Glutaredoxin-3 from Escherichia coli
AMINO ACID SEQUENCE, $^1$H AND $^{15}$N NMR ASSIGNMENTS, AND STRUCTURAL ANALYSIS*

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The primary and secondary structure of glutaredoxin-3 (Grx3), a glutathione-disulfide oxidoreductase from Escherichia coli, has been determined. The amino acid sequence of Grx3 consists of 82 residues and contains a redox-active motif, Cys-Pro-Tyr-Cys, typical of the glutaredoxin family. Sequence comparison reveals a homology (33% identity) to that of glutaredoxin-1 (Grx1) from E. coli as well as to other members of the thioredoxin superfamily. In addition to the active site cysteine residues, Grx3 contains one additional cysteine (Cys56) corresponding to one of the two non-active site (or structural) cysteine residues present in mammalian glutaredoxins. The sequence-specific $^1$H and $^{15}$N nuclear magnetic resonance assignments of reduced Grx3 have been obtained. From a combined analysis of chemical shifts, $^{3}$H NMR, coupling constants, sequential and medium range NOEs, and amide proton exchange rates, the secondary structure of reduced Grx3 was determined and found to be very similar to that inferred from amino acid sequence comparison to homologous proteins. The consequences of the proposed structural similarity to Grx1 are that Grx3, while possessing a largely intact GSH binding cleft, would have a very different spatial distribution of charged residues, most notably surrounding the active site cysteine residues and occurring in the proposed hydrophobic protein-protein interaction area. These differences may contribute to the observed very small (9–12 kDa), well characterized proteins capable of catalyzing thiol-disulfide exchange reactions. Representatives of at least one of these two protein families have been found in all organisms studied, indicating that proteins of this type are essential for cellular functions (for reviews, see Gleason and Holmgren (1988) and Holmgren (1989)). In the cell, glutaredoxin and thioredoxins differ in the manner they are reduced. Glutaredoxins are reduced via the ubiquitous tripeptide glutathione (GSH), whereas thioredoxins are reduced directly by the specific flavoenzyme thioredoxin reductase. In both cases, reducing equivalents are ultimately derived from NADPH. In vitro, thioredoxins are found to be general reductants of a number of different protein disulfides, whereas glutaredoxins are less capable in this respect, but are known to readily reduce mixed disulfides between proteins (or low molecular weight thiol-containing compounds) and GSH (Gravina and Mieyal, 1993). This activity is measured conventionally using the β-hydroxyethylene disulfide (HED) reduction assay (Holmgren, 1979a). Glutaredoxins can also reduce some specific protein disulfides, such as the redox-active disulfide of ribonucleotide reductase (Holmgren, 1979b; Bushweller et al., 1992).

Ribonucleotide reductases are key enzymes in the biosynthesis of deoxyribonucleotides. The aerobic classes of ribonucleotide reductases require the presence of a specific disulfide-reducing agent (e.g. Trx or Grx) for catalysis (for a recent review, see Reichard (1993)). Trx was thought to be unique in this role until a viable Escherichia coli mutant lacking Trx (trxA) was isolated, which eventually led to the discovery of Grx1 (Holmgren, 1976). The essential roles of Trx and Grx1 were then challenged through the viability of an E. coli trxA, Grx1 double mutant, A410 (Russel and Holmgren, 1988). From one such mutant, UC647 (Miranda-Vizuete et al., 1994), two novel E. coli glutaredoxins were identified, Grx2 and Grx3 (Åslund et al., 1994), and the gene for Grx3 was subsequently cloned. Grx3 was found to be an active, albeit inefficient, hydrogen donor for ribonucleotide reductase in comparison with Grx1.

Several three-dimensional structures of thioredoxins and glutaredoxins have been determined, the earliest being the crystal structure of oxidized E. coli thioredoxin (Holmgren et al., 1975). The first crystal structure of a glutaredoxin was that of glutaredoxin-1 of E. coli (Åslund et al., 1994). The structure of reduced Grx3 was determined and found to be very similar to that inferred from amino acid sequence comparison to homologous proteins. The consequences of the proposed structural similarity to Grx1 are that Grx3, while possessing a largely intact GSH binding cleft, would have a very different spatial distribution of charged residues, most notably surrounding the active site cysteine residues and occurring in the proposed hydrophobic protein-protein interaction area. These differences may contribute to the observed very small (9–12 kDa), well characterized proteins capable of catalyzing thiol-disulfide exchange reactions. Representatives of at least one of these two protein families have been found in all organisms studied, indicating that proteins of this type are essential for cellular functions (for reviews, see Gleason and Holmgren (1988) and Holmgren (1989)). In the cell, glutaredoxin and thioredoxins differ in the manner they are reduced. Glutaredoxins are reduced via the ubiquitous tripeptide glutathione (GSH), whereas thioredoxins are reduced directly by the specific flavoenzyme thioredoxin reductase. In both cases, reducing equivalents are ultimately derived from NADPH. In vitro, thioredoxins are found to be general reductants of a number of different protein disulfides, whereas glutaredoxins are less capable in this respect, but are known to readily reduce mixed disulfides between proteins (or low molecular weight thiol-containing compounds) and GSH (Gravina and Mieyal, 1993). This activity is measured conventionally using the β-hydroxyethylene disulfide (HED) reduction assay (Holmgren, 1979a). Glutaredoxins can also reduce some specific protein disulfides, such as the redox-active disulfide of ribonucleotide reductase (Holmgren, 1979b; Bushweller et al., 1992).

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of the phase T4 glutaredoxin (Söderberg et al., 1978). Together, these studies established the now typical thioredoxin/glutaredoxin poly peptide fold (Eklund et al., 1984). Subsequent studies by nuclear magnetic resonance (NMR) spectroscopy have revealed the structures of reduced and oxidized Grx1 (Sodano et al., 1991; Xia et al., 1992), and the structure of the mixed disulfide between Grx1 and glutathione, Grx1-SG (Bushweller et al., 1994). In the present work, we have determined the amino acid sequence of E. coli Grx3 and compared it to other members of the thioredoxin superfamily. As a first step toward characterizing the three-dimensional structure of this newest member of the glutaredoxin family, we have determined the sequence-specific 1H and 15N NMR assignments and the secondary structure elements of the reduced form of Grx3 in solution thereby supporting the proposed sequence alignments and the dose structural similarity of Grx3 to Grx1. Implications of this structural homology are discussed in light of the observed activities of Grx3.

**MATERIALS AND METHODS**

Protein Preparation for Sequence Analysis—E. coli Grx3 (150 μg) purified as described (Åslund et al., 1994), was reduced by incubation in 0.5 mM Tris-Cl, pH 8.0, for 4 h at 4 °C in the presence of 1 mM dithiothreitol (DTT). The reduction was carried out in a Microsep concentrator (3000Da cut-off; Filtron) during which time the material was concentrated to a final volume of 60 μl. Carboxymethylation was performed by the addition of 300 μl of neutralized 5 mM (15)iodoacetic acid (Amersham Corp., approximately 2400 cpmmol) in a solution containing 0.1 mM guanidine hydrochloride, 0.4 mM Tris-Cl, pH 8.0, 2 mM EDTA. The reaction was allowed to proceed for 4 h at 4 °C during continuous concentration. Reagents were removed by repetitive additions of 50 mM Tris-Cl, pH 7.5, followed by concentration. The resulting [15]Carboxymethylated Grx3 was desalted on a Vydac C4 reverse-phase HPLC column equilibrated in 0.1% (v/v) trifluoroacetic acid in water and eluted with a linear gradient of acetonitrile (0–40% during 60 min) containing 0.1% (v/v) trifluoroacetic acid at a flow rate of 1 ml/min.

Peptide Generation and Amino Acid Sequence Analysis—[15]Carboxymethylated Grx3 (5 nmol) was digested for 4 h at 37 °C with Lys-C endoprotease (Wako) at an enzyme to protein ratio of 1:10 in 0.1 M ammonium bicarbonate, pH 8.0, containing 1 mM guanidine hydrochloride. The resulting cleavage products were separated by reverse-phase HPLC as described above, and the peptide-containing fractions were labeled K1-K5. In a separate cleavage strategy, [15]carboxymethylated Grx3 (3 nmol) was treated with 70% (v/v) formic acid for 24 h at 37 °C to cleave Asp-Pro bonds. The cleavage products were also purified by HPLC and found to contain two peptides (F1 and F2).

Total guanine nucleotide compositions were determined by NMR analysis of purified protein or peptide samples for 24 h at 110 °C in evacuated tubes containing 6 mM HCl and 0.5% (w/v) phenol. Amino acid sequence analysis was performed using Edman degradation on the intact [15]carboxymethylated protein and the cleavage fragments using either a Milligen Prosequencer 6600 or an Applied Biosystems 470A sequencer.

Sequence homology between Grx3 and other glutaredoxins was probed with the aid of the computer program LASERGENE (Dnastar, Inc.) and the programs ALIGN, SSEARCH, and FASTA (Pearson, 1990).

Three-dimensional Modeling of Grx3—The three-dimensional structure of Grx3 was modeled using the previously determined NMR structures of 50 mM Tris-Cl (Sodano et al., 1991; Xia et al., 1992), and the Grx1-SG mixed disulfide complex (Bushweller et al., 1994). As the alignment presented in Fig. 2 leaves Grx3 with four residues not included in the Grx3 model, which therefore contains residues 1–79. The modeling of the oxidized form of Grx3 was performed using the program XEASY (Eccles et al., 1991; Bartels et al., 1995). Sequence-specific 1H and 15N resonance assignments were obtained for Grx3 at pH 6.8 and 28 °C using conventional methods (Wuthrich, 1986).

Secondary structure analysis using H-15N NMR chemical shifts was performed according to the technique of Wishart et al. (1992). Chemical shifts for reduced, unfolded Grx3 have been determined previously (Nordstrand et al., 1995). Expected 1H chemical shifts were calculated from the atomic positions of the Grx3 model according to the algorithm of Williamson et al. (1995).

**Insulin Disulfide Reduction Assay—Reduction of insulin disulfides was monitored spectrophotometrically as described previously (Luthman and Holmgren, 1982). Briefly, bovine pancreas insulin (Sigma, final concentration 1 mM) was added to cuvettes containing 0.5 mM 1 mM GSH, 0.2 mM NADPH, 10 μg/ml glutathione reductase, 1.0 mg/ml bovine serum albumin, and 50 mM Tris-Cl at pH 8.0. The reaction was started by the addition of 15N to a single, homonuclear two-dimensional total correlation spectroscopy spectrum was recorded after 1 h for a duration of 60 min. Amide protons still visible in this spectrum are classified as slowly exchanging (KHNH < 0.02 min⁻¹).

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**Ribonucleotide Reductase Activity—Ribonucleotide reductase activity was assayed essentially as described (Thelander et al., 1978; Holmgren, 1979b) by monitoring the conversion of [1H]CDP to [1H]dCDP by 10 μM of ribonucleotide reductase. Reducing equivalents were provided through 4 mM GSH, 1 mM NADPH, and 0.01 μg/ml glutathione reductase. Incubation reactions were performed in the presence of either 1 μM Grx1 or 0.35 μM Grx3. When only ribonucleotide reductase was tested for inhibition with sodium chloride, ammonium sulfate, or sodium acetate, 25 mM DTT was used as the hydrogen donor.
An intact $^{14}$C carboxymethylated Grx3 revealed the order of peptides obtained after treatment with 70% formic acid for 24 h at 37°C. Peptides obtained by cleavage with Lysendoprotease. Fragment F1 refers to analysis of undigested protein. Fragments K4 and K5 are N-terminal residues of the native protein or only cleavage site of the Lys-endoprotease. To resolve this ambiguity, intact $^{14}$C carboxymethylated Grx3 was treated with 70% formic acid to cleave the Asp-Pro peptide bond and generate the C-terminal peptide F1 (Fig. 1). The amino acid composition and sequence determination of peptide F1 confirmed Lys to be the C-terminal residue. The complete 82-amino acid sequence obtained (Fig. 1) is in good agreement with the total composition of Grx3 (Table I). Furthermore, the 82-residue amino acid sequence of Grx3 determined in this work corresponds exactly (with the absence of the initiator methionine residue) to a "glutaredoxin-like" protein found in an open reading frame of the chromosomal region 76.0–81.5 min on the E. coli chromosome (GenBank™ accession no. U00039 (1994)). Based on the amino acid sequence (Fig. 1), the molecular mass of the reduced Grx3 was calculated to be 9004 Da.

Sequence Homology—In general, if two protein sequences are more than 25–30% identical, there is almost certainly a structural similarity (Orengo et al., 1994; Rost and Sander, 1994). Amino acid sequence alignments between the two E. coli glutaredoxins, while indicative of homology, cannot be made without the introduction of a few short gaps in the Grx3 sequence. Residues 21–50 of Grx3 display little sequence homology with the redox protein (Fig. 4). While the sequential and medium range NOEs and $J_{HN}$, $J_{Ha}$, and amide proton exchange rates (Wüthrich, 1986) are measured for 66 of 70 non-Pro residues (Wüthrich, 1986). The occurrence of certain patterns of these NMR parameters along the polypeptide chain is characteristic of a particular secondary structure type (Billeter et al., 1982; Wüthrich et al., 1984; Wüthrich, 1986). Using the $^{1}H$ chemical shifts for Grx3 (Table II), a two-dimensional NOESY spectrum was analyzed for the presence of sequential $^{3}$J($i$,i+1) and medium range $^{3}$J($i$,i+2; i,i+3; i,i+4) NOEs characteristic of secondary structural elements in Grx3. Vinal spin–spin coupling constants, $^{3}$J($H_{N}H_{A}$), were measured for 66 of 70 non-Pro and non-Gly residues. A total of 20 residues having amide proton exchange rates slower than 0.02 min$^{-1}$ were identified.

The core of seven secondary structural elements within the Grx3 structure is defined in a straightforward and unambiguous manner from the data summarized in Fig. 4. The three $\alpha$ helices identified in Grx3 are labeled sequentially $\alpha_{1}$ (residues 13–24), $\alpha_{2}$ (38–47), and $\alpha_{3}$ (67–74) in Fig. 4. Many of the expected medium range NOEs could not be unambiguously assigned due to chemical shift degeneracies or cross-peak overlap. The four segments of extended structure identified in Grx3 are labeled sequentially $\beta_{1}$ (residues 3–7), $\beta_{2}$ (28–33), $\beta_{3}$ (53–57), and $\beta_{4}$ (60–62) in Fig. 4. These extended segments contain numerous interstrand NOE connectivities, enabling alignment of these four strands into a four-stranded $\beta$ sheet (Fig. 5). The short fourth strand, $\beta_{4}$, involving residues 60–62 is predicted from sequence homologies (Fig. 2) and appears from analysis of interstrand NOEs to $\beta_{5}$, but the small $^{3}$J($H_{N}H_{A}$) coupling constant of His 60 indicates a significant deviation from a regular $\beta$-like secondary structure (Fig. 4).

The sequential and medium range NOEs and $^{3}$J($H_{N}H_{A}$) coupling constants were successful in identifying the core of the sequence.
secondary structure elements, the exact positions of the ends of each of these structures are more difficult to define. Therefore, the N- and C-terminal extensions of the $\alpha$ helices and $\beta$ sheets in the three-dimensional structures could be up to three residues longer than indicated in Fig. 6 and 7 and still be consistent with the data presented here. The experimental determination of the $\beta$ strand alignment in Grx3 (Fig. 5) is in full agreement with the independently obtained sequence alignment (Fig. 2) and justifies both the position and the length of insertions and deletions in the sequence alignment with Grx1. Additionally, the conserved cis-proline (Pro53) present in each of the homologues in Fig. 2 is also present in reduced Grx3 as deduced from the presence of sequential $d^3$ daa and absence of sequential $d^3$ ada NOES involving this residue. Final confirmation of the precise boundaries of the secondary structure elements awaits the full three-dimensional structure determination currently in progress.

**NMR Chemical Shift Analysis**—The folding of a protein into its native conformation results in relatively large perturbations of the $^1$H chemical shift values relative to the unfolded or random coil values. Recently, there has been considerable progress in extracting the conformational information contained in chemical shifts (for a review, see Szilágyi (1995)). For example, chemical shifts of carbon-bound protons have been used successfully as conformational constraints in protein structure determination by NMR techniques (O¨ sapay et al., 1994) and as a measure of the quality of three-dimensional protein structure determinations (Williamson et al., 1995). Protons attached to the $C^\alpha$ atom ($H^\alpha$) have proved particularly useful in identifying the locations of $\alpha$ helices and $\beta$ sheets in the amino acid sequence of globular proteins (Wishart et al., 1992).

In order to test the validity of the proposed amino acid sequence homology of Grx3 with other members of the thioredoxin superfamily (Fig. 2) and to gain further insight into the secondary structure, we have analyzed the $H^\alpha$ chemical shifts of reduced Grx3 (Table II) using the procedure of Wishart et al. (1992). Fig. 6 displays a plot of the difference between the $H^\alpha$ chemical shift measured for reduced Grx3 and the $H^\alpha$ chemical shift reported for the same residue type in a "random coil" conformation, $\delta(H^\alpha_{\text{native}}) - \delta(H^\alpha_{\text{random}})$, at each position in the amino acid sequence. Regions in which the observed $H^\alpha$ chemical shifts are significantly (>0.1 ppm) shifted to lower field (negative differences) identify $\alpha$ helices and regions in which the observed $H^\alpha$ chemical shifts are shifted to higher field.
TABLE II

| Residue | $^1$$^3$N | NH | $^1$$^H$ | Others |
|---------|----------|----|----------|--------|
| 1 Ala   | 120.3    | 9.35 | 9.40    | 2.82, 2.97 |
| 2 Asn   | 120.2    | 9.32 | 9.47    | 2.19    |
| 3 Val   | 126.9    | 9.32 | 9.52    | 1.92, 2.03 |
| 4 Glu   | 118.4    | 8.70 | 5.50    | 1.41    |
| 5 Ile   | 129.7    | 9.03 | 5.48    | 2.95, 3.14 |
| 6 Tyr   | 108.4    | 7.93 | 5.35    | 4.33    |
| 7 Thr   | 116.2    | 7.55 | 5.04    | 1.52    |
| 8 Lys   | 124.5    | 10.16 | 4.08 | 2.05, 2.13 |
| 9 Glu   | 12.4    | 8.40 | 4.22    | 1.52    |
| 10 Thr  | 126.2    | 6.95 | 4.78    | 2.87, 2.91 |
| 11 Cys  | 126.2    | 6.95 | 4.78    | 2.87, 2.91 |
| 12 Pro  | 126.9    | 9.43 | 4.37    | 3.02, 3.25 |
| 13 Tyr  | 128.1    | 9.79 | 3.94    | 2.63, 3.60 |
| 14 Cys  | 118.8    | 8.09 | 4.20    | 3.23    |
| 15 His  | 119.8    | 8.09 | 4.20    | 3.23    |
| 16 Arg  | 119.5    | 8.16 | 4.17    | 1.92, 2.05 |
| 17 Ala  | 117.0    | 8.16 | 4.17    | 1.92, 2.05 |
| 18 Lys  | 116.0    | 8.03 | 3.26    | 1.87    |
| 19 Ala  | 120.7    | 8.21 | 4.08    | 1.45    |
| 20 Leu  | 119.5    | 8.21 | 4.08    | 1.45    |
| 21 Leu  | 118.3    | 7.31 | 3.73    | 1.25, 1.78 |
| 22 Ser  | 114.8    | 9.11 | 4.45    | 4.05    |
| 23 Ser  | 119.6    | 8.30 | 4.37    | 4.07, 4.13 |
| 24 Lys  | 119.5    | 7.43 | 4.34    | 1.63, 1.97 |
| 25 Gly  | 107.2    | 8.06 | 3.87, 4.07 | 2.06   |
| 26 Val  | 117.5    | 7.43 | 4.23    | 2.06    |
| 27 Ser  | 122.0    | 8.65 | 4.43    | 3.81    |
| 28 Phe  | 118.0    | 7.53 | 5.08    | 2.69    |
| 29 Gin  | 120.8    | 9.14 | 4.58    | 2.03, 2.10 |
| 30 Glu  | 128.4    | 9.04 | 4.90    | 1.92, 2.14 |
| 31 Leu  | 129.9    | 9.10 | 4.66    | 0.82, 1.19 |
| 32 Pro  | 113.5    | 9.10 | 4.75    | 2.11    |
| 33 Ile  | 117.9    | 8.65 | 4.33    | 2.55, 2.62 |
| 34 Asp  | 118.2    | 7.68 | 4.78    | 2.44, 2.98 |
| 35 Gly  | 127.3    | 8.91 | 4.14*   | 1.54*   |
| 36 Ala  | 120.0    | 8.40 | 4.22    | 1.52    |
| 39 Lys  | 120.4    | 7.83 | 4.18    | 1.89    |
| 40 Arg  | 119.7    | 8.04 | 3.85    | 1.99, 2.09 |
| 41 Glu  | 117.3    | 8.39 | 3.71    | 2.39, 2.17 |
| 42 Glu  | 117.3    | 8.39 | 3.71    | 2.39, 2.17 |
| 43 Met  | 119.3    | 8.04 | 2.82    | 1.36, 2.25 |
| 44 Ile  | 124.1    | 8.36 | 3.91    | 1.74    |
| 45 Lys  | 122.1    | 8.45 | 3.95    | 1.89    |
| 46 Arg  | 114.6    | 8.24 | 0.06    | 1.00    |
| 47 Ser  | 110.3    | 8.31 | 3.70    | 3.44    |
| 48 Gly  | 112.4    | 8.56 | 3.83, 4.13 | 2.44, 2.98 |
| 49 Arg  | 119.8    | 8.38 | 4.79    | 1.63, 1.71 |
| 50 Thr  | 101.7    | 8.10 | 4.25    | 4.36    |
| 51 Thr  | 110.3    | 7.80 | 4.53    | 4.26    |
| 52 Val  | 110.5    | 7.94 | 4.49    | 2.13    |
| 53 Pro  | 112.6    | 7.30 | 4.58    | 2.13, 2.89 |
| 54 Gin  | 112.6    | 7.30 | 4.95    | 1.86, 2.05 |
| 55 Ile  | 123.3    | 8.94 | 5.09    | 1.47    |
| 56 Phe  | 127.0    | 9.48 | 5.31    | 2.65, 3.07 |
| 57 Ile  | 120.0    | 8.93 | 4.78    | 1.62    |
| 58 Asp  | 131.8*   | 9.97 | 4.56    | 2.76, 3.00 |
| 59 Ala  | 113.4    | 9.41 | 3.72    | 1.65    |
| 60 Gin  | 120.8    | 8.31 | 4.61    | 2.58    |
| 61 His  | 126.1    | 9.30 | 3.92    | 2.99, 3.15 |
| 62 Ile  | 127.5    | 8.45 | 3.95    | 1.61    |
| 63 Gly  | 103.4    | 6.14 | 3.04, 4.66 | 2.58    |
| 64 Gly  | 108.6    | 9.05 | 3.77, 4.74 | 2.58    |
| 65 Cys  | 110.3    | 8.35 | 3.84    | 2.61, 3.30 |
| 66 Asp  | 118.9    | 7.58* | 4.37 | 2.65    |
| 67 Asp  | 121.7    | 8.36 | 3.76    | 1.34, 2.17 |
| 68 Leu  | 116.9    | 8.21 | 3.82    | 3.00, 3.02 |
| 69 Tyr  | 123.1    | 8.40 | 4.21    | 1.51    |
| 70 Ala  | 119.6    | 7.92 | 4.07    | 1.63, 1.78 |
| 71 Leu  | 118.0    | 7.89 | 4.46    | 2.44, 2.56 |
| 72 Asp  | 121.5    | 8.14 | 4.16    | 1.59    |
| 73 Ala  | 112.5    | 7.29 | 4.67    | 1.88, 2.05 |
| 74 Arg  | 103.6    | 8.35 | 3.45, 4.27 | 1.88, 2.05 |

Glutaredoxin-3 from E. coli

$^1$$^H$ and $^1$$^5$N chemical shifts for Grx3 at pH 6.8 and 28 °C.
positive differences) identify β strands (Wishart et al., 1992). With the exception of the short fourth strand of the β sheet (residues 60–62), each secondary structure element proposed by the sequence alignment to Grx1 (Fig. 2) is also indicated from this analysis of Grx3 (Fig. 6). Recently, the complete NMR resonance assignment of the reduced, unfolded Grx3 was determined (Nordstrand et al., 1995). When used in place of the average "random coil" values of ¹H chemical shifts proposed by Wishart et al. (1992), the experimentally determined ¹H chemical shifts for Grx3 produce a very similar pattern (Fig. 6).

Interestingly, an additional, short helix containing residues 78–82 is predicted when using the experimental random coil ¹H chemical shifts, but not by the average shift values of Wishart et al. (1992). The presence of this helical segment is further supported by the NMR parameters summarized in Fig. 4. The overall good correlation of the predictions from analysis of the ¹H chemical shifts (Fig. 6) with independently determined secondary structural elements (Figs. 4 and 5) further supports the secondary structure outlined in Fig. 4.

Having established a similar secondary structure content and location for Grx3 and Grx1, we were next interested whether the tertiary fold of Grx3 could be confirmed by similar means using the conformational information contained in the ¹H NMR chemical shifts. Exploiting the sequence homology of Grx3 with Grx1, for which three NMR structures exist (Sodano et al., 1991; Xia et al., 1992; Bushweller et al., 1994), we have constructed a three-dimensional model of oxidized Grx3, Grx3 model. The model contains the same basic secondary structure elements as found in Grx1 in the same relative orientation (Fig. 7). The secondary structure analysis of Grx3 model reveals a four-stranded β sheet containing residues 2–7 (β₁), 27–33 (β₂), 53–57 (β₃), and 60–62 (β₄) and three α helices containing residues 11–24 (α₁), 39–49 (α₂), and 64–75 (α₃). There is thus overall good agreement between the secondary structures present in Grx3 model and the experimentally determined secondary structure of Grx3 (Figs. 4–6). Apart from

| Residue | ¹H NH | ¹H | Others |
|---------|------|----|--------|
| Leu 77  | 122.0| 7.97| 4.46  |
| Residues in which the proton resonances were not observed at 28 °C but could be assigned from spectra recorded at 15 °C. |
| ¹H chemical shifts of these residues are reported for the Met-Grx3 form (see text for details). |
the minor displacement of helix 2 (Fig. 7, top), Grx3 model is very similar to the NMR solution structure of Grx1 upon which it is based. The truncated first helix of Grx3, suggested by both the sequence comparison to Grx1 and the secondary structure determination by NMR, contains one less helical turn at the C terminus than Grx1 (Figs. 1, 4, and 7) providing a shortened link to the second β strand. Analysis of Grx3 model reveals that the side chains of Grx3 are also well accommodated in the Grx1 structure with minimal backbone distortions.

1H NMR chemical shifts of oxidized Grx3 were calculated from the three-dimensional coordinates of Grx3 model using the algorithm of Williamson et al. (1995), which considers contributions from ring current, magnetic anisotropy, and electric field shifts. The resulting calculated Hα chemical shifts for Grx3 correlate well with those observed for Grx3 in solution. The standard deviation (S.D.) of calculated to observed Hα chemical shifts is 0.44 ppm. The accuracy of NMR solution structures with S.D. values around 0.3 ppm has been compared to x-ray crystal structures of 2.0-Å resolution (Williamson et al., 1995). S.D. values of 0.45 ppm are not without precedent among experimentally determined NMR solution and x-ray...
crystal structures (Williamson et al., 1995). Some discrepancies are expected from the fact that the experimental chemical shifts were obtained from reduced Grx3 whereas the model was built of the oxidized form. Thus, while a S.D. value of 0.44 ppm is, in these terms, clearly indicative of a structure of lower quality than a 2-Å resolution crystal structure, this value is sufficiently small to support the tertiary fold of Grx3 as presented in Grx3 model.

Activity of Grx3 with Protein Disulfides—Insulin reduction provides the basis for a classical assay used to test thioredoxin activity (Holmgren 1979c). Though much less potent than thioredoxin, both Grx1 and calf thymus glutaredoxin have been shown to reduce insulin disulfides in the presence of GSH (Luthman and Holmgren, 1982) with a $K_m$ value for insulin of $100 \mu M$. We found Grx3 to be much less efficient than Grx1 when tested with this disulfide substrate. At a 10 $\mu M$ concentration, the activity of Grx3 was only 6.2% that of Grx1 (Fig. 8). The very low activity of Grx3 was comparable to that of a site-directed mutant of E. coli Grx1, Grx1(C14S), which has been shown to reduce mixed disulfides with GSH but not the disulfide of ribonucleotide reductase (Bushweller et al., 1992). Thus, Grx3 might be able to reduce only mixed disulfides formed between insulin and GSH. This could also be true when Grx3 acts as hydrogen donor for ribonucleotide reductase, but needs experimental verification.

Since the structural differences between Grx3 and Grx1 involve differences in charge distribution around the active site cysteine residues (see below), the importance of these charges should decrease with increasing ionic strength. The activity of Grx3 versus that of Grx1 with ribonucleotide reductase was found to increase upon addition of sodium chloride or ammonium sulfate (data not shown). Since ribonucleotide reductase is known to be inhibited by these salts, the assays were performed with sodium acetate, which has no inhibitory effect on ribonucleotide reductase (Brown et al., 1984; Xia et al., 1994). We have observed dimers of Grx3 (Fig. 2), and it has been suggested that several of these residues form a surface for interaction with other proteins, such as ribonucleotide reductase (Eklund et al., 1984; Xia et al., 1992). Based on the sequence alignment in Fig. 2, these residues correspond to Lys$^9$, Cys$^{11}$, Pro$^{12}$, Tyr$^{13}$, Ile$^{23}$, Thr$^{31}$, Val$^{32}$, Pro$^{33}$, Gly$^{64}$, Cys$^{65}$, Asp$^{66}$, and Asp$^{67}$ in Grx3. Analysis of these and surrounding residues are of interest in relation to the factors behind the differential reactivity of Grx3 compared to that of Grx1 (Åslund et al., 1994).

A number of similarities and differences in the charged amino acids which map near the active site cysteines (Cys$^{11}$ and Cys$^{14}$) are noticed. Some differences are conservative as, for example, Lys$^9$ in the turn preceding the active site cysteine residues, which has arginine as a counterpart in Grx1. However, the negative charge of Glu$^9$ in Grx3 has no counterpart in Grx1. Likewise, there is no counterpart for Glu$^{12}$ of Grx1, just before the conserved cis-proline (Pro$^{13}$ in Grx3) and Asp$^{14}$ (Thr$^{23}$ in Grx1). It is also possible that the numerous amino acid differences observed in the loop containing the second helix (residues 32–53 in Grx3) are important determinants contributing to the low reactivity of Grx3 with ribonucleotide reductase compared to Grx1 (Fig. 9). Overall, based on the amino acid sequence, Grx3 is expected to be more basic than Grx1 with calculated pI values of 7.1 and 5.2, respectively.

Similar to mammalian glutaredoxins (Fig. 2), Grx3 contains a cysteine (Cys$^{65}$) residue after the two conserved glycine residues in the bend before the last helix. This residue corresponds to Tyr$^{72}$ of Grx1. The function of this single cysteine residue is not known. From the position of Cys$^{65}$ in the first turn of the third helix, one might expect this thiol to have an increased reactivity caused by the localized partial positive charge of the helix dipole (Hol et al., 1978). In addition, mammalian glutaredoxins have a second, non-active site, cysteine located three residues before the cysteine residue corresponding to Cys$^{65}$ in Grx3, whereas in prokaryotic glutaredoxins, there is a conserved histidine residue at this position (Fig. 2). In mammalian thioredoxins, an analogous internal cysteine pair can be found similar to those in mammalian glutaredoxins. A regulatory function of this cysteine pair in thioredoxins has been suggested (Ren et al., 1993). We have observed dimers of Grx3 following SDS-polyacrylamide gel electrophoresis under non-
reducing conditions (Åslund et al., 1994), presumably due to intermolecular disulfide bond formation.

The residues involved in binding of glutathione have been identified in the NMR solution structure of the mixed disulfide Grx1-SG (Bushweller et al., 1994) and are enclosed in boxes in Fig. 2. The interaction of GSH with Grx3 is likely to be very similar to that with Grx1, with the GSH molecule binding in a cleft on the surface of Grx3. The cysteine thiol of GSH would interact with Cys51 of Grx3 and the conserved Tyr53 would form one side of the binding site. A tyrosine or a phenylalanine residue is always found at this position in glutaredoxins (Nikkola et al., 1991). The conservation of the conserved cis-proline at position 53 should allow for main-chain hydrogen bonding between glutathione and the protein as it does for Grx1. The preceding residues (Thr51 and Val52) are also conserved in Grx3. The amino group of the γ-glutamyl group of one of the aspartic acid residues following Cys65 in Grx3, the distribution in this area is indeed important for GSH binding. De- 

To the two aspartic acid residues following Cys65 in Grx3, the intermolecular disulfide bond formation.

Concluding Remarks—The amino acid sequence of Grx3 has been determined by amino acid sequencing, and the resulting primary structure was found to be homologous to members of the thioredoxin superfamily, most notably Grx1 with 33% identity. In a first step toward characterizing the three-dimensional structure of Grx3, the secondary structure of reduced Grx3 was determined at pH 6.8 and 28 °C using NMR techniques. The secondary structure was found to contain three α helices labeled sequentially α1 (residues 13–24), α2 (38–47), and α3 (67–74) as well as four β strands labeled sequentially β1 (residues 3–7), β2 (28–33), β3 (53–57), and β4 (60–62), which could be aligned into a four-stranded β sheet. The resulting secondary structure is overall very similar to that determined for oxidized and reduced Grx1 (Xia et al., 1992; Sodano et al., 1991).

Despite a similar three-dimensional structure, Grx3 was found to have only a fraction of the capacity of Grx1 as a disulfide reductant despite an identical active site sequence (CPYC). The conformations of oxidized and reduced Grx1 have been shown by NMR to be very similar. The similar appearance of the circular dichroism spectra of oxidized and reduced Grx3 indicates little conformational change between the two forms. One possible explanation for the different reactivities of Grx1 and Grx3 in the observed differences in the distribution of charged residues in and around the proposed surface for interaction with other proteins. The increased activity of Grx3 in the ribonuclease reductase assay as a function of ionic strength supports this point. An increase in redox potential of Grx3 compared to the other hydrogen donors to ribonuclease reductase (e.g. Trx and Grx1) is also a possible explanation for the reduced activity. The effect of disulfide bonds on the conformational stability of globular proteins must necessarily reflect the reciprocal effect of the protein conformation on the stability of the disulfide bonds (Creighton, 1986). This linkage relationship, when applied to the oxidoreductases, proposes that differences in conformational stability between the oxidized and reduced forms of these proteins should effect the stability of the active site disulfide bond and hence affect the redox potential in a predictable manner. This relationship has been verified experimentally for the E. coli proteins Trx (Lin and Kim, 1989) and DsbA (Zapun et al., 1993; Wunderlich et al., 1993), where the oxidized form of the physiologically reducing Trx was found to be more stable than the reduced form whereas in the physiologically oxidizing DsbA, it is the reduced form that is more stable. In the framework of Grx3, an increased redox potential (less negative) could be achieved by increasing the stability of the reduced with respect to the oxidized form. This relatively straightforward mechanism could turn out to be a general phenomenon by which the process of evolution controls the gross redox function of these proteins.

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Glutaredoxin-3 from *Escherichia coli*: AMINO ACID SEQUENCE, 1H AND N NMR ASSIGNMENTS, AND STRUCTURAL ANALYSIS
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