Structural and Functional Characterization of the Mutant *Escherichia coli* Glutaredoxin(C14→S) and Its Mixed Disulfide with Glutathione

John H. Bushweller,[‡] Fredrik Åslund,[§] Kurt Wüthrich,*[,‡] and Arne Holmgren[§]

Department of Biochemistry, The Medical Nobel Institute, Karolinska Institute, S-10401 Stockholm, Sweden, and Institut für Molekularbiologie and Biophysik, ETH-Hönggerberg, CH-8093 Zürich, Switzerland

Received April 17, 1992; Revised Manuscript Received June 23, 1992

ABSTRACT: Glutaredoxin is essential for the glutathione (GSH)-dependent synthesis of deoxyribonucleotides by ribonucleotide reductase, and in addition, it displays a general GSH disulfide oxidoreductase activity. In *Escherichia coli* glutaredoxin, the active site contains a redox-active disulfide/dithiol of the sequence Cys11-Pro12-Tyr13-Cys14. In this paper, we have prepared and characterized the Cys14→Ser mutant of *E. coli* glutaredoxin and its mixed disulfide with glutathione. The Cys14→Ser mutant of glutaredoxin is shown to retain 38% of the GSH disulfide oxidoreductase activity of the wild-type protein with hydroxyethyl disulfide as substrate but to be completely inactive with ribonucleotide reductase, demonstrating that dithiol glutaredoxin is the hydrogen donor for ribonucleotide reductase. The covalent structure of the mixed disulfide of glutaredoxin(C14S) with GSH prepared with ^{15}N-labeling of the protein was confirmed with nuclear magnetic resonance (NMR) spectroscopy, establishing a basis for NMR structural studies of the glutathione binding site on glutaredoxin.

The protein glutaredoxin is required as a hydrogen donor for ribonucleotide reductase for the glutathione-dependent synthesis of deoxyribonucleotides. *Escherichia coli* glutaredoxin also acts as a hydrogen donor for the reduction of adenosine 3′-phosphate 5′-phosphosulfate and methionine sulfoxide (Holmgren, 1989). In addition to its protein disulfide oxidoreductase activity, glutaredoxin catalyzes GSH[1]-disulfide oxidoreduction reactions with low molecular weight substrates such as *S*-sulfocysteine and β-hydroxyethylene disulfide (Holmgren, 1979a). Glutaredoxin catalyzes oxidoreduction reactions with quite a different specificity from that of thioredoxin, which is a well-characterized, quite general protein disulfide reductase (Holmgren, 1979b, 1985). The active site of glutaredoxin contains a redox-active disulfide with the sequence Cys11-Pro12-Tyr13-Cys14. Glutaredoxin has two glutathione-dependent enzymatic reactions that are readily assayed: reduction of the catalytic disulfides of ribonucleotide reductase and general reduction of low molecular weight disulfides. In an efford to determine whether the two types of reactions utilize the same or differing mechanisms, we chose to construct a mutant of *E. coli* glutaredoxin in which one of the cysteines was replaced with a serine, yielding a monothiol glutaredoxin. The recently completed structural studies of *E. coli* glutaredoxin (Sodano et al., 1991b; Xia et al., 1992) show, in agreement with previous alkylation studies (O. Björnberg and A. Holmgren, unpublished data), that C11 is exposed at the protein surface, whereas C14 is buried in the protein core, indicating that C11 is the nucleophile in disulfide reduction chemistry with glutaredoxin. For this reason, we chose to mutate C14 to S. Indeed, Yang and Wells (1991a,b) have recently made an analogous mutation in pig liver glutaredoxin and demonstrated that this mutant retained GSH disulfide reductase activity; however, they did not examine the protein disulfide reductase activity of their mutant.

In addition to conventional biochemical techniques for characterization of the mutant protein, we used a variety of NMR experiments with ^{15}N-labeled glutaredoxin(C14S) and its mixed disulfide with unlabeled GSH to confirm the covalent structure. These data established a platform for NMR studies of the glutathione binding site, which are currently in progress in our laboratories.

MATERIALS AND METHODS

Materials. The expression plasmid, pMG524, and its host, *E. coli* N4830 [F⁻, su⁻, his⁻, ilv⁻, gal⁺, Δ8(λ CI857, ΔBAM, ΔHI)], were gifts from Prof. Britt-Marie Sjöberg. *E. coli* strains CU9276 [hsdR17, mcrAB, recA1, Δ(lac-proAB), (F′traD36, proAB⁺, lacᵠZdeltaM15)] and CJ236 [dut1, ung1, thi-1, relA1/pCJ105(camʳF′)] were used for mutagenesis. Polynucleotide kinase, T4 DNA polymerase, T4 DNA ligase, and restriction endonucleases were from Boehringer Mannheim. T7 DNA polymerase for sequencing was from Pharmacia and was used according to the manufacturer's instructions. SDS–polyacrylamide and isoelectric focusing gel electrophoreses were carried out using the Phastgel system of Pharmacia, employing 8–25% gradient SDS gels and IEF 4–6.5 gels. DTT, NADPH, glutathione, glutathione reductase, iodoacetamide, and iodoacetic acid were from Sigma. DE-52 anion-exchange resin was from Whatman. Sephadex G-50 and G-25 for gel chromatography were from Pharmacia. DNA oligonucleotides were prepared by solid-phase DNA synthesis by Scandinavian Gene Synthesis AB. Ribonucleotide reductase subunits B1 and B2 were gifts from Prof. Britt-Marie Sjöberg. All other reagents were of analytical grade.

Mutagenesis. The wild-type glutaredoxin expression plasmid, pAHOB-1 (Björnberg & Holmgren, 1991), was digested with *Eco*R1 and *Hin*dIII to give a 0.5-kb fragment containing

‡ ETH-Hönggerberg.

§ Karolinska Institute.

[1] Abbreviations: COSY, 2-dimensional correlated spectroscopy; 2QF, 3QF, 2-quantum filtered, 3-quantum filtered; CT-TR, calf thymus thioredoxin reductase; DTT, dithiothreitol; GSH, reduced glutathione; GSSG, oxidized glutathione; Grx, glutaredoxin; Grx(C14S), mutant *E. coli* glutaredoxin with C14 replaced by S; HED, β-hydroxyethylene disulfide; KP_i, potassium phosphate; NMR, nuclear magnetic resonance; NOESY, 2-dimensional nuclear Overhauser enhancement spectroscopy; PAPS, adenosine 3′-phosphate 5′-phosphosulfate; PMSF, phenylmethanesulfonyl fluoride; ppm, parts per million; RR, ribonucleotide reductase; TOCSY, 2-dimensional total correlation spectroscopy.

0006-2960/92/0431-9288$03.00/0

the glutaredoxin gene. This fragment was purified by agarose gel electrophoresis, recovered, and ligated into identically prepared M13mp19. This construction inserts the glutaredoxin gene in the antisense orientation. Mutagenesis was then carried out according to the method of Kunkel (1985; Kunkel et al., 1987) as described by Maniatis et al. (1989) employing a uracil-containing template. A mutagenic oligonucleotide of sequence 5′-CCT TAC TCT GTG CGT GCA-3′ with a single mismatch was designed and prepared by solid-phase DNA synthesis and used without further purification. This oligonucleotide was designed to replace C14 of wild-type glutaredoxin with a serine. Mutant plaques were screened by sequencing. The mutant phages were passaged a second time through *E. coli* CU9276 and sequenced again to confirm the mutation.

Construction of the Expression Vector for Mutant Glutaredoxin. The mutant M13mp19grx RF DNA was digested with *Eco*R1 and *Hin*dIII, and the resulting fragment was purified by agarose gel electrophoresis, recovered, and ligated into the identically prepared pMG524 plasmid. The resulting pMG524Grx(C14S) plasmid was then transformed into *E. coli* N4830. Crude extracts from cultures of several colonies were checked for protein expression by SDS–polyacrylamide gel electrophoresis. The plasmid from one colony was isolated and sequenced to reconfirm the mutation and check the integrity of the remainder of the gene.

Expression and Purification of Grx(C14S). The mutant protein was expressed and purified as described for the wild-type protein (Björnberg & Holmgren, 1991), except that 1 mDTT was added to all of the chromatography buffers.

Expression of ^{15}N-Labeled Grx(C14S). To produce uniformly ^{15}N-labeled protein, the plasmids pMG524GrxC14S and pNF2690 were transformed into the wild-type cell line C1a and grown with ampicillin and kanamycin selection on minimal media with $(^{15}NH_4)_2SO_4$ as the sole nitrogen source (Sodano et al., 1991a).

Purification of Grx(C14S) and Preparation of the Mixed Disulfide with Glutathione. All operations were carried out at 4 °C. A crude extract was prepared by resuspending 48 g of X-pressed *E. coli* pMG524GrxC14S cells in 250 mL of 0.1 M Tris-HCl (pH 7.5)/5 mM EDTA/1 mM PMSF and mixing at high speed in a blender for three 1-min intervals. The crude extract was centrifuged for 10 min at 15000*g* in an SS34 rotor and the supernatant collected. To the 225 mL of supernatant was added 155 g of ammonium sulfate (85% saturation) over 1 h with stirring, followed by an additional 1.5 h of stirring. The precipitate was collected by centrifugation for 15 min at 20000*g* in an SS34 rotor. The pellet was redissolved in 70 mL of 30 mM KP_i (7.3)/3 mM EDTA and then dialyzed against two changes of 2 L of 30 mM KP_i (pH 7.3)/3 mM EDTA for 2.5 h each, followed by 2 L of 30 mM KP_i (pH 7.3)/3 mM EDTA/2 mM DTT overnight. The dialyzed solution was loaded onto a 6 × 13 cm column of DE-52 in 30 mM KP_i (pH 7.3)/3 mM EDTA/1 mM DTT at ca. 3 mL/min, followed by washing with 2 column volumes (ca. 700 mL) of 30 mM KP_i (pH 7.3)/3 mM EDTA/1 mM DTT. The protein was eluted with a linear gradient of 2 × 2 L of 30 mM KP_i (pH 7.3)/3 mM EDTA/1 mM DTT and 350 mM KP_i (pH 7.3)/3 mM EDTA/1 mM DTT at 2.5 mL/min. The active fractions were pooled and concentrated by ultrafiltration on a YM3 ultrafiltration membrane to 70 mL. The DE-52 pool was loaded onto a 6.2 × 140 cm column of Sephadex G-50 in 50 mM Tris-HCl (pH 7.0)/1 mM EDTA/1 mM DTT and eluted with the same buffer at 2.2 mL/min. The active fractions were pooled and concentrated by ultrafiltration. To a 15-mL aliquot of 1.8 mM Grx(C14S) was added fresh DTT to a final concentration of 3 mM, and the solution was stirred for 15 min. Subsequently, 135 mL of deoxygenated 50 mM Trix-HCl (pH 7.0)/1 mM EDTA was added, and the solution was concentrated by ultrafiltration under nitrogen to 20 mL (1.63 mM protein concentration). To prepare the GSH mixed disulfide, 5 mL of this protein solution was mixed with 4 mL of buffer and 1 mL of 0.5 M GSSG and the solution stirred at 4 °C for 30 min. At this point, the remainder of the protein was frozen and stored at −20 °C. The excess GSSG was removed by desalting on a 3.5 × 17 cm column of Sephadex G-25 in Tris-HCl (pH 7.0)/1 mM EDTA eluted at 1 mL/min. The protein fractions were then pooled and exchanged into 0.1 M NH_4Ac (pH 6.1) by slow addition of 5 volumes of NH_4Ac buffer followed by concentration to 30 mL. The modified protein was then loaded onto a 1.8 × 18 cm column of DE-52 in 0.1 M NH_4Ac (pH 6.1) and eluted at 0.9 mL/min with a linear gradient of 2 × 400 mL of 0.1 M NH_4Ac and 0.5 M NH_4Ac. The protein fractions were pooled and exchanged by ultrafiltration into 0.1 M KP_i (pH 7.0)/0.1 mM EDTA by ultrafiltration and then desalted on a 3.5 × 17 cm column of Sephadex G-25 in 0.1 M KP_i (pH 7.0)/0.1 mM EDTA. The active fractions were concentrated by ultrafiltration, frozen, and stored at −20 °C.

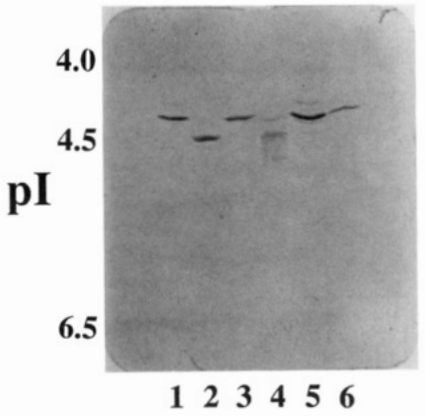

FIGURE 1: Isoelectric focusing gel of [^{15}N]Grx(C14S) treated with GSSG (lane 1), wild-type glutaredoxin (lane 2), grx(C14S) treated with iodoacetic acid (lane 3), grx(C14S) (lane 4), and Grx(C14S) treated with GSSG (lanes 5 and 6).

SDS and IEF Gel Electrophoresis. SDS–polyacrylamide gel electrophoresis was used with silver staining, as described by Pharmacia. For isoelectric focusing, grx(C14S) was treated with a 10-fold excess of iodoacetamide, iodoacetic acid, or, alternatively, 50 mM oxidized glutathione and desalted on a NAP-5 sizing gel column (Pharmacia). The IEF gels were stained with Coomassie as described by the manufacturer.

N-Terminal Sequencing. To further confirm the mutation, the N-terminal amino acid sequence of carboxymethylated grx(C14S) was determined on a gas-phase sequencer (Applied Biosystems) employing direct identification of PTH amino acids by on-line HPLC. The first 20 amino acids were determined and confirmed the mutation as well the integrity of the protein. We thank Prof. Uno Lindberg and Thomas Holt of the University of Stockholm for obtaining these data.

GSH Disulfide Oxidoreductase Assays and Activity Comparison. GSH disulfide reductase activity was determined with the HED assay of Holmgren (Holmgren, 1979a). An absorbance coefficient of 12 500 M^{-1} cm^{-1} (Björnberg & Holmgren, 1991) was used to determine the protein concentration of both wild-type and mutant proteins.

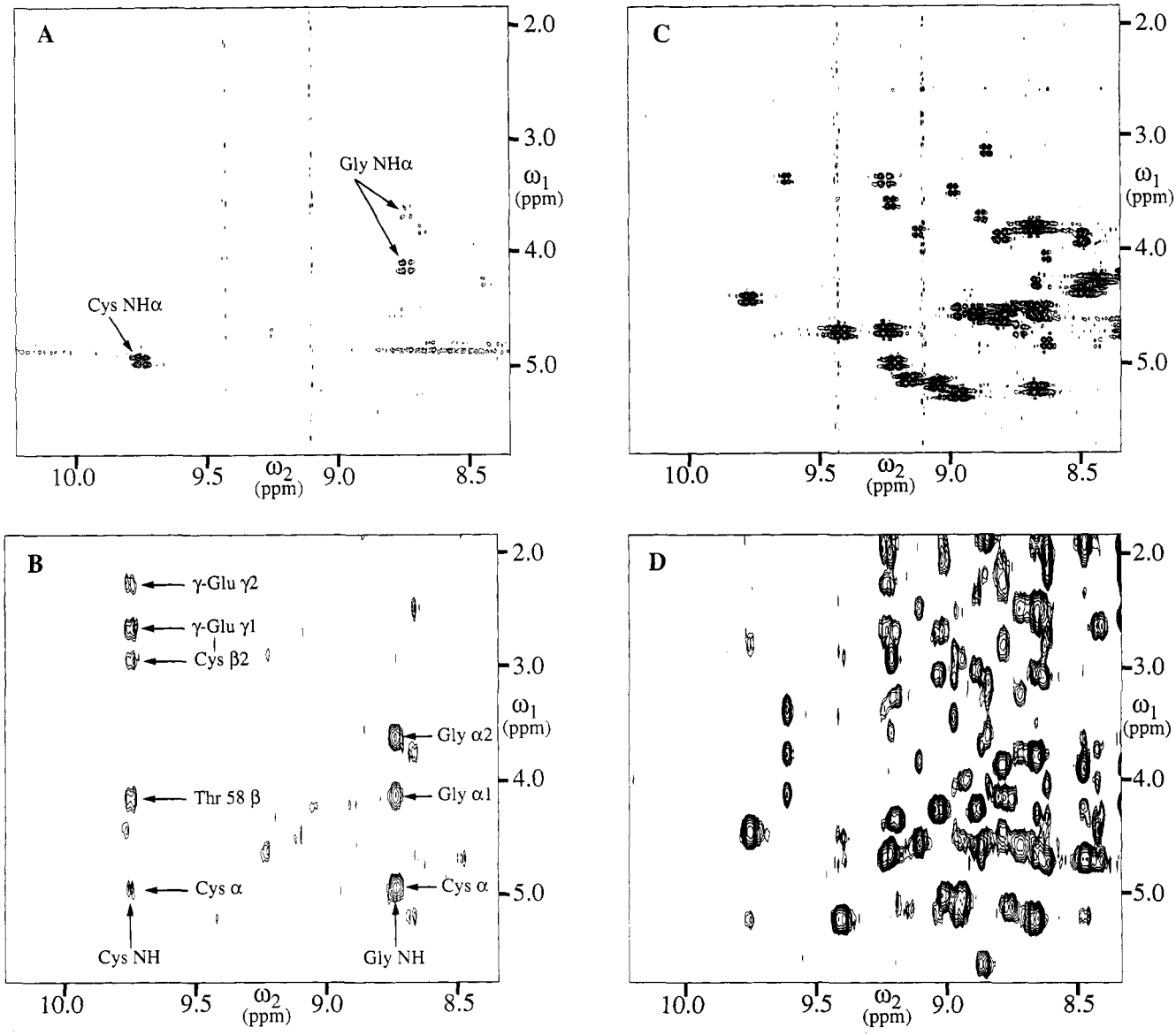

FIGURE 2: Fingerprint region (Wüthrich, 1986) of 2D ^{1}H NMR spectra of the mixed disulfide of uniformly ^{15}N-labeled Grx(C14S) and unlabeled GSH. (A, B) Subspectra from ^{15}N-(ω_2)-half-filtered 2D [^{1}H,^{1}H]-COSY and [^{1}H,^{1}H]-NOESY experiments, respectively, showing cross peaks between pairs of protons that are both not bound to ^{15}N, i.e. the glutathione amide protons and carbon-bound protons. (C, D) Subspectra from ^{15}N-(ω_2)-half-filtered 2D [^{1}H,^{1}H]-COSY and [^{1}H,^{1}H]-NOESY experiments showing cross peaks involving at least one ^{15}N-bound proton, i.e., between the amide protons of the protein and carbon-bound protons.

Ribonucleotide Reductase Assays. Assays for ribonucleotide reductase activity were performed as described by Reichard (1961) and Holmgren (1979a,b) using 4 mM glutathione with 1 mM NADPH and glutathione reductase, 1 mM DTT, or 330 nM CT-TR with 1 mM NADPH as reducing sources.

NMR Measurements. NMR measurements were carried out on Bruker AMX 500 and AMX 600 spectrometers. For assignment purposes, 2QF-COSY spectra (Rance et al., 1983), clean-TOCSY spectra (Griesinger et al., 1988) with a mixing time of 80 ms, and NOESY spectra (Anil Kumar et al., 1980) with a mixing time of 100 ms were recorded in both D_2O and H_2O. In addition, DQ spectra (Rance et al., 1989) were recorded in D_2O and H_2O, and a 3QF-COSY spectrum (Müller et al., 1986) was recorded in D_2O. COSY and NOESY experiments with ^{15}N(ω_2)-half-filter were recorded according to the method of Otting and Wüthrich (1990). All experiments were performed in the phase-sensitive mode with phase incrementation of the first pulse according to the method of States et al. (1982). In H_2O samples, the strong water signal was suppressed by selective saturation (Wider et al., 1983). The 2D NMR data sets were multiplied in both dimensions with phase-shifted sine-bell functions (DeMarco & Wüthrich, 1976). Baseline distortions were eliminated by a baseline correction with a third-order polynomial.

RESULTS AND DISCUSSION

Preparation and Biochemical Characterization of Grx(C14S) and Its Mixed Disulfide with GSH. The mutant glutaredoxin, Grx(C14S), prepared as described under Materials and Methods, and its mixed disulfide with GSH were found to be homogeneous preparations using several different criteria. The mixed disulfide was prepared from thoroughly reduced Grx(C14S) by treatment with an excess of oxidized GSH, followed by size-exclusion chromatography to remove the excess glutathione. To facilitate the removal of any remaining impurities, this step was carried out after the initial chromatography on DE-52 and Sephadex G-50 but before the final anion-exchange chromatography on DE-52 (for details see Materials and Methods). For an initial characterization, we examined isoelectric focusing gels of the mixed disulfide along with wild-type glutaredoxin and Grx(C14S)

alkylated with iodoacetamide or iodoacetic acid. The rationale was to first compare wild-type glutaredoxin and the mutant treated with iodoacetamide. Alkylation of the thiol with iodoacetamide removes the negative charge of the thiol and should therefore result in identical isoelectric points of the wild-type and mutant proteins. Comparison of the wild-type protein and Grx(C14S) alkylated with iodoacetamide did indeed show the isoelectric points to be identical (data not shown). Next, we compared the mixed disulfide of Grx(C14S) and GSH and Grx(C14S) treated with iodoacetic acid, which should also alkylate the free thiol. If, as expected, the mixed disulfide has only one glutathione attached, these species will show identical behavior since both iodoacetic acid and glutathione add one additional negative charge to the protein. As can be seen in Figure 1, this is the case. Isoelectric focusing of the mutant alone (Figure 1) does not give a single band due to oxidation of the protein under the conditions employed to run the gel.

In addition to confirmation of the protein sequence from DNA sequencing, the N-terminal polypeptide segment of 20 residues was sequenced. It was found to be identical to the wild-type protein with the exception of the residue at position 14, which was confirmed to be serine.

NMR Spectra of Grx(C14S) and the Mixed Disulfide with GSH. The three-dimensional NMR solution structures of reduced and oxidized *E. coli* Grx were recently determined (Sodano et al., 1991b; Xia et al., 1992). Using the fully assigned NMR spectra of the wild-type protein as a reference, corresponding spectra recorded with uniformly ^{15}N-labeled and unlabeled Grx(C14S) (not shown) not only presented additional evidence for the integrity of the mutant protein and the expected amino acid sequence but indicated further that the two proteins have very similar 3D structures, since identical ^1H chemical shifts were found for most corresponding residues (Wüthrich, 1986).

For the mixed disulfide formed with uniformly ^{15}N-labeled protein and unlabeled glutathione, the resonances of the glutathione were distinguished from those of the protein using homonuclear 2D ^1H NMR experiments recorded with a ^{15}N-(ω_2)-half-filter (Otting & Wüthrich, 1990). The fingerprint regions of the $^{15}N(\omega_2)$-half-filtered COSY and NOESY spectra are shown in Figure 2. Parts A and B of Figure 2 show the subspectra containing cross peaks between proton resonances that are not bound to ^{15}N, whereas parts C and D show those subspectra that contain exclusively peaks involving at least one ^{15}N-bound proton. Figure 2A shows only two COSY peaks in this region, which were attributed to Gly and to an AMX spin system from analysis of COSY, TOCSY, and DQ spectra. These resonances therefore correspond to the amide proton signals of the glycyl and cysteinyl residues of the glutathione. The $d_{\alpha N}$ NOE connectivity (Wüthrich, 1986) between the glycine NH at 8.7 ppm and the cysteine αH at 4.9 ppm is observed in Figure 2B along with the sequential NOEs of the cysteine NH at 9.7 ppm to the γ-methylene protons of the γ-glutamate of the glutathione at 2.3 and 2.7 ppm. The sequential connectivity to the γ-methylene protons led also to the elucidation of the γ-glutamate spin system via analysis of COSY, TOCSY, and 3QF-COSY spectra. The chemical shifts for glutathione in the mixed disulfide with Grx(C14S) are given in Table I. In addition, we have assigned all of the ^1H and ^{15}N resonances of Grx(C14S) in the mixed disulfide, thereby enabling us to proceed with the collection of data for a complete structure determination of the mixed disulfide.

Table I: ^1H Chemical Shifts of Glutathione in the Mixed Disulfide with Grx(C14S)

	chemical shift[a]			
residue	NH	αH	βH	others
γ-Glu		3.32	0.41, 1.80	γCH_2 2.32, 2.70
Cys	9.72	4.89	2.97, 4.22	
Gly	8.74	3.63, 4.10		

[a] The solution contained 2 mM Grx(C14S)-GS in 100 mM phosphate buffer at pH 6.5 and 20 °C. The chemical shifts are relative to internal sodium 2,2,3,3-tetradeuterio-3-(trimethylsilyl)propionate.

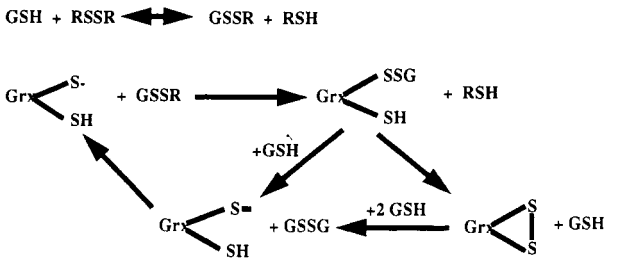

FIGURE 3: Proposed mechanism for the reduction of low molecular weight disulfides by glutaredoxin.

GSH Disulfide Oxidoreductase Activity of Grx(C14S). The GSH disulfide oxidoreductase activity was assayed using the HED assay of Holmgren (1979a). Grx(C14S) was shown to retain 38% of the activity of the wild-type protein. Earlier work (O. Björnberg and A. Holmgren, unpublished data) demonstrated that the preferred substrate of glutaredoxin in this assay is a mixed disulfide with glutathione, i.e., that there is a binding interaction between glutaredoxin and glutathione. On the basis of these observations, along with the fact that Grx(C14S) retains activity in this assay, we propose the mechanism depicted in Figure 3 for the GSH disulfide reductase activity of glutaredoxin. Clearly, Grx(C14S) will be able to support the reduction chemistry depicted in Figure 3, with the exception of the reoxidation of the protein to the disulfide form, possibly explaining the reduction in specific activity that was observed. This mechanism differs from that proposed by Yang and Wells (1991b) in that we propose an initial equilibration of glutathione and HED to form the mixed disulfide, which is thought to be the actual substrate for the reduction. This is quite reasonable on the basis of the enhanced rate of reduction observed with disulfides of glutathione relative to those not containing glutathione (O. Björnberg and A. Holmgren, unpublished data).

Protein Disulfide Reductase Activity of Grx(C14S). Since the mutant retains its GSH disulfide reductase activity, it was then of interest to examine the protein disulfide reductase activity. Since glutaredoxin has been identified as a hydrogen donor for ribonucleotide reductase, perhaps the primary donor (Holmgren, 1979c), we chose to examine the activity with this enzyme. The electron transfer from the hydrogen donor system (glutaredoxin or thioredoxin) to the active-site thiols of ribonucleotide reductase is known to be mediated via an outer pair disulfide (Aberg et al., 1989), so this provides an excellent test system for protein disulfide reduction by Grx(C14S). As seen in Figure 4, the mutant is inactive as a hydrogen donor for ribonucleotide reductase employing either GSH, NADPH, and glutathione reductase or DTT as the ultimate source of reducing equivalents. The inactivity of the mutant shows that both thiols of glutaredoxin are necessary for protein disulfide reduction.

The simplest mechanism one can envision for protein disulfide reduction of glutaredoxin involves the dithiol form of glutaredoxin reacting with the oxidized disulfide of the

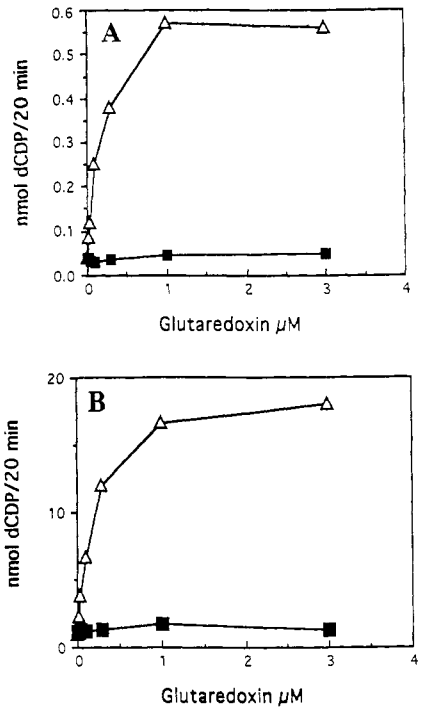

FIGURE 4: (A) Activity of wild-type and mutant glutaredoxin with ribonucleotide reductase employing 4 mM GSH, NADPH, and glutathione reductase as the source of reducing equivalents, with 1 μg of RR per assay: (△) Wild-type glutaredoxin; (■) Grx(C14S). (B) Activity of wild-type and mutant glutaredoxin with ribonucleotide reductase employing DTT as the source of reducing equivalents with 10 μg of RR per assay: (△) Wild-type glutaredoxin; (■) mutant glutaredoxin.

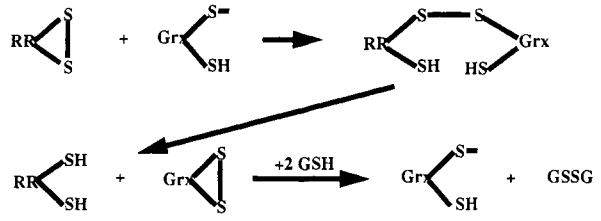

FIGURE 5: Proposed mechanism of protein disulfide reduction by glutaredoxin.

substrate as depicted in Figure 5. Clearly, this is the operative mechanism when DTT is employed as the ultimate source of reducing equivalents. However, the large amounts of glutathione present in vivo, as well as the inhibition of deoxyribonucleotide production with glutaredoxin as hydrogen donor that was observed with analogues of glutathione (Höög et al., 1982), require one to consider possible mechanisms involving glutathione. No difference in K_m was observed between the assays conducted with DTT or with GSH, NADPH, and glutathione reductase, so there appears to be no significant effect of GSH on the binding of glutaredoxin to ribonucleotide reductase. We therefore examined whether there was any effect on the overall rate due to the presence or absence of glutathione. Previous work has demonstrated that glutaredoxin can be reduced by calf thymus thioredoxin reductase in the absence of glutathione (O. Björnberg and A. Holmgren, unpublished data), which enabled us to examine the maximal rate in the absence of added thiol as well as in the presence of GSH. The rates under these two conditions were found to be identical (data not shown), so all presently available evidence indicates a dithiol mechanism not requiring GSH for protein disulfide reduction by glutaredoxin. Whether this mechanism will turn out to be generally true for protein disulfide reduction by glutaredoxin remains to be shown. Examination of the reactivity of the mutant with other protein substrates, such as PAPS-reductase, should answer this question. The highly conserved active-site sequence of glutaredoxins always contains two cysteines separated by two amino acids, so clearly the most vital reactions of glutaredoxin are dependent on both cysteines.

The enhanced rate of reduction of glutathione-containing disulfides (O. Björnberg and A. Holmgren, unpublished data), as well as the previously mentioned inhibition of deoxyribonucleotide production (Höög et al., 1982), must then be due to the aforementioned binding interaction of glutaredoxin with glutathione. Indeed, evidence has been shown for a binding interaction of glutathione with the glutaredoxin produced by the phage T4, and a binding site for GSH on T4 glutaredoxin has been proposed on the basis of modeling and mutagenesis studies (Nikkola et al., 1991). Grx(C14S) now provides an interesting opportunity to examine the binding site of glutathione on *E. coli* glutaredoxin, using NMR studies with isotope-labeled mixed disulfides with GSH (see above).

In conclusion, we have demonstrated that dithiol glutaredoxin is the relevant reductant for protein disulfide reduction and that glutathione is not required. In addition, we have prepared a mixed disulfide of Grx(C14S) with glutathione which should allow us to identify with NMR techniques the glutathione binding site on glutaredoxin and to identify the interactions responsible for this binding.

ACKNOWLEDGMENT

This investigation was supported by grants from the Swedish Medical Research Council (13X-3529), the Swedish Cancer Society (961), and the Schweizerischer Nationalfonds (31.25174.88). J.H.B. was supported by a postdoctoral fellowship from the U.S. National Institutes of Health. Special thanks are given to Barbro Ehn for technical assistance.

REFERENCES

Aberg, A., Hahne, H., Karlsson, M., Larsson, A., Ormö, M., Ahgren, A., & Sjöberg, B.-M. (1989) *J. Biol. Chem. 264*, 12249–12252.

Anil-Kumar, Ernst, R. R., & Wüthrich, K. (1980) *Biochem. Biophys. Res. Commun. 95*, 1–6.

Björnberg, O., & Holmgren, A. (1991) *Protein Expression Purif. 2*, 287–295.

DeMarco, A., & Wüthrich, K. (1976) *J. Magn. Reson. 24*, 201–204.

Griesinger, C., Otting, G., Wüthrich, K., & Ernst, R. R. (1988) *J. Am. Chem. Soc. 110*, 7870–7872.

Holmgren, A. (1979a) *J. Biol. Chem. 254*, 3664–3671.

Holmgren, A. (1979b) *J. Biol. Chem. 254*, 9113–9119.

Holmgren, A. (1979c) *J. Biol. Chem. 254*, 3672–3678.

Holmgren, A. (1985) *Annu. Rev. Biochem. 54*, 237–271.

Holmgren, A. (1989) *J. Biol. Chem. 264*, 13963–13966.

Höög, J., Holmgren, A., D'Silva, C., Douglas, K. T., & Seddon, P. (1982) *FEBS Lett. 138*, 59–61.

Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488–492.

Kunkel, T. A., Roberts, J. D., & Zakour, R. A. (1987) *Methods Enzymol. 154*, 367–382.

Müller, N., Ernst, R. R., & Wüthrich, K. (1986) *J. Am. Chem. Soc. 108*, 6482–6492.

Nikkola, M., Gleason, F., Saarinen, M., Joelson, T., Björnberg, O., & Eklund, H. (1991) *J. Biol. Chem. 266*, 16105–16112.

Otting, G., & Wüthrich, K. (1990) *Q. Rev. Biophys. 23*, 39–96.

Rance, M., Sørensen, O., Bodenhausen, G., Wagner, G., Ernst, R. R., & Wüthrich, K. (1983) *Biochem. Biophys. Res. Commun. 117*, 479–485.

Rance, M., Chazin, W. J., Dalvit, C., & Wright, P. (1989) *Methods Enzymol. 176*, 114–134.

Reichard, P., Baldesten, A., & Rutberg, L. (1961) *J. Biol. Chem. 236*, 1150–1157.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning*, Cold Spring Harbor Laboratory Press, Plainview, NY.

Sodano, P., Chary, K. V. R., Björnberg, O., Holmgren, A., Kren, B., Fuchs, J., & Wüthrich, K. (1991a) *Eur. J. Biochem. 200*, 369–377.

Sodano, P., Xia, T., Bushweller, J. H., Björnberg, O., Holmgren, A., Billeter, M., & Wüthrich, K. (1991b) *J. Mol. Biol. 221*, 1311–1324.

States, D. J., Haberkorn, R. A., & Ruben, D. J. (1982) *J. Magn. Reson. 48*, 286.

Wider, G., Hosur, R. V., & Wüthrich, K. (1983) *J. Magn. Reson. 52*, 130–135.

Wüthrich, K. (1986) *NMR of Proteins and Nucleic Acids*, Wiley, New York.

Xia, T., Bushweller, J. H., Sodano, P., Billeter, M., Björnberg, O., Holmgren, A., & Wüthrich, K. (1992) *Protein Sci. 1* (3), 310–321.

Yang, Y., & Wells, W. W. (1991a) *J. Biol. Chem. 266*, 12759–12765.

Yang, Y., & Wells, W. W. (1991b) *J. Biol. Chem. 266*, 12766–12771.

Registry No. GSH–disulfide oxidoreductase, 9068-62-6; ribonucleotide reductase, 9040-57-7.