcy, Hcy-thiolactone (HTL) (*4*). HTL is a product of methionyl-tRNA synthetase editing reaction. Because Hcy is structurally similar to methionine, methionyl-tRNA synthetase misactivates Hcy and forms homocysteinyl adenylate. In the next step, the side chain thiolate of Hcy reacts with the activated carboxyl group of Hcy, yielding HTL. The thioester bond of HTL is highly prone to reactions with ϵ-amino groups of protein lysine residues (*5*, *6*). Most of Hcy in human blood (about 70%) is N-linked to proteins, and N-linked Hcy has been identified and quantified in individual blood proteins (*7*). It has been shown that protein modification by HTL (N-homocysteinylation) has detrimental effects on protein structure and function. For example, N-homocysteinylation causes aggregation of many proteins, such as low-density lipoprotein (*8*), fibrinogen, cytochrome *c*, myoglobin, RNase A, transferrin, γ-globulin, and hemoglobin (*5*). N-Homocysteinylation of enzymes such as trypsin, methionyl-tRNA synthetase, or paraoxonase leads to diminished activity or complete inactivation depending on the extent of modification (*5*, *9*). Protein N-homocysteinylation is linked to two important aspects of human pathobiology: activation of the immune response (*10*, *11*) and thrombogenesis (*12*, *13*). Studies on the effects of N-homocysteinylation on protein structure and function may lead to a better understanding of the Hcy pathobiology.

Experiments with human and animal cell cultures show that, although HTL is synthesized inside cells, it accumulates also in extracellular fluids and modifies both intracellular and extracellular proteins (*14*, *15*). These findings are consistent with exceptionally low pK = 6.67 of HTL (*16*) which makes it neutral at physiological pH and thus able to diffuse through cell membranes (*6*).

Cytochrome *c* is a peripheral membrane mitochondrial protein that mediates single electron transfer between protein complexes in the respiratory chain of eukaryotes. Cytochrome *c* consists of a polypeptide chain of 104 amino acid residues and covalently attached heme group. Positively charged lysine residues are exposed on the surface of the

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[1] Abbreviations: CD, circular dichroism; CAM, carboxyamidomethyl; DTT, dithiothreitol; Hcy, homocysteine; HTL, Hcy-thiolactone; IAA, iodoacetic acid; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel elecrophoresis.

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molecule and play an important role in cytochrome *c* oxidase and reductase binding. Because of the cationic amino acid side chains clustered at the surface of cytochrome *c*, it is very basic with an isoelectric point near 10. Heme is essential for the physiochemical activity of cytochrome *c*. It is attached to polypeptide through two thioether bonds with cysteine residues 14 and 17. The iron atom oscillates between the ferrous (Fe^{II}) and ferric (Fe^{III}) states. The axial coordination sites are occupied by two ligands: the imidazole nitrogen atom of histidine 18 and the sulfur atom of methionine 80. Besides its function as an electron carrier, cytochrome *c* has been also found to play a role in apoptosis, in which it is involved in caspase activation (*17*−*19*). Cytochrome *c* is very susceptible to the modification by HTL; forms containing <1 N-linked Hcy/mol protein are prone to multimerization (*5*).

The aim of the present study was to examine the effect of N-homocysteinylation on the structure and function of cytochrome *c*. Here we report that four out of 19 Lys residues in cytochrome *c* are preferentially modified by HTL and that the modification causes subtle structural changes manifested in diminished susceptibility to proteolysis. We also show that N-homocysteinylation affects the redox status of cytochrome *c* by rendering the heme iron reduced.

MATERIALS AND METHODS

Preparation of Modified Proteins. *N*-Hcy-cytochrome *c* was prepared by incubating horse heart cytochrome *c* (type IV) with *L*-Hcy-thiolactone (both from Sigma-Aldrich) at 20 °C for 22 h as described previously (*5*).

To prepare *N*-(Hcy-SH)-albumin-Cys34-*S*-CAM, 10 mg/mL human serum albumin (Sigma-Aldrich) was first incubated with 1 mM DTT at room temperature for 5 min. Liberated thiol group of Cys34 was alkylated with 8 mM IAA for 30 min in the dark. Excess IAA was removed by ultrafiltration through a 10 kDa cutoff membrane (Millipore). The albumin-Cys34-*S*-CAM was than N-homocysteinylated with 0.4 mM HTL for 16 h at 37 °C (*2*). Excess HTL was removed by ultrafiltration.

MALDI-TOF−*Mass Spectrometry*. Protein and peptide mass analyses were carried out on a MALDI-TOF Autoflex instrument (Bruker Daltonics). *N*-Hcy-cytochrome *c* was dissolved at concentration 2 mg/mL in water, and applied in volume 0.5 μL to the target plate (MTP 384 massive target T, Bruker Daltonics), followed by addition of 0.5 μL of sinapinic acid, and allowed to evaporate. For peptide mixtures α-cyano-4-hydroxycinnaminic acid was used as a matrix. The sample was ionized with a laser ray of 337 nm. The instrument was calibrated with protein mass standards (Bruker Daltonics). Spectra of proteins were acquired in the linear mode while peptide maps were collected in the reflectron mode.

Tryptic Digest of N-Hcy-Cytochrome c. One nanomole of *N*-Hcy-cytochrome *c* was reduced with 45 mM DTT in 100 mM ammonium bicarbonate at 55 °C for 45 min. Subsequently sulfhydryl groups were alkylated with 100 mM IAA at 20 °C, for 15 min, in the dark. Bovine pancreatic trypsin (Worthington Biochemical Corporation) was added to obtain a final trypsin−cytochrome *c* ratio (mass−mass) of 1:50. Incubation was carried out at 37 °C for 24 h. After this time tryptic peptides were fractionated on reversed phase C18 microcolumn (ZipTip, Millipore) using 10, 30, 50, and 100% acetonitrile (Merck) for elution. Each fraction was directly applied to the Prespotted Anchorchip (Bruker Daltonics), allowed to evaporate, and subjected to MALDI-TOF analysis. In parallel, trypsin was incubated without *N*-Hcy-cytochrome *c* and treated as above to obtain trypsin autolytic peptide masses.

Computer Data Analysis. Based on the list of peptide masses, protein identification was performed by MASCOT (http://www.matrixscience.com). Parameters of the analysis were as follows: peptide mass tolerance, 0.2 Da; fixed modification, carbamidomethylation of Cys; variable modification, oxidation of Met and allow up to 2 missed cleavages. Mass increase due to the N-homocysteinylation of Lys by carbamidomethylated Hcy is 174 Da. Peptides with mass increase equal to one or two Hcy residues attached were identified by PepModSearch (http://bioinfo.pl/∼kajla/pepmodsearch/index.php). This program was created for identification of a post-translational modification in a protein, based on increase in peptide mass due to the modification. In order to exclude peptides resulting from the autolysis of trypsin, we used PepListComp, a tool available in the PepModSearch service, to subtract a trypsin autolytic mass list from the *N*-Hcy-cytochrome *c* mass list. To perform the search for modified fragments, PepModSearch requires the amino acid sequence and the experimental peptide mass list of the analyzed protein. In order to select peptide masses corresponding to modified peptides, PepModSearch compares the result of an *in silico* digestion (carried out by MS-Digest: http://prospector.ucsf.edu) with the list of experimental peptide masses given by the user, looking for mass differences equal to an integer multiple of the mass increase due to modification.

Spectrophotometric Assays. Spectral changes were monitored with a Beckman DU-65 spectrophotometer in the 450−650 nm range. Cytochrome *c* (20 mg/mL) was incubated with 20 mM HTL in 100 mM potassium phosphate buffer, pH 7.4, 0.2 mM EDTA at 24 °C. At 1 h time intervals 4.5 μL aliquots of the incubation mixture were diluted 100-fold with 100 mM potassium phosphate buffer, pH 7.4, 0.2 mM EDTA and the spectrum recorded. All readings were taken in 1 cm cells against a reagent blank.

In another experiment cytochrome *c* was incubated with *N*-(Hcy-SH)-albumin-Cys34-*S*-CAM (Hcy/protein molar ratio 1:1) in 100 mM potassium phosphate buffer, pH 7.4, 0.2 mM EDTA, for 77 h at 24 °C. As control, cytochrome *c* was incubated with albumin-Cys34-*S*-CAM or without albumin. Absorption spectra were acquired after 24 and 77 h incubation at 24 °C.

Susceptibility of N-Hcy-Cytochrome c to Proteolysis. Native cytochrome *c* and cytochrome *c* (2 mg/mL) modified with 1-, 2-, 4-, and 19-fold molar excess of HTL were digested with trypsin (Worthington Biochemical Corporation) at an enzyme−substrate ratio of 1:5 in 100 mM ammonium bicarbonate for 1 or 6 h at 37 °C, chymotrypsin (Merck) or Pronase (Koch-Light Laboratories Ltd.) at an enzyme−substrate ratio of 1:50 in 100 mM potassium phosphate buffer, pH 7.4 for 2 h at 37 °C. The digestion with Pronase was carried out in the presence of 5 mM $CaCl_2$. Digests were mixed 1:1 with SDS−PAGE sample buffer containing 1% 2-mercaptoethanol, denatured for 5 min at 99 °C, and subjected to SDS−PAGE on 12% gels. Protein bands were

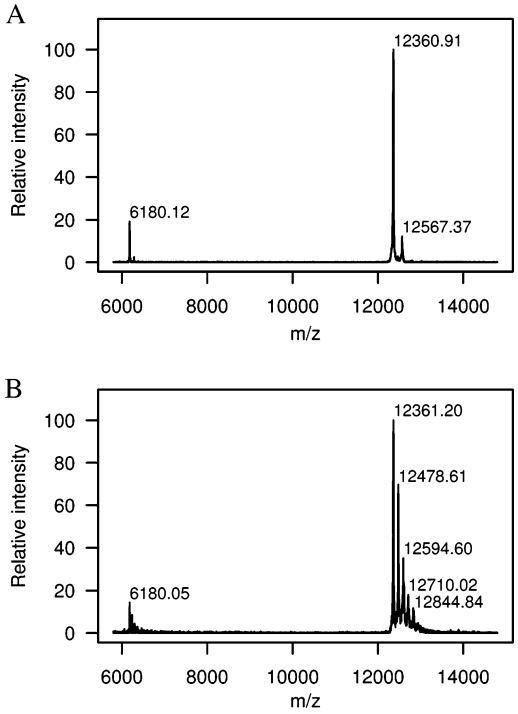

FIGURE 1: Mass spectra of cytochrome *c*: (A) native; (B) modified with 4-fold molar excess of HTL.

visualized by staining with Coomassie Brilliant Blue R-250 (Fluka) and quantified by densitometry.

Circular Dichroism. Cytochrome *c* modified with 1- to 8-fold molar excess of HTL was desalted on 10 kDa cutoff filters (Millipore) and diluted to 0.2 mg/mL in 10 mM potassium phosphate buffer, pH 7.4, before measurement. Three accumulations of each CD spectrum were recorded with a Jasco J-810 spectrometer at 20 °C. The cell lengths were 0.1 and 1 cm for 180−300 and 300−650 nm regions, respectively. The data were collected at wavelength intervals of 0.5 nm, with a speed of 100 nm/min. Background (10 mM potassium phosphate buffer, pH 7.4) was recorded and subtracted from the original spectra. Molar concentration of the protein was calculated using the molar extinction coefficient $\epsilon_{410} = 106\,100\ M^{-1}\ cm^{-1}$ (*20*). Cytochrome *c* secondary structure analysis was performed using algorithms: SELCON3 (*21*), CONTIN (*22*), and CDSSTR (*23*), available on an online server, DICHROWEB (*24*). The final percentage content of α helix, *β* sheet, turns, and unordered is a mean value of results obtained from those algorithms. Dataset 7 was used as a reference for estimation of the secondary structure element content.

RESULTS

N-Homocysteinylation of Cytochrome c. Cytochrome *c* was modified with a 4-fold molar excess of HTL. To determine the extent of protein N-homocysteinylation, native and HTL-modified cytochrome *c* were subjected to MALDI-TOF mass spectrometric analysis. Two signals, at *m*/*z* of 12 360.91 and 12 567.37, were observed in mass spectra of the native protein (Figure 1A). The first signal comes from unmodified cytochrome *c*, while the latter signal corresponds to an adduct of the protein with sinapinic acid matrix. N-Homocysteinylation results in a protein mass increase of 117.17 Da per each modified Lys residue. As shown in Figure 1B, cytochrome *c* modified with a 4-fold molar excess of HTL is a mixture of native molecules and molecules with 1, 2, 3, and 4 N-linked Hcy residues.

Identification of N-Homocysteinylated Lys Residues. To identify sites susceptible to modification by HTL, native cytochrome *c* and cytochrome *c* modified with a 2- or 4-fold molar excess of HTL were reduced with DTT, treated with IAA to block sulfhydryl groups, and digested with trypsin. Tryptic peptides were subjected to MALDI-TOF mass spectrometric analysis. Sequence coverages for native cytochrome *c* and cytochrome *c* modified with a 2- or 4-fold molar excess of HTL, by tryptic peptides, measured by MASCOT were 67 ± 5.4% ($n = 4$) and 81 ± 2.8% ($n = 2$) or 81 ± 2.8% ($n = 4$), respectively. Analyses of peptide mass list of cytochrome *c* modified with a 2-fold molar excess of HTL have revealed that Hcy is preferentially attached to Lys8 or -13 and Lys86 or -87. In the presence of a 4-fold molar excess of HTL over cytochrome *c*, Lys99 and Lys100 were also modified (Table 1, Figure 2).

Iron Redox State. Two bands, at $\lambda = 520$ and 550 nm, characterize the visible absorption region of ferrocytochrome *c*, while ferricytochrome *c* exhibits one broad band with maximum at 530 nm. The absorption spectra of cytochrome *c* incubated with increasing concentrations of HTL suggest that the reduction of heme iron has occurred as a result of the modification (Figure 3). During the incubation of cytochrome *c* with HTL, the ratio A_{550}/A_{520}, indicator of the reduction of heme iron, is time- (Figure 4A) and HTL concentration-dependent (Figure 4B). The reduction of cytochrome *c* heme iron was observed also after incubation with *N*-(Hcy-SH)-albumin or *N*-(Hcy-SH)-albumin-Cys34-*S*-CAM, while albumin-Cys34-*S*-CAM did not affect a redox state of heme iron (not shown). Because residual HTL was removed from the modified albumin preparation, this effect is caused by *N*-(Hcy-SH)-albumin-Cys34-*S*-CAM and not by HTL.

When *N*-Hcy-cytochrome *c* (prepared by modification with 19 mol of HTL/mol of protein and purified by gel filtration) was oxidized with potassium ferricyanide (1.1 mol/mol of protein), the absorption bands at 520 and 550 nm disappeared and a broad band at 530 nm appeared within a few minutes, consistent with the transition from ferro- to ferricytochrome *c*. However, this spectral change was transient and the heme iron became fully reduced within 30 min (not shown).

The reduction of heme iron after incubation of cytochrome *c* with HTL was confirmed by analysis of a CD spectrum in the Soret region (350−490 nm). The shape of the CD spectrum of the *N*-Hcy-cytochrome *c* in this range was significantly different from that of the unmodified protein (Figure 5). The CD spectrum of the native cytochrome *c* is similar to other spectra previously reported for ferricytochrome *c* (*25*). The major Soret peaks are at 404 nm (positive) and 415 nm (negative). N-Homocysteinylation leads to gradual decline in the positive peak and a replacement of the negative peak with a positive one that moves toward longer wavelengths (423 nm). The latter peak, together with a negative peak at 330 nm, is typical for ferrocytochrome *c*.

Susceptibility to Proteolysis. To determine whether modification with HTL affects the structure of cytochrome *c*, the susceptibility of *N*-Hcy-cytochrome *c* and native cytochrome *c* to proteolytic digestion was examined. We found that

Table 1: Peptides from *N*-Hcy-cytochrome *c* Identified as Carrying N-Linked Hcy[a]

peptide mass, Da	*N*-Hcy-peptide mass, Da	sequence	variable modification
1875.96	2049.75 ± 0.13 ($n = 7$)	8-**K**IFVQ**K**CAQCHTVEK-22	
1051.63	1225.57 ± 0.12 ($n = 5$)	80-MIFAGI**KK**K-88	1Met-ox[b]
1507.80	1855.90 ± 0.02 ($n = 2$)	92-EDLIAYL**KK**ATNE-104[c]	

[a] Lysine residues carrying N-linked Hcy are indicated in bold. [b] Met-ox: oxidation of Met. [c] Peptide with two modified lysine residues.

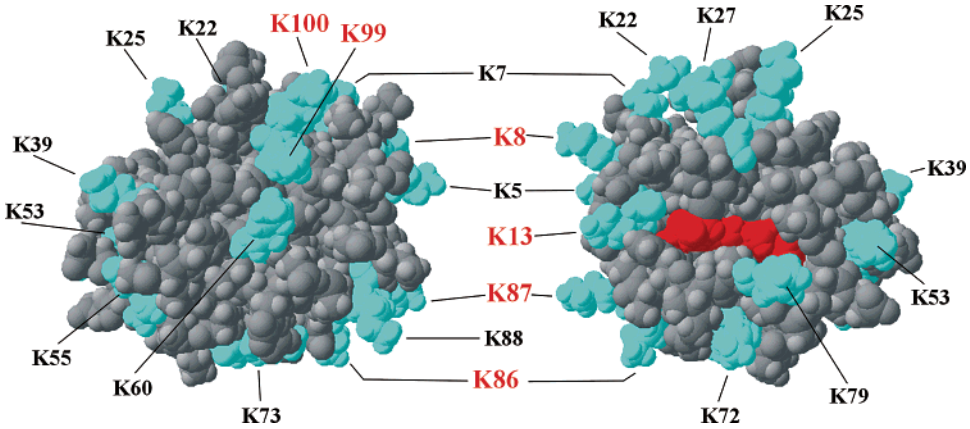

FIGURE 2: The structure of cytochrome *c* (*39*). Lysine residues susceptible to the modification by HTL are indicated by red symbols. Unreactive lysine residues are indicated by black symbols. The heme moiety is colored red. The distances between heme iron and the reactive lysine residues are from 13.68 Å to 23.65 Å.

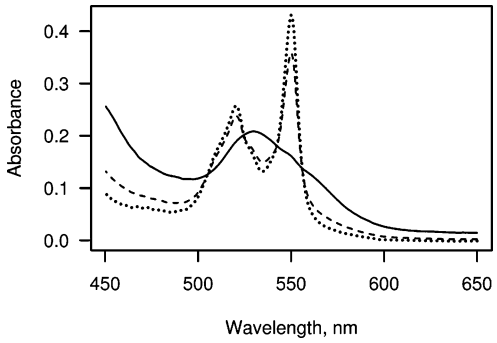

FIGURE 3: Absorption spectra of native cytochrome *c* (solid line) and cytochrome *c* modified with an equimolar concentration of HTL (dashed line) and a 2-fold molar excess of HTL (dotted line).

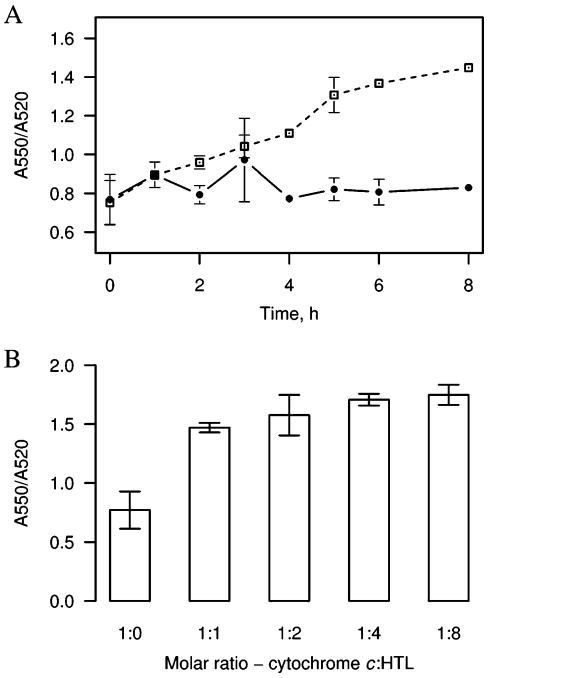

FIGURE 4: (A) Time-dependent changes in A_{550}/A_{520} ratio of cytochrome *c* during modification with an equimolar concentration of HTL (dotted line). As a control, A_{550}/A_{520} ratios of cytochrome *c* incubated without HTL are shown (solid line). (B) Dependence of A_{550}/A_{520} on cytochrome *c*/HTL ratio during modification. Data are means ± SD of three experiments.

cytochrome *c* and *N*-Hcy-cytochrome *c* exhibited different susceptibilities to proteolysis by trypsin, chymotrypsin, or Pronase, with the N-homocysteinylated form being less susceptible to degradation than the unmodified form (Figure 6). To determine whether the extent of N-homocysteinylation affects proteolytic degradation, we modified cytochrome *c* with increasing amounts of HTL (from 1 to 19 mol of HTL/mol of protein) and subjected it to digestion by trypsin. As expected, the susceptibility to digestion by trypsin was HTL concentration-dependent: the higher the number of Lys residues modified, the greater was the resistance of the protein to trypsin (not shown). Taken together, these results show that N-homocysteinylation renders cytochrome *c* more resistant to proteolytic digestion.

Secondary Structure. The effect of N-homocysteinylation on cytochrome *c* polypeptide chain conformation was monitored by CD spectrometry. The CD spectra of proteins are divided into wavelength ranges, based on the energy of the electronic transitions that dominate in the region. Below 250 nm peptide contributions dominate, and this region provides the information about secondary structure composition of the protein. The 350−550 nm region, where extrinsic chromophores contribute, is useful for studying the interac-

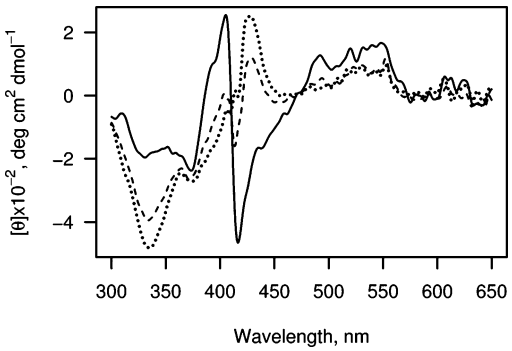

FIGURE 5: CD spectra of native cytochrome *c* (solid line) and cytochrome *c* modified with an equimolar concentration of HTL (dashed line) and a 2-fold molar excess of HTL (dotted line) in the Soret and visible regions.

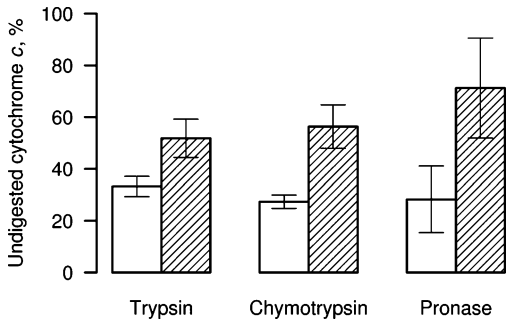

FIGURE 6: N-Homocysteinylation affects susceptibility of cytochrome *c* to proteolytic degradation. Percentages of undigested cytochrome *c* remaining after treatments with trypsin, chymotrypsin, and Pronase are shown. Open bars, native cytochrome *c*; hatched bars, *N*-Hcy-cytochrome *c*.

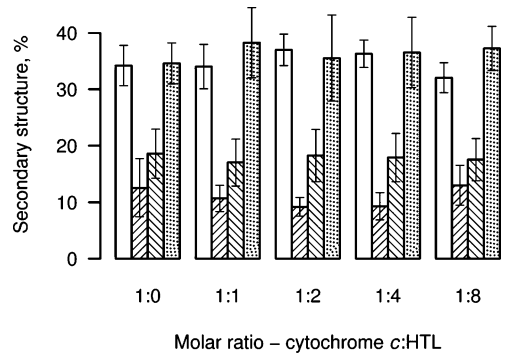

FIGURE 7: Secondary structure composition of *N*-Hcy-cytochrome *c* modified with increasing molar excess of HTL. The mean percentages of each structural element, α-helix (empty bars), β-sheet (//), turns (\\), and unordered (dotted bars) in unmodified and HTL-modified cytochrome *c*, calculated from corresponding CD spectra, are shown.

tion of the protein chain with prosthetic group.

The CD spectrum of horse heart cytochrome *c* in the far UV spectral region, with minimum at 208 and 222 nm, typical for proteins that contain α-helix in abundance, was not significantly affected by N-homocysteinylation (not shown). DICHROWEB analysis (*24*) of data did not indicate any significant secondary structure composition change upon treatment with increasing concentration of HTL (Figure 7).

DISCUSSION

Cytochrome *c* is an electron transfer protein, which functions on the surface of the inner mitochondrial membrane. Lysine residues have an important role in cytochrome *c* function by providing electrostatic bonds for the interaction with cytochrome *c* oxidase (*26*). Modification of the amino groups of Lys residues with HTL leads to a change of the charge on the protein surface. The highly basic ϵ amino group of lysine ($pK_a = 10.5$) is replaced by a less basic α amino group of N-linked Hcy ($pK_a \sim 6.7$) (*5*, *6*, *16*). Acetylation of cytochrome *c* causes a decrease in p*I* value, which in turn prevents cytochrome *c* from interacting with cytochrome *c* reductase and oxidase (*27*). The activity of cytochrome *c* decreases with increasing degree of acetylation (*28*). N-Homocysteinylation is also expected to inactivate cytochrome *c* by decreasing the positive charge on the protein's surface. However, as shown in the present work, a major effect of N-homocysteinylation, because of the incorporation of a thiol group, is the interference with the redox state of cytochrome *c*.

Scheme 1

$$\text{Cyt } c(Fe^{+3}) + \text{HTL} \rightarrow N\text{-(Hcy-SH)-Cyt } c(Fe^{+3}) \rightarrow N\text{-(Hcy-S•)-Cyt } c(Fe^{+2}) \rightarrow$$
$$\rightarrow [\text{Cyt } c(Fe^{+2})]\text{-}N\text{-Hcy-S-S-Hcy-}N\text{-}[\text{Cyt } c(Fe^{+2})]$$

The present work identifies four Lys residues as preferential sites of N-homocysteinylation in cytochrome *c in vitro*. It also provides evidence for subtle structural alterations manifested by increased resistance of *N*-Hcy-cytochrome *c* to proteolytic degradation. The Lys residues constituting preferential sites for the modification by HTL, namely, Lys8 or -13, Lys86 or -87, Lys99, and Lys100, are located within turns of the polypeptide chain, with one exception, Lys8, which is located in α-helix. These lysine residues are also susceptible to modifications by other elecrophilic agents, such as acetic anhydride (*29*), trinitrobenzene sulfonate (*30*), pyridoxal phosphate (*31*), or 4-hydroxy-2-nonenal (*32*). Analyses of N-homocysteinylation sites in albumin (*33*) and fibrinogen (*13*), together with the results obtained in the present study for cytochromee *c*, suggest that factors other than the secondary structure elements determine which Lys residues are modified.

During the incubation of cytochrome *c* with HTL a change in the color of the solution was observed. The color of cytochrome *c*, as well as other heme proteins, is due to the transitions within the π electron system of the heme porphyrin. The factors that influence these transitions include the oxidation state of the iron, the peripheral substitution pattern of the individual porphyrins, the axial ligation of the iron, and the polarity of the heme environment within the folded protein chain. Heme protein spectra show two characteristic bands. One is in the 390−450 nm wavelength range and is called the Soret. The other band or bands lie in the visible, 450−700 nm, range. Both bands are allowed transitions $\pi \rightarrow \pi^*$. The visible bands are more sensitive to oxidation state and/or ligation of the iron. The redox state of cytochrome *c* can be determined from the absorption spectrum in this region.

Low molecular weight thiols, as well as sulfhydryl groups of proteins, are oxidized by transition metal ions, for example Fe^{+3}. During this reaction Fe^{+3} iron is reduced to Fe^{+2}. As shown here, N-homocysteinylation of ferricytochrome *c* resulted in its conversion to a ferrous form. Our data are consistent with the mechanism depicted in Scheme 1. Reaction of HTL with any of the four susceptible lysine

residues of ferricytochrome *c* affords *N*-(Hcy-SH)-Cyt *c*(Fe^{+3}). The heme iron in the product undergoes reduction by the thiolate of N-linked Hcy, to afford a modified ferrocytochrome *c*, *N*-(Hcy-S•)-Cyt *c*(Fe^{+2}). The reduction occurs in trans between different molecules of *N*-(Hcy-SH)-Cyt *c*(Fe^{+3}), and can also occur with other *N*-(Hcy-SH)-protein. For example, a similar reduction of heme-Fe^{+3} was also observed during incubation of ferricytochrome *c* with *N*-(Hcy-SH)-albumin-Cys34-*S*-CAM. An intramolecular reduction is unlikely, because the sites of N-homocysteinylation are located too far from the heme iron (Figure 2). Dimerization between the thiyl radicals from different molecules of the Hcy-modified ferrocytochrome *c*, *N*-(Hcy-S•)-Cyt *c*(Fe^{+2}), leads to the formation of multimeric forms of N-homocysteinylated cytochrome *c* that are observed on nonreducing SDS−PAGE gels (*5*). Interestingly, oxidation of extensively N-homocysteinylated cytochrome *c* with equimolar ferricyanide leads to a transient formation of the ferric form, which is subsequently reduced back to the ferrous form by an N-linked Hcy thiol of the modified cytochrome *c*.

The present study shows that *N*-Hcy-cytochrome *c* is more resistant to proteolytic degradation by trypsin, chymotrypsin, and Pronase than the native counterpart. The increased resistance of *N*-Hcy-cytochrome *c* to proteolysis could result from the conversion of ferricytochrome *c* to a more thermodynamically stable ferrocytochrome *c*, known to be more resistant to protelysis (by proteinase K) than ferricytochrome *c* (*34*). However, N-homocysteinylation can also increase resistance of cytochrome *c* to proteolysis, independently of the structural transition associated with the change of its redox status, as has been shown for *N*-Hcy-albumin (*33*). It is believed that the pathophysiological processes of aging and the development of certain human diseases may be caused by inefficient removal of damaged proteins (*35*). *N*-Hcy-protein may represent a novel class of damaged proteins that are resistant to proteolysis (ref *33* and this work) and may also fail to be efficiently removed by the human body's proteolytic systems, thereby causing pathophysiological effects.

N-Homocysteinylation appears not to cause any major secondary structure changes in proteins. We did not find evidence in CD spectra for any significant changes in the conformation of the main polypeptide chain of cytochrome *c* in response to N-homocysteinylation. This may not be surprising, given that cytochrome *c* is known to be a very stable protein, and even high temperature does not change its secondary structure (transition temperature of ferrocytochrome *c* 100.6 ± 0.3 °C) (*36*). Moreover, the structure of the reduced cytochrome *c* is much more stable toward unfolding than the oxidized form, mainly due to the enhanced bonding between heme iron and Met80 in ferrocytochrome *c* (*37*). Similarly, no gross structural changes in N-homocysteinylated forms of LDL (*38*), myoglobin, transferrin, and ferritin (Perła-Kaján, unpublished data) were observed.

In conclusion, our data show that N-homocysteinylation of susceptible lysine residues in cytochrome *c* has important structural and functional consequences, manifested by increased resistance to proteolysis and change in iron redox state. A thiol of the N-linked Hcy introduced by N-homocysteinylation changes the redox state of the heme ligand of cytochrome *c* by rendering it reduced. Our data suggest that N-homocysteinylation may interfere with the function of heme-containing proteins.

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