Reduction of Mutant Phage T4 Glutaredoxins by *Escherichia coli* Thioredoxin Reductase*

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Fifteen mutant T4 glutaredoxins (previously T4 thioredoxin) have been assayed for activity with *Escherichia coli* thioredoxin reductase. The mutations include substitutions in the region of the active site, in the 2 cysteines, and in the 2 residues between the cysteines forming the active-site disulfide bridge. Mutant thioredoxins where substitutions have been made in charged residues around the active site show the biggest differences in activity. The positive residues Lys-13 and Lys-21 were found to be important for efficient binding to thioredoxin reductase. Substitution of the aspartic acid at position 80 with a serine produced a glutaredoxin with superior activity. This mutant glutaredoxin has earlier been shown to be more efficient than the wild type in thiol transferase activity (Nikkola, M., Gleason, F. K., Saarinen, M., Joelson, T., Björnberg, O., and Eklund, H. (1991) J. Biol. Chem. 266, 16105–16112). Even the glutaredoxin P66A, where the active-site cis-proline has been substituted, could be efficiently reduced by thioredoxin reductase. Glutaredoxins lacking one or both cysteines were not active.

Thioredoxin and glutaredoxin are small disulfide-containing redox proteins. Both can function in ribonucleotide reduction by transferring electrons from NADPH to the reductase. Thioredoxin is reduced by the flavoprotein thioredoxin reductase and serves as a general protein disulfide reductase. Glutaredoxin is reduced by glutathione and this protein can reduce a number of small molecules such as cystine and dehydroascorbate (Wells et al., 1990). The active site of both proteins consists of a 14-membered disulfide ring. However, there is little additional amino acid similarity between the two proteins, and except for electron transfer in ribonucleotide and sulfur reduction, little overlap in function (for a recent review, see Holmgren (1989)).

A unique glutaredoxin is produced in *Escherichia coli* by bacteriophage T4. It serves as a reducing agent for the phage-induced ribonucleotide reductase. The viral glutaredoxin actually has the properties of both types of redox proteins. It can be efficiently reduced by glutathione and will, in turn, reduce other small disulfides (Nikkola et al., 1991). It can also be reduced by NADPH and the host thioredoxin reductase.

Because T4 glutaredoxin has a more positive redox potential than *E. coli* thioredoxin (−230 and −260 millivolts, respectively), it can also be reduced by thioredoxin, especially under alkaline conditions (Berglund and Holmgren, 1975). Thus the viral glutaredoxin can channel much of the cell’s reducing power into deoxynucleotide synthesis for viral DNA replication.

T4 glutaredoxin has been crystallized and the structure solved (Sjöberg and Söderberg, 1976; Söderberg et al., 1978). The structure of the wild type protein has been refined at 2.0-Å resolution (Fig. 1). Recently, the crystal structure for a mutant glutaredoxin has been solved and refined at 1.45-Å resolution (Eklund et al., 1992). Both crystal and NMR structures have been reported for *E. coli* thioredoxin (Katti et al., 1990; Dyson et al., 1990). The phage glutaredoxin has a tertiary structure which is similar to that of *E. coli* thioredoxin (Eklund et al., 1984). However, its amino acid sequence shows considerable homology to *E. coli* glutaredoxin and other thiol transferases (Nikkola et al., 1991). Since these latter proteins are not substrates for *E. coli* thioredoxin reductase, a question arises as to what features of T4 glutaredoxin enable it to accept electrons from both the flavoprotein and glutathione. We have produced altered T4 glutaredoxins by site-directed mutagenesis and previously identified residues involved in glutathione binding (Nikkola et al., 1991). Residues in the active-site disulfide ring were also changed to determine their effect on redox activity (Joelson et al., 1990). These and additional mutant glutaredoxins are described in this paper.

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and their activity as substrates for E. coli thioredoxin reductase is further elucidated.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Mutant Proteins, Plasmids, Phages etc.—**Bacterial strains, plasmids, phages, and the construction of most of the mutant glutaredoxins have been described (Joelson, 1988; Joelson et al., 1990; Nikkola et al., 1991). The mutant glutaredoxins K13S and C14S/C17S were constructed in a similar fashion using the Amersham sit-directed mutagenesis kit. The mutagenic primers were synthesized by Syntycem AB, Umeå, Sweden.

**Protein Purification—**Thioredoxin reductase was isolated from an overproducing strain containing the trxB gene on a pUC vector which was kindly provided by J. Fuchs, University of Minnesota. The enzyme was purified by chromatography on DEAE-Sepharose and 2',5'-ADP-Sepharose as described by Pigiet and Conley (1977). In the final step, a Mono Q column (Pharmacia LKB Biotechnology Inc.) was substituted for hexylamine-agarose. The reductase was eluted from the column with a gradient of 0 to 0.3 M NaCl in 20 mM Tris-HCl buffer pH 7.5, containing 1 mM EDTA. The protein used was homogeneous after this step as determined by polyacrylamide gel electrophoresis on 10% native gels run at alkaline pH.

**E. coli** thioredoxin was purified from an overproducing strain containing the trxA gene on a pUC-type plasmid. Purification was done by chromatography on DEAE-Sepharose and Sephadex G-50 as previously described (Limb et al., 1986).

Wild-type and mutant glutaredoxins were purified as described elsewhere (Nikkola et al., 1991). Mutant glutaredoxins were modeled using Frodo (Jones, 1982) and O (Jones et al., 1990).

**Kinetic Studies—**For kinetic measurements, the thioredoxin or T4 glutaredoxin catalyzed reduction of 5,5'-dithiobis(2-nitrobenzoic acid) was monitored in the presence of E. coli thioredoxin reductase and NADPH (method 4 in Latham and Holmgren (1982)). Assay mixtures contained 100 mM Tris-HCl with the pH adjusted to 7.0, 0.0, or 9.0, 2 mM EDTA, 50 μg of bovine serum albumin, 0.5 mg of 5,5'-dithiobis(2-nitrobenzoic acid) dissolved in ethanol, and 0.24 mM NADPH in a final volume of 0.5 ml. Glutaredoxins and thioredoxin were added to the reaction cuvette in a concentration range of 0.03 to 50 μM. The reaction was initiated by adding E. coli thioredoxin reductase to both cuvettes at a concentration of 13 nM as determined from the flavin absorbance at A445 (Thiellander, 1967). The increase in absorbance at 412 nm was monitored at 25 °C in a Hewlett-Packard 8450A "double beam" spectrophotometer. Kinetic parameters were obtained from unweighted least squares analyses of Lineweaver-Burk plots. Constants are reported as a mean of 3–5 separate analyses.

Thioredoxin and glutaredoxin concentrations were estimated from the absorbance at 280 nm. Previously reported extinction coefficients for thioredoxin (Kalis and Holmgren, 1980), and wild type and mutant glutaredoxins (Joelson et al., 1990) were used.

**RESULTS**

All of the altered T4 glutaredoxins with 2 cysteines were able to serve as substrates for E. coli thioredoxin reductase and were reduced by NADPH via the flavoprotein.

All of the proteins were assayed at pH 8.0 and the results are presented in Table I. At this pH, T4 glutaredoxin was a better substrate than E. coli thioredoxin as reflected in the 6-fold higher catalytic efficiency. This is due mainly to a lower Kyna for the viral protein. Turnover numbers were approximately the same for both proteins. T4 glutaredoxins in which the residues between the active-site cysteines were altered showed 80–100% of the activity of the wild type protein. The one exception is the mutant protein in which both residues are changed to prolines, V16P/Y16P. The presence of adjacent prolines or glycines in the disulfide ring is reflected in the Km for the mutant protein. The mutant protein reduction is comparable. Similarly, mutation of the cis-proline residue at position 66 to an alanine raises the Kyna without changing turnover.

The most dramatic changes in catalytic efficiency are observed when charged residues are mutated. Changing the histidine residue at position 12 or the lysine at position 12 to serine leads to large increases in the Kyna values. When Lys-21 is replaced by a glutamine residue, both Kyna and Kcat are altered. This mutant protein has only 3% of the activity of the wild type. In contrast, mutation of the aspartic acid at position 80 to a serine improves the activity of the glutaredoxin as a substrate for thioredoxin reductase. The D80S glutaredoxin binds more tightly and is reduced more rapidly than the wild type. Lineweaver-Burk plots are shown for wild type glutaredoxin and mutant glutaredoxins D80S and K13S in Fig. 2. Mutant glutaredoxins with altered active-site cysteines were inactive in this assay system.

The ability of thioredoxin and glutaredoxin variants to serve as substrates for thioredoxin reductase is pH dependent. As seen in Table II, E. coli thioredoxin is a better substrate than the viral protein at pH 7.0. The reaction with the altered glutaredoxins also shows lower catalytic efficiency as compared to pH 8.0. This is due mainly to higher Kyna values. Turnover rates are generally increased at the lower pH.

### Table I

| Protein | Kyna | Kcat | Catalytic efficiency | Catalytic efficiency |
|---------|------|------|----------------------|----------------------|
| E. coli trx | 2.17 ± 0.95 | 1435 ± 278 | 661 | 0.09 1990 |
| T4 wt | 0.43 ± 0.10 | 1653 ± 204 | 3844 | 0.03 1990 |
| Y16P | 0.30 ± 0.06 | 1557 ± 219 | 3114 | 0.01 1990 |
| V15P | 0.38 ± 0.12 | 1519 ± 113 | 4219 | 1.0 1.0 |
| V15G | 0.47 ± 0.05 | 1700 ± 153 | 3617 | 0.94 1.0 |
| V15G; Y16P | 0.35 ± 0.03 | 1638 ± 65 | 4680 | 1.22 1.0 |
| V15F; Y16P | 0.96 ± 0.09 | 1611 ± 16 | 1678 | 0.44 1.0 |
| V15G; Y16G | 0.71 ± 0.09 | 2245 ± 180 | 3162 | 0.82 1.0 |
| P66A | 0.69 ± 0.21 | 1650 ± 320 | 2750 | 0.72 1.0 |
| H12S | 1.74 ± 0.17 | 2064 ± 200 | 1186 | 0.31 1.0 |
| K13S | 2.93 ± 0.52 | 1554 ± 168 | 530 | 0.14 1.0 |
| K21Q | 8.07 ± 0.99 | 1155 ± 150 | 127 | 0.03 1.0 |
| D80S | 0.28 ± 0.04 | 1990 ± 284 | 7107 | 1.85 1.0 |
| H12S; D80S | 0.97 ± 0.11 | 2161 ± 195 | 2225 | 0.58 1.0 |

**Fig. 2.** Lineweaver-Burk plots of wild type T4 glutaredoxin and mutant glutaredoxins D80S and K13S as substrates for E. coli thioredoxin. Assays were done at pH 8.0 as described under "Experimental Procedures." Thioredoxin reductase concentration was 13 nM. The line was drawn from a least-squares analysis of the data points. ●, T4 glutaredoxin; ○, D80S; △, K13S.
Reduction of Mutant T4 Glutaredoxins by Thioredoxin Reductase

The ability of T4 glutaredoxins to serve as substrates for E. coli thioredoxin reductase at pH 7.0

All assays were run in 100 mM Tris-Cl, pH 7.0. Other reaction conditions are described under "Experimental Procedures." Thioredoxin reductase concentration was 13 nM.

| Protein | $K_m$ (μM) | $k_{cat}$ (min⁻¹) | Catalytic efficiency | Catalytic efficiency mutant:wt |
|---------|------------|-------------------|---------------------|------------------------------|
| E. coli trx | 0.77 ± 0.32 | 4250 ± 165 | 3182 | |
| T4 wt | 4.33 ± 1.72 | 3600 ± 296 | 831 | 1.00 |
| K13S | 46.1 ± 15.2 | 4338 ± 105 | 94 | 0.10 |
| D80S | 1.52 ± 0.35 | 2854 ± 471 | 1746 | 2.10 |
| H12S | 15.5 ± 1.0 | 3900 ± 223 | 252 | 0.30 |
| H12S; D80S | 20.4 ± 6.6 | 2335 ± 531 | 114 | 0.14 |
| V15P | 5.23 ± 2.6 | 1723 ± 125 | 329 | 0.40 |

Table III

Ability of T4 glutaredoxins to serve as substrates for E. coli thioredoxin reductase at pH 9.0

All assays were run in 100 mM Tris-Cl, pH 9.0. Other reaction conditions are described under "Experimental Procedures." Thioredoxin reductase concentration was 13.6 nM.

| Protein | $K_m$ (μM) | $k_{cat}$ (min⁻¹) | Catalytic efficiency | Catalytic efficiency mutant:wt |
|---------|------------|-------------------|---------------------|------------------------------|
| E. coli trx | 4.45 ± 1.57 | 593 ± 116 | 133 | |
| T4 wt | 0.40 ± 0.13 | 857 ± 116 | 2143 | 1.00 |
| Y16A | 0.61 ± 0.14 | 2321 ± 41 | 3805 | 1.78 |
| V15P | 0.24 ± 0.08 | 1089 ± 12 | 413 | 2.06 |
| H12S | 0.39 ± 0.09 | 797 ± 91 | 2554 | 1.05 |
| K13S | 1.28 ± 0.15 | 793 ± 47 | 820 | 0.29 |
| D80S | 0.04 ± 0.01 | 735 ± 6 | 18380 | 857 |
| H12S; D80S | 0.55 ± 0.19 | 1065 ± 191 | 1936 | 0.90 |

At pH 9.0, E. coli thioredoxin is a poor substrate for the reductase (see Table III). Activity with this protein is highest at pH 7.0 and steadily declines as the pH is raised. Conversely, T4 glutaredoxin is a much better substrate at alkaline pH. Reductase activity with some of the mutant proteins is also improved at this pH. The overall increase in catalytic efficiency is greatest for glutaredoxin D80S. This is due to a 10-fold decrease in the $K_m$ suggesting a much tighter binding to the reductase.

The data on the activity of the mutant glutaredoxins clearly show that charged residues have an effect on binding to the reductase. Therefore, the ionic strength of the buffer should add to the reductase. The reactions were run in 10 mM Tris-Cl, pH 7.0. Other additions are as described under "Experimental Procedures." The ionic strength of the buffer should influence activity by affecting interaction with the reductase, i.e., changing the $K_m$. Some of these predictions have been confirmed by mutagenesis studies with E. coli thioredoxin.

The ionic strength dependence was determined for glutaredoxins at 0.22 μM; O, 0.13 μM D80S; Δ, 0.54 μM H12S; Δ, 0.56 μM K13S.

At pH 9.0, E. coli thioredoxin is a poor substrate for the reductase (see Table III). Activity with this protein is highest at pH 7.0 and steadily declines as the pH is raised. Conversely, T4 glutaredoxin is a much better substrate at alkaline pH. Reductase activity with some of the mutant proteins is also improved at this pH. The overall increase in catalytic efficiency is greatest for glutaredoxin D80S. This is due to a 10-fold decrease in the $K_m$ suggesting a much tighter binding to the reductase.

The data on the activity of the mutant glutaredoxins clearly show that charged residues have an effect on binding to the reductase. Therefore, the ionic strength of the buffer should influence activity. As illustrated in Fig. 3, the activity of thioredoxin reductase with T4 glutaredoxins is highest at low ionic strength, and declines as salt is added to the reaction. The ionic strength dependence was determined for glutaredoxins with charges in charged residues. It can be concluded that charged residues at the surface of the glutaredoxin are important in optimizing the interaction. Most of the mutant glutaredoxins tested show a similar ionic strength dependence, with the exception of glutaredoxin K13S.

**DISCUSSION**

Thioredoxin reductase from E. coli will accept thioredoxins from a number of different bacteria as substrates. For example, thioredoxin from the cyanobacterium Anabaena sp. 7118, with 48% amino acid identity to the E. coli protein, is efficiently reduced, although the $K_m$ for the heterologous reaction is higher (Lim et al., 1986). However, protein recognition cannot be confined solely to the thioredoxin active-site sequence, Cys-Gly-Pro-Cys, since many homologous proteins do not interact with E. coli thioredoxin reductase (see for example, Gleason (1992a)). It has been suggested that a number of additional residues in the active-site region outside the disulfide ring are actually more important in protein recognition and specificity. These include a conserved cis-proline at position 76 and neighboring residues, and a non-polar region including Val-91, Gly-92, and Ala-93 (Eklund et al., 1984). Assuming relatively minor changes in the overall structure, mutations in these residues would be expected mainly to alter activity by affecting interaction with the reductase, i.e., changing the $K_m$. Some of these predictions have been confirmed by mutagenesis studies with E. coli thioredoxin.

Changing Gly-92 to an aspartic acid or serine makes the mutant thioredoxin a poor substrate for the reductase (Holmgren et al., 1981; Russel and Model, 1986). In addition, changing the lysine residue at position 36 next to the disulfide ring to a glutamic acid slows binding to thioredoxin reductase (Navarro et al., 1990).

Fig. 4 compares the backbone structures of E. coli thioredoxin and T4 glutaredoxin. Although the viral protein is smaller, there is considerable similarity in the region of the active-site disulfides. Unlike the relatively open structure in E. coli thioredoxin, T4 glutaredoxin has a binding cleft to accommodate glutathione and other small molecules. Tyr-16, located in the disulfide ring, has been shown to be especially important in binding glutathione (Nikkola et al., 1991). However, mutation of Tyr-16 to a proline as described previously (Joelson et al., 1990) or a small alanine residue has only a slight effect on interaction with thioredoxin reductase. Likewise, mutant glutaredoxins with substitutions at Val-15 have wild type characteristics (Joelson et al., 1990). Our results confirm this. Mutant glutaredoxins in which both residues in the ring were replaced by prolines or glycines still had 44 and 82%, respectively, of the activity of the wild type protein. Mutation of Pro-34 to a serine in E. coli thioredoxin does not change the kinetics with the reductase. It can be concluded that the residues between the cysteines are not essential for binding to thioredoxin reductase. Residues at this position seem to primarily affect the activity of the reduced protein (Joelson et al., 1990; Gleason et al., 1990) and may influence the redox potential (Krause et al., 1991).
A cis-proline at position 76 in the beginning of a β-strand is conserved in all thioredoxins (Eklund et al., 1991). The equivalent position in T4 glutaredoxin is occupied by Pro-66. Mutation of Pro-76 in E. coli thioredoxin to an alanine yields a relatively unstable protein (Kelley and Richards, 1987). Its $K_m$ with thioredoxin reductase is five times higher than with wild type protein, but the $k_{cat}$ is similar (Gleason, 1992b). In like manner, T4 glutaredoxin P66A has 72% of the activity of the wild type due to a higher $K_m$. The cis-proline occupies a structurally important position behind the active-site Cys-31 of the wild type due to a higher $K_m$. The cis-proline occupies an exposed position on the surface of T4 glutaredoxin corresponding to the highly conserved Trp-31 in E. coli thioredoxin (Eklund et al., 1992). Since this residue is on the surface, one can assume its $pK_a$ is around 6–7. Proximity to lysines 13 and 40 may change this value somewhat. The kinetic data in Table I was obtained at pH 8.0. Mutation of this histidine to a serine causes a 4-fold increase in $K_m$, suggesting that this mutant binds poorly to the reductase. Turnover is not affected. At pH 7.0 (Table II), T4 glutaredoxin is a poorer substrate for the reductase than E. coli thioredoxin. Again, at this pH, the His-12 mutant protein shows a similar 4-fold increase in $K_m$. At pH 9.0, however, there is no difference in the $K_m$ values for the wild type and glutaredoxin H12S. Thus the positive charge caused by the partial protonation of the histidine at near-neutral pH may improve the interaction between glutaredoxin and thioredoxin reductase. Mutant glutaredoxin H12S has even decreased affinity for glutathione (Nikkola et al., 1991). Recent mutagenesis studies on E. coli thioredoxin show that position 31 is important for thioredoxin-protein interactions. Thioredoxin W31H is approximately 60% as effective a substrate for the reductase as the wild type. Thioredoxin W31A has slightly lower catalytic efficiency, while thioredoxin W31Y activity is unchanged. The major effect of replacing the tryptophan, however, is on the protein interactions of reduced thioredoxin (Krause and Holmgren, 1991).

Replacement of the positively charged lysine at position 13 with a neutral serine raises the $K_m$ approximately 7-fold with no effect on electron transfer. Lys-13 lies in the proposed interaction area in T4 glutaredoxin and in a patch of positive charges which includes residues His-12 and Lys-40. Lys-13 is closest to the active-site and seems to be important for interaction with thioredoxin reductase (Fig. 5). This is reflected in the ionic strength dependence of the reaction. As seen in Fig. 3, activity declines at high ionic strength where the charge would be masked. When Lys-13 is mutated to serine, activity increases as salt is added, indicating that the positive charge at position 13, together with other charged residues responsible, is responsible for electrostatically guiding T4 glutaredoxin to the reductase-binding site.

Mutation of the lysine residue at position 21 causes the most dramatic decline in activity. In this case, both $K_m$ and $k_{cat}$ are altered suggesting that this residue is important not only for binding but may also function in facilitating the disulfide exchange. The position of Lys-21 in T4 glutaredoxin corresponds to that of a highly conserved Lys-57 found in bacterial thioredoxins. We predict that a similar mutation of this residue in E. coli thioredoxin will yield a protein with relatively little redox activity, especially if this residue is critical for protein stability as predicted by Langsetmo et al. (1991a, 1991b).

In contrast to the negative effects of mutating the positively charged residues, altering Asp-80 to a serine improves the interaction with thioredoxin reductase. Asp-80 lies at the beginning of the last α-helix in T4 glutaredoxin and although rather removed from the active-site disulfide, it is still on the same face of the protein. A similar area in E. coli thioredoxin is the proposed nonpolar interaction area around residues 91–93 (Eklund et al., 1984). No negative charges occur in this area in the bacterial protein. Thus the elimination of Asp-80 in T4 glutaredoxin makes it more like E. coli thioredoxin, thus improving its ability to function as a substrate for the reductase. T4 glutaredoxin D80S also exhibits tighter binding of glutathione. The negative charge at a similar position is conserved in other glutaredoxins. This residue may facilitate interaction with other proteins such as ribonucleotide reductase or play a role in protein stability.

The reduction of thioredoxins or T4 glutaredoxin by the reductase is a simple disulfide exchange. A question arises as to how specificity is achieved since not all disulfide-containing proteins or even all thioredoxins are substrates. There is a similar problem in examining the interactions of all redox proteins. In cytochromes, for example, specificity can be controlled by a combination of factors including, redox potentials,
protein conformation, i.e. steric factors, and electrostatic effects (Tollin et al., 1986).

From a comparison of the x-ray structures (Fig. 4) it can be seen that T4 glutaredoxin and E. coli thioredoxin have common tertiary structural features (Ekland et al., 1984). However, considering the differences in the active-site region and comparing the primary structures, it would seem unlikely that both could be reduced by the same reductase with equal efficiency. Redox potentials do not seem to play a large role here since these are in the range of -230 to -260 mV which is comparable to the two-electron reduced reductase (ODonnell and Williams, 1983).

E. coli thioredoxin reductase is a rather unusual disulfide reductase in that both cysteines at the active site can act in disulfide exchange (Prongay et al., 1989). The two binding sites are reported to be relatively shallow depressions at the subunits rather than a cleft seen in other reductases (Kuriyan et al., 1991). Docking experiments indicate that the reduction of glutaredoxin may require conformational changes.

Although ionic strength of the buffer was reported to have little effect on thioredoxin reductase activity, mutant studies of E. coli thioredoxin indicate that charged residues such as lysine 36 play a role in the interaction (Navarro et al., 1990). The reductase is a negatively charged molecule (Thelander, 1987). E. coli thioredoxin also has a low isoelectric point. Two prominent areas of positive charge can be seen, one at Lys-36 which is known to function in binding (Navarro et al., 1990) and another at Lys-57, which in T4 glutaredoxin corresponds to Lys-21. Other than that, T4 glutaredoxin with its many basic residues differs from E. coli thioredoxin. Nevertheless, the pH and ionic strength dependence indicate that electrostatics are still important for interaction with the reductase. The mutant studies confirm that charged residues in positions comparable to those in E. coli thioredoxin affect binding. Considering the differences in structure between T4 glutaredoxin and E. coli thioredoxin, electrostatics may actually be more of a factor in protein recognition and binding than steric interactions.

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**Fig. 5.** Stereo view of the active site of T4 glutaredoxin with some of the residues substituted in this study.