Protein Science (1994), *3*:428–434. Cambridge University Press. Printed in the USA.

The primary structure and properties of thioltransferase (glutaredoxin) from human red blood cells

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(RECEIVED October 29, 1993; ACCEPTED January 10, 1994)

Abstract

Thioltransferase (glutaredoxin) was purified from human red blood cells essentially as described previously (Mieyal JJ et al., 1991a, *Biochemistry 30*:6088–6097). The primary sequence of the HPLC-pure enzyme was determined by tandem mass spectrometry and found to represent a 105-amino acid protein of molecular weight 11,688 Da. The physicochemical and catalytic properties of this enzyme are common to the group of proteins called glutaredoxins among the family of thiol:disulfide oxidoreductases that also includes thioredoxin and protein disulfide isomerase. Although this human red blood cell glutaredoxin (hRBC Grx) is highly homologous to the 3 other mammalian Grx proteins whose sequences are known (calf thymus, rabbit bone marrow, and pig liver), there are a number of significant differences. Most notably an additional cysteine residue (Cys-7) occurs near the N-terminus of the human enzyme in place of a serine residue in the other proteins. In addition, residue 51 of hRBC Grx displayed a mixture of Asp and Asn. This result is consistent with isoelectric focusing analysis, which revealed 2 distinct bands for either the oxidized or reduced forms of the protein. Because the enzyme was prepared from blood combined from a number of individual donors, it is not clear whether this Asp/Asn ambiguity represents inter-individual variation, gene duplication, or a deamidation artifact of purification.

Keywords: erythrocyte; glutaredoxin; glutathione; human red blood cell; matrix-assisted laser desorption; primary sequence; tandem mass spectrometry; thioltransferase

Circulating red blood cells are exposed to oxidative stress by potentially toxic dietary constituents, drugs, and environmental chemicals and by toxic oxygen species formed during inflammatory reactions and ischemia/reperfusion events of cardiovascular trauma. Such oxidative stress may alter the redox status of sulfhydryl groups that are vital to the physiological functions of the RBCs. Thus, Hb has a reactive sulfhydryl group on each β-subunit (Cys-β93) whose modification alters O_2 and heme binding at the conformationally sensitive dimer interface (Makino & Sugita, 1982; Craescu et al., 1986). Similarly, oxidation of SH groups on phosphofructokinase inactivates this critical metabolic control enzyme (Valentine et al., 1987) and thereby interferes with energy production in RBCs. Disulfide crosslinking of SH groups on integral membrane proteins like spectrin (Snyder et al., 1988) alters RBC permeability and deformability (Wagner et al., 1988).

We isolated and purified a thioltransferase enzyme from human RBCs that has properties identifying it as a member of the superfamily of thiol:disulfide oxidoreductases, including glutaredoxins and thioredoxins. Coupled to GSSG reductase, the hRBC thioltransferase was shown to catalyze the GSH-dependent reduction of Hb*S*-*S*-glutathione mixed disulfide and the reductive reactivation of disulfide-modified phosphofructokinase in vitro (Mieyal et al., 1991a, 1991b). Later, Terada et al. (1992) also isolated this enzyme and reported evidence for thioltransferase-catalyzed regeneration of RBC membrane sulfhydryl groups that were modified by oxidation. Thus, the hRBC thioltransferase enzyme displays activities consistent with a physiological role in sulfhydryl homeostasis in RBCs. In further stud-

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Abbreviations: ACCA, α-cyano-4-hydroxycinnamic acid; ACN, acetonitrile; CID, collision-induced dissociation; DTT, dithiothreitol; E, *Escherichia coli*; FAB, fast atom bombardment; Grx, glutaredoxin; h, human; Hb, hemoglobin; IEF, isoelectric focusing; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MS, mass spectrometry; RBC, red blood cell; Trx, thioredoxin.

ies to dissect the mechanism of catalysis, we discovered that thioltransferase appears to be highly selective for glutathionyl mixed disulfide substrates. Catalysis of GSH-dependent dethiolation of GS-containing mixed disulfides displayed 2 substrate kinetic patterns consistent with a ping-pong mechanism involving a thioltransferase-SSG intermediate. This finding suggests potential physiological roles for thioltransferase beyond repair of oxidatively modified protein SH-groups (Gravina & Mieyal, 1993).

A high degree of homology has been noted among the primary amino acid sequences of a number of the mammalian thioltransferase and Grx enzymes (Hopper et al., 1989; Papayannopoulos et al., 1989), leading to the supposition that "thioltransferase" and "glutaredoxin" represent alternative names for the same family of enzymes (Mannervik et al., 1989). In order to show that the thioltransferase protein from hRBCs is actually a Grx by homology to the other known mammalian proteins and to characterize it further, it was of interest to determine its primary structure. Here we report a high degree of sequence homology among all the mammalian Grx's including the hRBC protein; however, unique amino acid differences distinguish the human Grx.

Results

The primary structure of human Grx isolated from erythrocytes was determined solely by tandem MS (Hopper et al., 1989). The protein was digested with a number of proteolytic enzymes, either separately or in combination, followed by partial fractionation of the resulting peptides by reversed-phase HPLC. The peptides in each HPLC fraction were ionized by fast atom bombardment, which generates protonated peptide ions, $(M + H)^+$, that are mass analyzed by the first section (MS-1) of a tandem mass spectrometer (Sato et al., 1987). Sequence information can then be obtained by using MS-1 to mass select individual peptides, as their protonated molecules $(M + H)^+$, in each fraction followed by fragmentation of the peptide via collision-induced dissociation with helium in the field-free region between MS-1 and the second mass spectrometer (MS-2). The CID fragments are mass-analyzed by MS-2 and the amino acid sequence can be derived from the resulting mass spectrum (Biemann, 1988, 1990).

Prior to the enzymatic digestion, the molecular weight of hRBC Grx was determined by using matrix-assisted laser desorption time-of-flight MS (Hillenkamp et al., 1991). Sinapinic acid was chosen as the matrix for the MALDI-TOF experiments because it provided the best signal intensity and resolution for the Grx at the 337-nm frequency generated by the N_2 laser. The resultant $(M + H)^+$ ions corresponding to the protonated Grx were mass analyzed by a time-of-flight mass spectrometer. The MALDI-TOF mass spectrum (shown in Fig. 1A) of the hRBC Grx exhibited singly and doubly charged $(M + H)^+$ ions of the protein molecule. The structure of these peaks (in contrast to the sharper and better resolved peaks of the horse heart myoglobin used as an internal standard) implied that the material was not homogeneous and that the major component had an M_r of 11,841 (the observed peak is the $(M + H)^+$ ion, which is 1 Da higher). Reduction with either triethylphosphine (Rügg & Rudinger, 1977) or dithiothreitol resulted in a decrease in the mass of the protein to 11,688 Da. The mass spectrum (shown in Fig. 1B) exhibits a somewhat broadened peak because the reduced Grx ionizes poorly (i.e., requires a higher laser power).

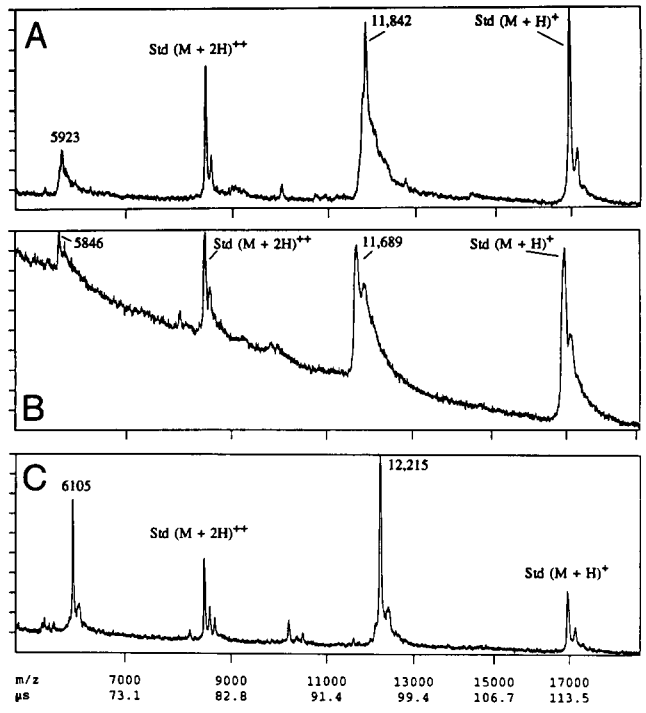

Fig. 1. MALDI-TOF mass spectra of hRBC Grx. The ions labeled Std $(M + H)^+$ and Std $(M + 2H)^{++}$ at m/z 16,952.5 and m/z 8,476.8, respectively, are due to the internal standard (horse heart myoglobin). **A:** Mass spectrum of the Grx as isolated by the procedure described in the Materials and methods. **B:** Mass spectrum of the native hRBC Grx after reduction with triethylphosphine. **C:** Mass spectrum obtained following reduction and *S*-ethylpyridylation of the native protein. The matrix was sinapinic acid and the low intensity signals adjacent to the major peaks are adducts of the matrix.

Based on this result, the heterogeneity of the original sample is attributed to the modification of some of the cysteines in the isolation and purification process (see Discussion). For digestion, the reduced hRBC Grx was *S*-alkylated (in 2 separate experiments) with 4-vinylpyridine and iodoacetamide, respectively. A MALDI-TOF mass spectrum of the modified proteins was obtained after purification by reversed-phase HPLC. The mass spectrum of the *S*-ethylpyridylated hRBC Grx shown in Figure 1C now exhibits a single sharp peak for the $(M + H)^+$ ion. The alkylated Grx ionized as easily as the original material, which provides additional support for the hypothesis that the cysteines of the native protein had been at least partly modified. The M_r (determined by MALDI-TOF-MS) of both the ethylpyridylated and the carbamidomethylated Grx increased to 12,214 for the former and 11,973 for the latter, respectively. Based on the M_r of the reduced protein (11,688), these increases in mass correspond to 526 Da and 285 Da, respectively, which correlate well with the calculated values for the alkylation by 5 ethylpyridyl groups (525 Da) or 5 carbamidomethyl groups (285 Da).

In order to determine the primary structure of the human erythrocyte Grx, *S*-carbamidomethylated protein was first digested with trypsin. After partial fractionation by reversed-phase HPLC, the molecular weight and amino acid sequence of the tryptic peptides were determined by FAB-MS and tandem MS, respectively. Three of the tryptic peptides $(M + H)^+ = m/z$

2,309, *m/z* 2,854, and *m/z* 3,292 were too large to be unambiguously sequenced by tandem MS and were further digested with endo-Asp-N. The sequence of the resulting peptides permitted (together with overlapping sequences derived separately from chymotryptic peptides) the assembly of the sequences of these 3 tryptic peptides. In another experiment, *S*-ethylpyridylated Grx was digested with α-chymotrypsin, and the resulting peptides were partially fractionated by reversed-phase HPLC. The molecular weights of the chymotryptic peptides were determined by FAB-MS and their sequences deduced from the CID spectra.

The complete amino acid sequence of hRBC Grx derived from the peptides generated by the various enzymatic digests of the protein is shown in Figure 2. The position of each peptide in the final sequence was determined from the overlapping sequences generated by the tryptic and chymotryptic enzymatic digests of hRBC Grx. The digestion of the *S*-ethylpyridylated Grx with endo-Lys-C aided this process by producing larger peptides, which confirmed the alignment deduced from the tryptic and chymotryptic peptides. Lys and Gln, which have the same nominal mass, were identified based on the specificity of endo-Lys-C to differentiate between these 2 amino acids or alternatively, by acetylation, which adds 42 Da only to Lys and the N-terminal amino group, but not to Gln.

As shown in Figure 2, position 51 was identified as both Asp and Asn. This result indicates that the essentially pure enzyme preparations contained a mixture of 2 proteins that should be distinguishable by isoelectric focusing. Accordingly, hRBC Grx at various states of purification was subjected to agarose IEF. The gel was sliced into fractions, the proteins were eluted into buffer, and fractions containing thioltransferase activity were identified. Both oxidized and reduced forms of hRBC thioltransferase displayed 2 pI peaks: pH 7.5 and 8.8, and pH 7.1 and 8.5, respectively. Like rat liver thioltransferase (Gan & Wells, 1986) and unlike the pig liver enzyme (Gan & Wells, 1987), hRBC thioltransferase showed only a small shift in pI (pH 7.1–7.5, and pH 8.5–8.8) upon oxidation. The lower and higher pH forms of thioltransferase activity were not interconvertible by oxidizing and reducing agents, suggesting they might be isoforms of the enzyme. Western blot analysis confirmed that the 2 pI forms of activity were recognized by antibodies raised against pure hRBC thioltransferase (see Materials and methods). In separate experiments the pure protein was subjected to isoelectric focusing under denaturing conditions. Two pI forms were observed at pH values corresponding to those predicted for the primary sequence shown in Figure 2 with either Asp or Asn at position 51 (pI values *observed*: 7.7 ± 0.2 and 8.15 ± 0.05 [$n = 2$]; pI values *predicted*[3]: sequence with Asp-51: 7.74; sequence with Asn-51: 8.15).

Discussion

The Grx isolated from human erythrocytes is highly homologous to the other mammalian Grx's of known primary structure (Hopper et al., 1989; Papayannopoulos et al., 1989; Yang et al., 1989). Like the Grx's from other mammalian species, hRBC Grx also contains an acetylated alanine at the N-terminus, which was determined from the CID mass spectrum of the N-terminal tryptic peptide $(M + H)^+ = m/z$ *1,085.7*[4] from *S*-ethylpyridylated protein shown in Figure 3. The presence of the b_1[5] ion, *m/z 114.0*, indicated that acetylated alanine (rather than Leu or Ile, which have the same mass) is at the N-terminus because b_1 ions in CID mass spectra of peptides with a free (basic) N-terminal

[3] PC Gene software program.

[4] The *m/z* values in italics denote monoisotopic mass, i.e., the sum of ^{12}C, ^{1}H, ^{14}N, ^{16}O, etc., isotopes only. This distinguishes these measurements from polyisotopic *m/z* measurements indicated in roman type.

[5] For an explanation of the notation used in the interpretation of CID mass spectra of peptides see Biemann (1988) or Papayannopoulos and Biemann (1992). A scheme of the notation is also included on the Diskette Appendix.

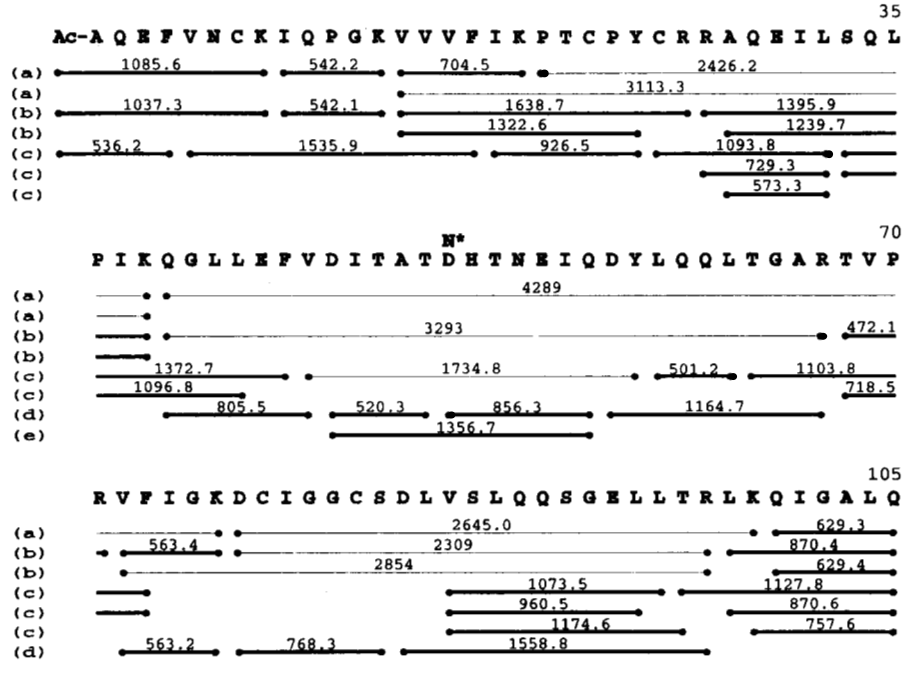

Fig. 2. Amino acid sequence of hRBC Grx. **a**, Endo-Lys-C peptides; **b**, tryptic peptides; **c**, chymotryptic peptides; **d**, peptides resulting from digestion of tryptic HPLC fractions with endo-Asp-N; **e**, an additional peptide from tryptic/endo-Asp-N digestion having Asn instead of Asp as indicated by the asterisk. The $(M + H)^+$ value (obtained by FAB-MS) is displayed above the line segments representing the peptides. Heavy underlining denotes sequence data obtained from CID mass spectra. Light underlining represents peptides for which the molecular weight was determined.

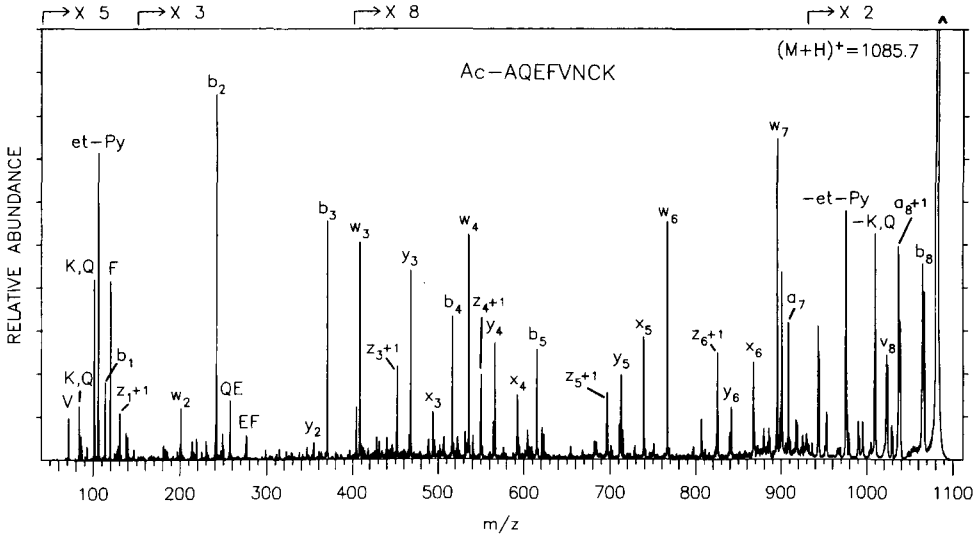

Fig. 3. CID mass spectrum of the N-terminal tryptic peptide $(M + H)^+ = 1,085.7$ from hRBC Grx.

amino acid are generally not observed (Hopper et al., 1989). In addition, the absence of Leu or Ile is also supported by the lack of a significant peak at *m/z 86*, the immonium ion of these 2 isomeric amino acids.

Despite the similarities to the other Grx's, there are a number of differences. Of particular interest is the discovery of an additional Cys near the N-terminus in the human Grx. As evident in Figure 3, the N-terminal tryptic peptide contains an abundant ion at *m/z 106.0* corresponding to the ethylpyridyl group and a high mass ion at *m/z 979.2*, which arises from the loss of the ethylpyridyl moiety from the $(M + H)^+$ ion. Because other mammalian Grx's do not contain Cys near the N-terminus, several experiments were carried out to confirm this finding further. A MALDI-TOF mass spectrum of an endo-Lys-C digest solution of *S*-ethylpyridylated Grx confirmed the peptide molecular weights determined by FAB-MS. One of the ions present in the mass spectrum of this digest was *m/z* 1,086, which is equivalent to the monoisotopic ion *m/z 1,085.7* (obtained by FAB-MS) for the N-terminal tryptic peptide of *S*-ethylpyridylated Grx shown in Figure 3. Conversely, a MALDI-TOF mass spectrum obtained from an endo-Lys-C digest solution of the native protein reduced with triethylphosphine did not contain the *m/z* 1,086 ion but instead exhibited a new peak at *m/z* 981. The mass difference of 105 Da between *m/z* 1,086 and *m/z* 981 corresponds to the addition of 1 vinylpyridine molecule to the Cys side chain. Finally, tryptic digestion of *S*-carbamidomethylated Grx gave an analogous ion $(M + H)^+ = m/z\ 1,037.3$, and the subsequent CID mass spectrum of this peptide provided additional confirmatory evidence for the deduced sequence.

The unique occurrence of an additional Cys residue near the N-terminus (Cys-7) in the human enzyme could be an incidental silent mutation or a functionally significant change. This "extra" Cys might serve to facilitate protein-protein disulfide association of thioltransferase with other sulfhydryl proteins while retaining catalytic activity at its active site (Cys-22/Cys-25). For example, such interprotein disulfide formation could anchor the human Grx to the RBC membrane as a first line of defense at the interface with the oxidizing extracellular milieu. It is interesting in this context that human thioredoxin also has a fifth cysteine residue compared to the other mammalian Trx (calf thymus) that has been sequenced. In recent studies this "extra" Cys (Cys-68) was found to facilitate disulfide dimerization of hTrx without affecting its catalytic activity (Ren et al., 1993), and Wollman et al. (1988) have suggested that an equilibrium between monomeric reduced hTrx and dimeric oxidized hTrx may exist physiologically. Previously we observed dimer and monomer forms of hRBC Grx via Western blot analysis of the partially purified enzyme, and the immunoreactive 23-kDa (dimer) band disappeared upon treatment of the preparation with DTT and iodoacetate (Gravina, 1993).

Although the human Grx contains a His, its position is not homologous with His-89 in the Grx's from pig liver and calf thymus. The CID mass spectrum of the tryptic/endo-Asp-N peptide $(M + H)^+ = m/z\ 856.3$ (shown in Fig. 4) exhibits an abundant ion at *m/z 110.3*, the immonium ion of His. The a_2 and b_2 ions (*m/z 225.4* and *m/z 253.4*, respectively) correspond to a peptide with Asp and His at the amino-terminus. The Asp was identified as the N-terminus of this peptide (finally assigned to position 51) on the basis of the specificity of endo-Asp-N and the presence of the y_6 and $z_6 + 1$ ions (*m/z 741.4* and *m/z 725.4*, respectively). Thus, His is the second residue in this peptide and was finally assigned to position 52 in the final amino acid sequence. Another peptide generated from the tryptic/endo-Asp-N digest, $(M + H)^+ = m/z\ 1,356.7$, provided confirmatory evidence for the position of His in the final sequence of the human Grx. This peptide also revealed a microheterogeneity in the protein because Asn instead of Asp was identified at position 51 from the CID spectrum of this peptide. Further confirmation of this finding came from the fact that the measured *m/z* of this peptide was *0.9* Da less than that expected from a peptide with an Asp at position 51 that resisted enzymatic cleavage. (The residue masses of Asp and Asn are *115.0* and *114.0* Da, respectively.) Finally, the assignment of Asn rather than Asp (except at the N-terminus) in this peptide is consistent with the fact that endo-Asp-N cleaves at the peptide bond N-terminal to Asp but not at Asn.

Due to the very close homology exhibited by all of the known mammalian Grx's, the discovery of both Asp and Asn at the same position (51) in the human Grx sequence was somewhat surprising. There are several conceivable explanations for the

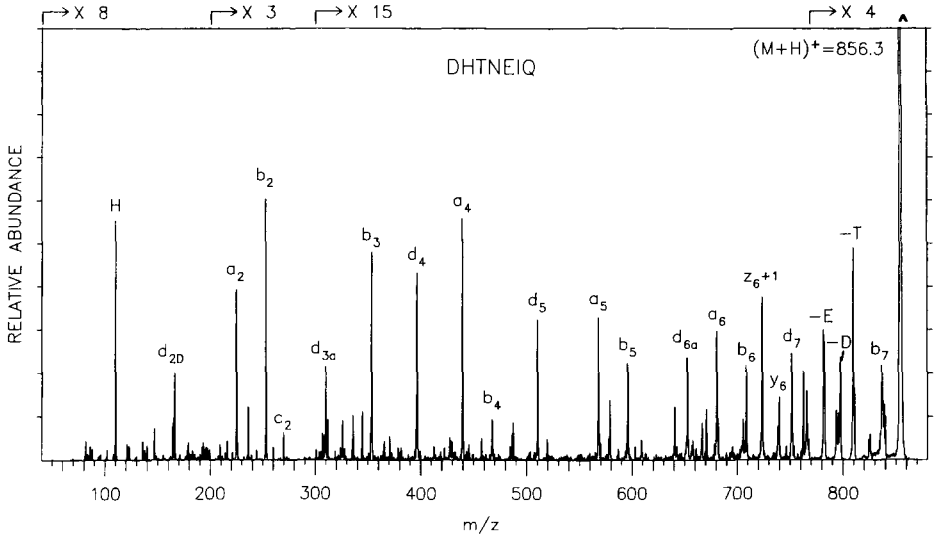

Fig. 4. CID mass spectrum of a His-containing tryptic/endo-Asp-N peptide $(M + H)^+ = 856.3$ from hRBC Grx. The peak labeled d_{2D} is a "secondary d_2 ion" (Johnson et al., 1988).

ambiguity at position 51. The fact that the 2 amino acids are Asp and Asn suggests that the observed Asp could have been generated artifactually by deamidation of the Asn presumably during isolation and purification of the protein. If this occurred, it would represent a unique reactivity at that site in the protein, because 11 other Asn and Gln residues were not affected, including a near neighbor Asn-54. Alternatively, because the purified protein was isolated from a mixture of blood from multiple individual donors, the occurrence of 2 amino acids at the same position could represent interindividual genetic variation. Gene duplication expressed in all individuals is also conceivable.

The human Grx is unique among the Grx's of known primary structure in the absence of Met at position 88, which is in agreement with the amino acid analysis and lack of CNBr reactivity, which indicated the absence of this amino acid (Mieyal et al., 1991a). The CID mass spectrum of the tryptic/endo-Asp-N peptide $(M + H)^+ = m/z\ 1{,}559.0$ (shown in Fig. 5) has a nearly complete w_n ion series. The b_5 ion and the abundant w_{10} ion (*m/z 528.6* and *m/z 1,085.7*, respectively) together identify Leu as the amino acid at position 88; the w_{10} ion also serves to differentiate it from Ile.

No tryptophan was detected by sequence analysis. This result supercedes the estimate of 2 Trp residues that was reported previously (Mieyal et al., 1991a) based on relative absorbance at 280 nm of dilute solutions of purified hRBC Grx and *Escherichia coli* Trx in 6 M guanidine HCl (ETrx has 1 Trp residue). The reason for the erroneous 280 nm absorbance that suggested Trp content in hRBC Grx is not known.

Potential difficulties in amino acid assignment by simple backbone cleavage are presented when the nominal masses are equal as in the case of the isomeric Ile and Leu (nominal mass of 113) and the isobaric Lys and Gln (nominal mass of 128). The identity of all 21 Ile and Leu residues could be determined based on the d_n (Johnson et al., 1988) or w_n (Johnson et al., 1987) ions,

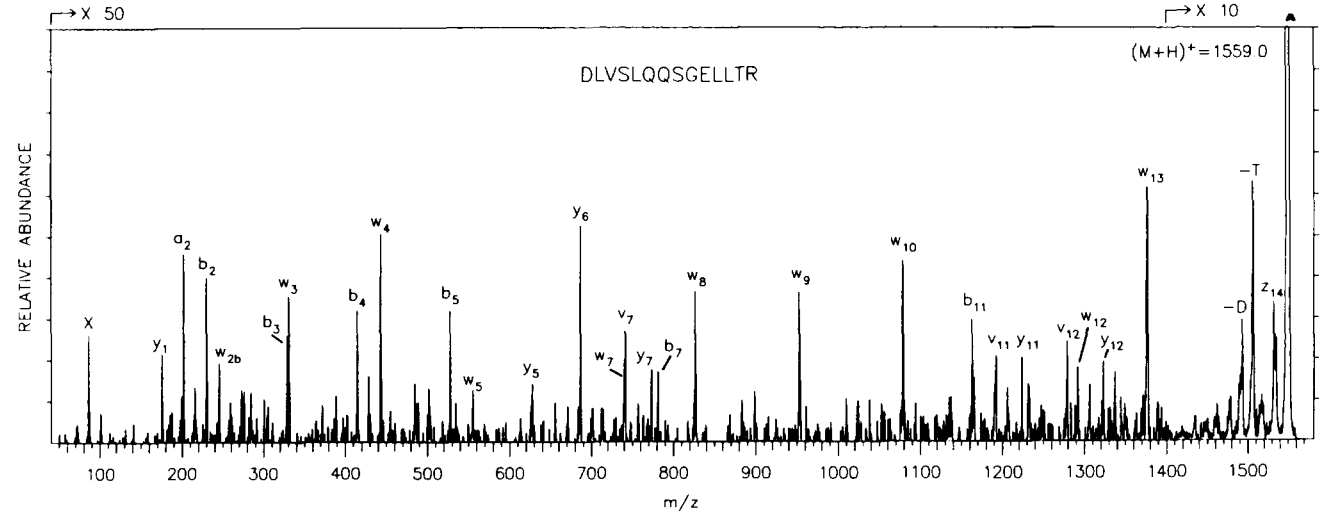

Fig. 5. CID mass spectrum of a tryptic/endo-Asp-N peptide $(M + H)^+ = 1{,}559.0$ from the hRBC Grx.

which involve cleavage of the side chains at the β,γ-carbon bond. As noted previously, Lys and Gln were differentiated on the basis of the specificity of endo-Lys-C. The only exception was the C-terminal Gln, which, of course, could not be assigned from the enzymatic specificity. It was identified by *N*-acetylation of the C-terminal tryptic peptide of *m/z 629.4*, which shifted to *m/z 671.4* due to reaction of the free N-terminal amino group, rather than to *m/z 713.4*, the value expected if a Lys was part of this molecule.

Finally, the experimentally determined M_r (see Results) of the reduced Grx (11,688) is in close agreement with the calculated M_r (11,686.6) based on the mass spectrometrically derived sequence (for Asn at position 51) of hRBC Grx. Similarly, the M_r (also determined by MALDI-TOF-MS) of the *S*-carbamidomethylated (11,973) and of the *S*-ethylpyridylated (12,214) proteins also agrees well with the calculated values of 11,971.9 and 12,212.3, respectively. These results establish the mass of the human Grx protein and clarify the ambiguity that existed previously because the molecular weight of human placental thioltransferase was estimated to be 6,500 Da (Larson et al., 1985).

It remains to be explained why the protein as isolated (see Materials and methods section) has a molecular weight higher than that of the native protein to which it can be converted by reducing agents. The reduction in mass by 153 Da corresponds to the conversion of 2 -S-S-CH_2-CH_2-OH groups to -SH (calculated difference: 2 × 76 = 152 Da). The shape of the peak in Figure 1A does not exclude the possibility that 1, 2, and 3 cysteines have reacted in a 1:2:1 ratio rather than 2 completely modified cysteines. Figure 6 provides a plausible explanation for this observation. Thus, treatment of the reduced enzyme with hydroxyethyldisulfide, which is done routinely at the last step of purification, could give the distribution of protein forms shown. This model is based on the supposition that Cys-7 would readily form a mixed disulfide, whereas the Cys-22/Cys-25 pair is known to be poised for intramolecular disulfide formation. Cys-78 and Cys-82 are interpreted to be less prone to oxidation and intramolecular disulfide formation, so that a mixture of 4 states may occur.

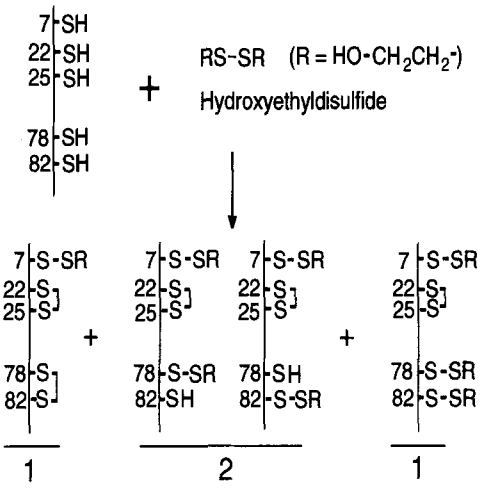

Fig. 6. Plausible explanation for the possibility that 1, 2, and 3 cysteines have reacted to form mixed disulfides with mercaptoethanol in a 1:2:1 ratio rather than 2 completely modified cysteines.

Materials and methods

Purification of hRBC thioltransferase (Grx)

The purification of hRBC thioltransferase to apparent homogeneity was described in detail by Mieyal et al. (1991a).

Anti-hRBC thioltransferase antibody preparation

Purified thioltransferase (200 μg) was mixed with Freund's complete adjuvant (50 μL thioltransferase solution/0.4 mL adjuvant), and the mixture was injected at 4 separate sites on the shaven, antiseptic back of a New Zealand White rabbit. Four months later a boost of 200 μg of pure thioltransferase mixed with Freund's incomplete adjuvant was injected, and 4 weeks later 50 mL of blood was collected. This regimen was repeated twice more at 4-month intervals with 50–100 μg of thioltransferase in Freund's incomplete adjuvant. At termination, 150 mL of serum was recovered from the rabbit.

Preparation of peptides for MALDI-TOF-MS

Approximately 1 nmol of *S*-ethylpyridylated hRBC Grx was digested with endo-Glu-C (100:1 substrate:enzyme) in 2 M urea, 100 mM ammonium acetate (pH 4.0) for 12 h at 37 °C. In addition, approximately 1 nmol of *S*-ethylpyridylated Grx was digested with endo-Asp-N in 2 M urea, 100 mM ammonium acetate (pH 7.8) for 12 h at 37 °C. Aliquots from the reaction solutions were diluted with MALDI matrix solution and analyzed (no HPLC fractionation) directly by MALDI-TOF-MS.

FAB-MS

FAB-MS of the proteolytic peptides was performed utilizing the first section (MS-1) of a tandem high resolution mass spectrometer (JEOL HX110/HX110). Single scans were acquired at a scan speed of 2.2 min to scan the range from *m/z* 100 to 6,000. The resolution was approximately 1:3,000 using 300-Hz filtering and an accelerating voltage of 10 kV. Under these conditions the isotope multiplets resolved at least up to *m/z* 3,000. The *m/z* values noted therefore correspond to the monoisotopic mass shown in italics (see Footnote 4). The cesium gun was operated at 20–25 kV.

Tandem MS

The first mass spectrometer (MS-1) of the tandem instrument of $E_1B_1E_2B_2$ configuration (JEOL HX110/HX110) was used to mass select individual peptides as their ^{12}C-only protonated molecules, $(M + H)^+$. CID with He occurred in the field-free region between MS-1 and the second mass spectrometer (MS-2), and the resulting fragments were mass analyzed by MS-2 using linked scans (0.8 min from *m/z* 50 to 2,000). The resolution of both instruments was set to 1:1,000 (i.e., static resolution of MS-1 is 1:2,000; dynamic resolution of MS-2 is 1:1,000). Because all CID fragment ions are derived from the ^{12}C-only precursor ion they are monoisotopic and the corresponding *m/z* values are shown in italics (see Footnote 4). Both MS-1 and CID profile scans were recorded with a JEOL DA5000 data system.

MALDI-TOF-MS

Matrix solutions of either sinapinic acid (3,5-dimethoxy-4-hydrocinnamic acid) or α-cyano-4-hydroxycinnamic acid were prepared in mixtures of acetonitrile and H_2O. The sinapinic acid solution was a saturated solution prepared in 30% ACN, whereas the ACCA solution was approximately 10 g/L in 50% ACN. Sample preparation for MALDI-TOF-MS involved dilution of the intact Grx or Grx digest solution with one of the matrix solutions to give a final sample concentration of typically between 1 and 10 pmol/μL. About 1 μL of this solution was applied to the probe target and allowed to evaporate. MALDI-TOF mass spectra were acquired using a modified Vestec VT2000 LD-TOF (Vestec Corp., Houston, Texas) and a Laser Science N_2 laser (LSI, Inc., Cambridge, Massachusetts). The N_2 laser (337 nm) has a pulse width of 8 ns and was operated at a repetition rate of 5 Hz. Laser irradiation of the sample produced protonated (mostly singly and doubly charged) ions that were analyzed by the TOF mass spectrometer using 30 kV accelerating voltage. The resolution is generally between 1:100 and 1:300, which is not sufficient to resolve isotopic multiplets. The resulting *m/z* values are thus derived from the polyisotopic envelope at full-width of the peak at half height and printed in normal type (see Footnote 4).

Supplementary material on Diskette Appendix

Additional Materials and methods text, hRBC Grx IEF data, and homology of hRBC Grx to other mammalian Grx's of known primary structure can be found on the Diskette Appendix. Tables (1S, 2S, and 3S) containing mass spectrometric data for peptides generated by tryptic, chymotryptic, and endo-Lys-C, respectively, digestion of hRBC Grx are also included.

Acknowledgments

This work was supported by research grants from the National Institutes of Health (GM 05472 and RR 00317 to K.B.) and from the American Heart Association (#91014450 to J.J.M.). This publication is also identified with the Protein Group at Case Western Reserve University.

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