Modifications of the Active Center of T4 Thioredoxin by Site-directed Mutagenesis*

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The active site sequence of T4 thioredoxin, Cys-Val-Tyr-Cys, has been modified in two positions to Cys-Gly-Pro-Cys to mimic that of Escherichia coli thioredoxin. The two point mutants Cys-Gly-Tyr-Cys and Cys-Val-Pro-Cys have also been constructed. The mutant proteins have similar reaction rates with T4 ribonucleotide reductase as has the wild-type T4 thioredoxin.

Mutant T4 thioredoxins with Pro instead of Tyr at position 16 in the active site sequence have three to four times lower apparent $K_M$ with E. coli ribonucleotide reductase than wild-type T4 thioredoxin. The $K_M$ values for these mutant proteins which do not have Tyr in position 16 are thus closer to E. coli thioredoxin than to the wild-type T4 thioredoxin. The bulky tyrosine side chain probably prevents proper interactions to E. coli ribonucleotide reductase. Also the redox potentials of these two mutant thioredoxins are lower than that of the wild-type T4 thioredoxin and are thereby more similar to the redox potential of E. coli thioredoxin. Mutations in position 15 behave more or less like the wild-type protein.

The kinetic parameters with E. coli thioredoxin reductase are similar for wild-type and mutant T4 thioredoxins except that the apparent $k_{cat}$ is lower for the mutant protein with Pro instead of Tyr in position 16.

The active site sequence of T4 thioredoxin has also been changed to Cys-Pro-Tyr-Cys to mimic that of glutaredoxins. This change does not markedly alter the reaction rate of the mutant protein with T4 ribonucleotide reductase or E. coli thioredoxin reductase, but the redox potential is lower for this mutant protein than for wild-type T4 thioredoxin.

Thioredoxins and glutaredoxins are small redox active proteins which have been isolated and characterized from widely different species. The Escherichia coli proteins have been most thoroughly studied (Holmgren, 1985; Holmgren et al., 1986). These proteins have one active site diithiol per molecule. Their main function in cells is probably to reduce ribonucleotide reductase in each activity cycle. This enzyme catalyzes the reduction of ribonucleotides to deoxyribonucleotides for DNA synthesis (for reviews see Thelander and Reichard, 1979; Lammers and Follmann, 1983). Other functions for thioredoxins except that the apparent $k_{cat}$ and $k_{cat}/K_M$ are determined (for a review see Gleason and Holmgren, 1988). The identity between molecules is high in the active site regions. In other parts of the molecules the homology is often low. Most thioredoxins have no detectable amino acid homology to glutaredoxins, except at the carboxyl end. The cysteine residues involved in the redox reactions are in all thioredoxins and glutaredoxins separated by 2 residues. Generally, thioredoxins have the sequence Cys-Gly-Pro-Cys in their active sites. Two thioredoxins have been found in Corvnebacterium nephridii. One has the consensus sequence, and the other has an Ala instead of Gly (Lim et al., 1987). The glutaredoxins have the amino acid sequence Cys-Pro-Tyr-Cys in their active sites (Hoog et al., 1983; Klintrot et al., 1984; Papayannopoulos et al., 1989). T4 thioredoxin differs from the two other families and has the active site sequence Cys-Val-Tyr-Cys (Holmgren and Sjöberg, 1972).

The three-dimensional structures of T4 thioredoxin and E. coli thioredoxin have been determined (Söderberg et al., 1978; Holmgren et al., 1975). The T4 thioredoxins have a similar fold despite the lack of sequence homology (Sjöberg and Holmgren, 1972). The active site is located at the amino-terminal end of a helix in both cases. The location of the active site in T4 thioredoxin is shown in Fig. 1.

We have initiated an investigation on structure and function of T4 thioredoxin using site-directed mutagenesis. Since the residues between the cysteine residues are highly conserved within the thioredoxin and glutaredoxin families, respectively, these residues were chosen as the primary target for our studies. In this study we have changed the active site residues of T4 thioredoxin to mimic the local structure in this region of the thioredoxins and of the glutaredoxins. Here we report our activity measurements of the mutant proteins with thioredoxin reductase and ribonucleotide reductase.

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FIG. 1. Stereo drawing of the α-carbon chain of T4 thioredoxin, the amino acid residues at the active site are shown in thick lines.

TABLE I

Bacterial strains, plasmids, and phages

| Strain   | Genotype                                                                 |
|----------|---------------------------------------------------------------------------|
| C600     | thr-1, leu-6, thi-1, supE44, lacY1, gal, pro, rpsL, hsr−, hsm*             |
| 5K       | hasdR−, derivative of C600                                                |
| JM103    | Δ(lac-pro), thi, strA, supE, endA, sbeB15, hsdR4/F ′ traD36, proAB, lacIg, lacZ Δ M15 |
| JM105    | thi, rpsL, endA, sbeB15, hsdR4, Δ(lac-proRΔ)B/ \ F ′ traD36, proAB, lacIg, lacZ Δ M15 |
| HG2154   | K12 ara, Δ(lac-pro), mutL::Tn10, thi/F ′ proA“B”, lacIg, lacZ Δ M15       |
| HB2151   | K12 ara, Δ(lac-pro), mutL::Tn10, thi/F ′ proA“B”, lacIg, lacZ Δ M15       |
| N4830    | F− su−, his−, ilv−, gal−, Δ 8 (λ c857, ΔBAM, ΔH1)                         |

| Phage    | Genotype                        |
|----------|---------------------------------|
| T4alc7   | am51(g56), N50060(donB−RII), am87(g42) ale7                              |
| M13mp9   | Cloning vector                  |
| M13K19   | Mutagenesis vector              |
| M13mp18  | Cloning vector                  |

| Plasmids | Genotype                        |
|----------|---------------------------------|
| pMG524   | Expression vector (ΔP, Amp')     |
| pBR322   | Cloning vector (Amp', TeT')      |

MATERIALS AND METHODS

Bacterial Strains, Phages, Plasmids, and Enzymes—Restriction endonucleases Asp718, TaqI, BamHI, HindIII, DraI, EcoRI, Rsal, AccI, and Smal were purchased from Boehringer Mannheim GmbH or New England Biolabs and were used according to directions from the manufacturers. Bacteriophage T4 DNA ligase was a kind gift from Prof. G. Magnusson, Department of Virology, University of Uppsala. The Klenow fragment of E. coli DNA-polymerase I was from Pharmacia Biotechnology International. Ribonucleotide reductase from E. coli was prepared according to Sjöberg et al. (1986). T4 ribonucleotide reductase was prepared according to Berglund et al. (1969). E. coli thioredoxin reductase was a kind gift from Prof. A. Holmgren, Department of Physiological Chemistry, Karolinska Institutet, Stockholm.

Oligonucleotides were synthesized by G. Englund, Department of Immunology, University of Uppsala. Deoxynucleotides, dideoxynucleotides, [α−32P]thio-dATP, and [γ−32P]ATP were from Amersham Corp. Deoxynucleotides for extension reactions in site-directed mutagenesis were from Pharmacia Biotechnology International.

The bacterial strains, phages, and plasmids used are listed in Table I.

Cloning of T4 Thioredoxin—Since the nucleotide sequence of T4 thioredoxin was not known when we started our investigation we had to construct a degenerate probe for cloning of the gene. From the amino acid sequence of T4 thioredoxin (Holmgren and Sjöberg, 1972) possible nucleotide sequences were translated using the program Backtranslate, part of the University of Wisconsin Genetic Computer Group program package (Devereux et al., 1984). From the possible translations, the following degenerate oligonucleotide1 was chosen and synthesized by KabiGen, Stockholm:

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16 TyrCysAspAsnAla 20

5′ TAYTGYGAYAAYGC 3′
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The cloning of the T4 thioredoxin gene is described in Fig. 2. The phage T4alc7 was propagated in E. coli 5K and was prepared according to a slightly modified protocol for "Preparing stocks of bacteriophage λ," and DNA was purified according to Maniatis et al. (1982). Plating was done according to Messing (1983). 1200 plaques were screened by hybridization with the 32P-labeled probe. One strong positive clone was found. The size of the cloned fragment was about 1.8 kilobases.

Expression of the Gene—Sodium dodecyl sulfate-gel electrophoresis and Western blotting (Blake et al., 1984) were used to detect expression of the T4 thioredoxin gene. No expression could be detected from the 1.8 kilobase fragment cloned in pBR322. A smaller, 438-base pair RsaI fragment, containing the gene, was isolated and cloned in the Smal site of M13mp18 (Fig. 2). T4 thioredoxin was only expressed when the nrdC gene was in the same orientation as the lacZ gene. For efficient expression, a 520-base pair fragment containing the nrdC gene was transferred from M13K19 to the expression plasmid pMG524 containing the heat-inducible XPL promoter (Fig. 2). The plasmid was transformed into the E. coli strain N4830.

Site-directed Mutagenesis—For mutagenesis a 0.45-kilobase Asp718-BamHI fragment was transferred from M13mp18 to the

1 Y is T or C.
Mutagenic primers contained two to five mismatches, and common codons for highly expressed E. coli proteins were chosen in M13K19 (Sanger and Coulson, 1975) for site-directed mutagenesis. Asp718 and BamHI were used to clone the mutagenized fragments into the tetracycline resistance gene of pBR322. A 438-bp fragment containing the gene was cut with RsaI, which was cloned in M13mp18 which had been cut with SmaI. In the next step the fragment was moved to the vector M13K19 with EcoRI and HindIII. The mutagenized fragments were cloned in the EcoRI-HindIII-digested plasmid pMG524, which contains a heat-inducible XPL promoter for efficient expression of the mutant proteins. The scale of the picture is increased from top to bottom. DNA from phage T4alc7 (C) was cut with TaqI and the fragments were cloned in M13mp9, which had been cut with AclI. The resulting library was screened with a radioactive probe. DNA from a phage that gave positive signal was cleaved with BamHI and HindIII and the resulting 1.8-kilobase fragment (B) was cloned into the tetracycline resistance gene of pBR322. A 458-bp base pair fragment containing the gene (E) was cut out with RsaI, this fragment was cloned in M13mp18 which had been cut with SmaI. In the next step the fragment was moved to the vector M13K19 with Asp718 and BamHI for site-directed mutagenesis. The mutagenized fragments were cloned in M13K19 (Fig. 2). Mutagenesis was performed according to Carter et al. (1985). The mutagenic primers are listed in Table II. Mutagenic primers contained two to five mismatches, and there were generally nine bases flanking the mismatches at each side. The most common codons for highly expressed E. coli proteins were chosen in each case. Mutations were confirmed byideoxy sequencing in M13K19 (Sauger and Coulomb, 1975).

For efficient expression of mutated nrdC genes a 520-base pair EcoRI-HindIII fragment was transferred to EcoRI-HindIII-restricted pMG524 in each case. The nucleotide sequence of the mutant was again confirmed with plasmid sequencing (Chen and Seeburg, 1985).

**Active Site Mutations of T4 Thioredoxin**

**Growth and Heat Induction**—The E. coli strain N4830 containing a pMG524 derivative was grown in 2 liters of 2 × YT (Maniatis et al., 1982). Overnight cultures were diluted to an A600 = 0.1 and grown at 30 °C with vigorous shaking to an A600 = 0.5. Heat induction was performed at 39 °C and the cultures were grown with vigorous shaking to an A600 = 4. Typically 12 g of bacteria (wet weight) were obtained.

**Purification of the Protein**—The purification of wild-type and mutant T4 thioredoxin was performed as described previously (Berglund and Sjoberg, 1970; Sjöberg, 1972) with some modifications. The following column steps were used: Sephadex G-25 for desalting, and DEAE- and CM-cellulose (Whatman, DE52 diethylaminoethyl-cellulose and CM32 carboxymethyl-cellulose) for ion-exchange chromatography. A DIAFLO concentration cell with YM-5 membranes was used for concentration of the proteins. Typical yields were 25–50 mg of protein from 12 g of wet-weight bacteria.

**Determination of Protein Concentration**—T4 thioredoxin contains 4 tyrosines and no tryptophan. Two of the mutant proteins have only 3 tyrosine residues and thus have lower molar absorbance than the wild-type. Based on titration of the sulphydryls with thioredoxin reductase and NADPH (Laurent et al., 1984) we found that 1 A280 unit corresponds to the following concentrations of T4 thioredoxin protein: wild-type (1.52 mg/ml), V15G;Y16P (1.80 mg/ml), Y16P (1.60 mg/ml), V15P (1.47 mg/ml), and V15G (1.44 mg/ml). According to Berglund and Sjöberg (1970) 1 A280 unit = 1.65 mg of protein/ml based on refractometry.

Assays—Enzyme kinetics were monitored with a Shimadzu UV-260 double-beam spectrophotometer equipped with a recorder. The dialidic reductase activity using DTNB was done according to Holmgren (1978) with mutant proteins as substrates. In the assay, the formation of DTNB was measured:

\[
\text{NADPH} + H^+ + \text{TrxRed} = \text{NADP}^+ + \text{TrxRed(SH)}_2
\]

\[
\text{TrxRed(SH)}_2 + \text{TrxS}_{2} = \text{TrxRedS}_2 + \text{Trx(SH)}_2
\]

\[
\text{Trx(SH)}_2 + \text{DTNB}_{2} = \text{TrxS}_2 + \text{DTNB}_{2}\text{red}
\]

where TrxRed is thioredoxin reductase, RibRed is ribonucleotide reductase, S2 is a disulfide bridge, and (SH2) is a reduced dithiol.

The conditions are designed such that thioredoxin (Trx), which is oxidized at the start of the reaction, cycles between oxidized (ox) and reduced (red) forms and provides steady-state rate measurements. The assay is performed at pH 8, the pH optimum for the reaction. The concentrations of the chemicals were 0.08 mg/ml DTNB, 0.1 M Tris-HCl (pH 8), 5 mM EDTA (pH 8), 0.24 mg/ml bovine serum albumin, and 0.12 mM NADPH. Thioredoxin or thioredoxin mutant concentrations were varied between 1.25 and 20 μM. Thioredoxin reductase concentration was 2.1 nM. To be able to assay the mutants in position 16, 4.2 mM thioredoxin reductase had to be used. The concentrations of the chemicals were 0.08 mg/ml DTNB, 0.1 M Tris-HCl (pH 8), 5 mM EDTA (pH 8), 0.24 mg/ml bovine serum albumin, and 0.12 mM NADPH. Thioredoxin or thioredoxin mutant concentrations were varied between 1.25 and 20 μM. Thioredoxin reductase concentration was 2.1 nM. To be able to assay the mutants in position 16, 4.2 mM thioredoxin reductase had to be used.

The concentrations in the assay of E. coli ribonucleotide reductase with the thioredoxins were 1.5 mM ATP, 12 mM MgCl2, 0.62 mM NADPH, 39 mM HEPES (pH 7.6), 77 μM EDTA, 0.58 mM CDP, 3.9 μM E. coli ribonucleotide reductase, and 0.6 μM thioredoxin reductase. In the assay of T4 ribonucleotide reductase we tested only one concentration, due to shortage of T4 ribonucleotide reductase. The concentrations used in the assay of E. coli ribonucleotide reductase with the thioredoxins were 1.5 mM ATP, 12 mM MgCl2, 0.62 mM NADPH, 39 mM HEPES (pH 7.6), 77 μM EDTA, 0.58 mM CDP, 3.9 μM E. coli ribonucleotide reductase, and 0.6 μM thioredoxin reductase.

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**Assays—Enzyme kinetics were monitored with a Shimadzu UV-260 double-beam spectrophotometer equipped with a recorder.**

**The reduced form of DTNB (red) is two molecules of 5-mercapto-2-nitrobenzoic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.**
Redox equilibria were determined essentially according to Berglund and Sjöberg (1970). The net reduction of thioredoxin with NADPH in the presence of thioredoxin reductase is reversible:

\[ \text{NADPH} + \text{H}^+ + \text{Trx}S_0 = \text{NADP}^+ + \text{Trx}(SH)_0 \]

and the redox potential of the disulfides in the mutant proteins can be determined (Moore et al., 1964; Porquè et al., 1970; Berglund and Sjöberg, 1970). Thioredoxin is initially in the oxidized state. The equilibrium between thioredoxin and NADPH is first driven toward reduced thioredoxin. Then surplus NADP\(^+\) is added to reverse the reaction to establish a new equilibrium. Redox potential of the disulfide in the thioredoxin molecule can be calculated using the known oxidation-reduction potential of the NADP\(^+\)/NADPH couple which is \(-0.305\) V at pH 7 and 20°C. The concentrations in the start solution were 0.1 mM Tris-HCl (pH 7.0) and 1 mM EDTA. Thioredoxin or mutant thioredoxin was first added to the solution and the concentration (≈50 μM) of the protein was determined by measuring the absorption at 280 nm. In the next step NADPH was added, and its concentration (≈70 μM) was determined at 340 nm. To start the reaction 0.5 μM thioredoxin reductase was added. The reaction was monitored at 340 nm with a recorder. When the reaction was completed NADP\(^+\) was added to 1.2 mM concentration to reverse the reaction. The absorption at the new equilibrium was then determined. The reaction was monitored with the start solution in the blank. The absorption of thioredoxin reductase and NADP\(^+\) at 340 nm was independently determined during the assay and corrected for in the calculations.

**RESULTS**

**Construction of Active Site Mutants**—The nucleotide sequence of the cloned structural gene for T4 thioredoxin confirmed the known amino acid sequence of the protein (Holmgren and Sjöberg, 1972). In addition the nucleotide sequence is identical to the independently determined nucleotide sequence of the gene (LeMaster, 1986; Tomaszewski and Rüger, 1987). Four different mutants of T4 thioredoxin were constructed by site-directed mutagenesis (Table II). In one series of mutant T4 thioredoxins the wild-type active site sequence -Cys-Val-Tyr-Cys- was modified to mimic that of E. coli thioredoxin: -Cys-Gly-Pro-Cys-. The single mutants -Cys-Gly-Tyr-Cys- and -Cys-Val-Pro-Cys- were part of this series. In another approach the wild-type sequence was modified to mimic the active site sequence of glutaredoxin: -Cys-Pro-Tyr-Cys-. Model building of the mutant proteins was made on an Evans & Sutherland PS330 display with the FRODO program (Jones, 1978, 1982).

The mutant proteins behaved similarly to the wild-type during purification, and were obtained in good yield. The lower specific absorbance for the two mutant proteins with Pro instead of Tyr is an in situ proof that the proteins are different from the wild-type.

**Activity of the Mutated Thioredoxins**—T4 thioredoxin works as an electron carrier between thioredoxin reductase and ribonucleotide reductase in the enzymatic reduction of ribonucleoside diphosphates. Below we present data concerning the efficiencies of the mutant T4 thioredoxins to be reduced by E. coli thioredoxin reductase. The mutant T4 thioredoxins were then investigated for their ability to reduce T4 ribonucleotide reductase and E. coli ribonucleotide reductase.

**The interaction between thioredoxin and thioredoxin reductase can be studied separately by using DTNB which in reduced form gives rise to a chromophore. The results of a DTNB assay are presented in Table III. The mutant proteins gave essentially wild-type characteristics, except the Y16P thioredoxin which showed a significantly lower apparent \( k_{\text{cat}} \).**

This means that, except for the Y16P protein, there is no major alteration in the efficiency of electron transfer from thioredoxin reductase to the mutant thioredoxins. Assays with the complete enzymatic systems contain several orders of magnitude higher concentrations of thioredoxin reductase. Thus, the alterations of catalytic efficiency of mutant thioredoxins demonstrated below will predominantly reflect their interaction with the ribonucleotide reductase enzymes. Furthermore, thioredoxin reductase shows significantly lower apparent \( K_M \) and higher \( k_{\text{cat}} \) values with the thioredoxins than the two ribonucleotide reductases.

The E. coli ribonucleotide reductase reaction appears to follow ping-pong kinetics (Thelander, 1974). The general rate equation for ribonucleotide reductase with CDP as one substrate and thioredoxin as the other is as follows:

\[ v = \frac{V_{\text{max}}}{1 + \frac{K_{M,\text{CDP}}}{[\text{CDP}]} + \frac{K_{M,\text{Trx}}}{[\text{Trx}]} } \]

The concentration of either CDP or thioredoxin is varied in the assay, keeping the concentration of the other constant. Our present investigation shows that T4 ribonucleotide reductase behaves similarly (data not shown) and exhibits reciprocal plots characteristic of ping-pong kinetics (Cleland, 1970). The following values were calculated for T4 ribonucleotide reductase: \( K_{M,\text{CDP}} = 1.7 \times 10^{-4} \) M and \( K_{M,\text{Trx}} = 4.6 \times 10^{-4} \) M. The corresponding values for E. coli ribonucleotide reductase, based on values from Thelander (1974) with dTTP as the effector, were \( K_{M,\text{CDP}} = 2.7 \times 10^{-4} \) M and \( K_{M,\text{Trx}} = 2.9 \times 10^{-4} \) M.

The rates of reaction for the mutant and wild-type thioredoxins with T4 ribonucleotide reductase (Table IV) were determined. The rates were very similar for all mutants. The E. coli thioredoxin showed weak activity with T4 ribonucleotide reductase at the concentration of enzyme used in the T4 thioredoxin assay system. The activity comparable to that of T4 thioredoxin was reached at about an order of magnitude higher concentration of E. coli thioredoxin (data not shown).

For the reaction with E. coli ribonucleotide reductase, two of the mutants, V15G;Y16P and Y16P, gave three and four times lower apparent \( K_M \) values, respectively, than the wild-type protein (Table V and Fig. 3). The catalytic efficiency \( (k_{\text{cat}}/K_M) \) of these two mutant proteins is significantly higher.
than that of the wild-type protein. In contrast, V15P and V15G had higher apparent $K_M$ than the wild-type protein. However, the accuracy of the V15G $K_M$ value is less than for the others, due to its very low oxidation of NADPH.

The equilibrium constants for the reaction have been calculated for the wild-type and mutant T4 thioredoxins and are given in Table VI. The redox potential for the Y16P mutant approaches the redox potential for the E. coli thioredoxin. The double mutant and the V15P mutant have intermediate redox potentials.

**DISCUSSION**

Sequence comparisons of thioredoxins have shown that the 2 residues Gly-Pro, which separate the active site cysteines, are largely conserved among species. Similarly, the sequence Pro-Tyr has been found in the corresponding position for glutaredoxins. In T4 thioredoxin, however, instead of the Gly-Pro consensus sequence Val-Tyr is found. The present study investigates the extent to which these residues influence the activity of thioredoxin in its redox reactions with ribonucleotide reductase and thioredoxin reductase. We have modified the active site of T4 thioredoxin to mimic the local structure in other thioredoxins and glutaredoxins. Such changes will of course not give perfect resemblance to the other proteins since the surroundings of the disulfide bridge differ.

The interaction areas for thioredoxin reductase and ribonucleotide reductase most probably extend beyond the 4 active site residues. Other residues must contribute to binding or catalysis. Mutations of E. coli thioredoxin demonstrate that residues far away in the primary structure but close to the active site area strongly affect its activity (Model and Russel, 1986). Residues in this area around the active site differ between members of the thioredoxin family and even more so for glutaredoxins (Eknlund et al., 1984; Klintrot et al., 1984; Papayannopoulos et al., 1989). In E. coli thioredoxin, the residues of the disulfide bridge form a short helix, which is tilted relative to the following long helix due to a kink caused by Pro-40. Furthermore, loops around the active site differ in length between T4 and E. coli thioredoxins. Structural comparisons of T4 and E. coli thioredoxin have led to the identification of a hydrophobic surface on one side of the disulfide bridge which was suggested to interact with thioredoxin reductase and ribonucleotide reductase (Eknlund et al., 1984). The main structural difference between T4 thioredoxin and E. coli thioredoxin in this region is Tyr-16. The side chain of Tyr-16 protrudes into solvent in T4 thioredoxin whereas the surface of the E. coli thioredoxin is flat.

The mutant T4 thioredoxins, where Tyr-16 has been substituted have kinetic parameters which deviate from those of the wild-type T4 thioredoxin. For the reaction with E. coli ribonucleotide reductase, which is the most specific of the enzymatic reactions, these two mutants had lower apparent $K_M$ than wild-type T4 thioredoxin, 20 and 30 µM, respectively, compared to 83 µM. The mutants were made more similar to E. coli thioredoxin in this respect and thus had one of the desired effects. An apparent $K_M$ of 1.25 µM (Holmgren 1979) has been reported for E. coli thioredoxin. The lowered $K_M$ for the mutants may be interpreted as stronger binding to E. coli ribonucleotide reductase caused by the change of tyrosine to the less bulky hydrophobic side chain of proline. Val-15 on the other hand is positioned relative to the enzyme interaction area so that the side chain points away from it. Thioredoxins which were changed in position 15 behave more or less like the wild-type protein. The exception is in the E. coli ribonucleotide reductase reaction, where both mutants give higher apparent $K_M$ compared to the wild-type T4 thioredoxin.

Although thioredoxin reductase exhibits narrow substrate specificity it reduces thioredoxins as different as those from T4 and E. coli (Holmgren, 1985). The $K_M$ for thioredoxin reductase differs only by a factor of 2 for the two thioredoxins (Berglund, 1969). Our mutations have rather small effects on the interactions with thioredoxin reductase. The activities of the mutant T4 thioredoxins with T4 ribonucleotide reductase are similar to that of the wild-type thioredoxin. This indicates that a change to smaller residues in the investigated positions has little effect on enzyme-thioredoxin interactions.

A single change from Tyr to Pro in position 16 shifts the redox potential from −0.23 to −0.25 V which is remarkably close to −0.26 V for E. coli thioredoxin. There is a preference for Pro in the sequences of the active site tetrapeptide of thioredoxins and glutaredoxins, and also a preference for Gly in the thioredoxins. Obvious questions are how these residues affect the stability of the active site, and the relationship between their structure and activity. Residues 15 and 16 in T4 thioredoxin adopt helical conformation. Pro in either of these positions need not change the conformation of the main chain, prolines are common in the first turn of a helix. Although Pro can easily fit in positions 15 and 16 in structures
similar to that of oxidized wild-type thioredoxin, it would make the main-chain more rigid. As a result, the switch from one state to the other would be hindered. In contrast, a Gly residue, which in most thioredoxins is present in the equivalent position to 15, would impart greater flexibility to the main-chain.

In the reduced form of thioredoxin, the active site has to adopt a more open conformation since the disulfide bridge is broken. A proline in position 16 would make the main-chain more rigid than any other residue type in this position. It should stabilize the helical conformation of the active site peptide and thus favor the oxidized form of the protein. Consistent with our hypothesis we observe a lower redox equilibrium for mutant T4 thioredoxins with Pro at position 16. This effect can be partly reduced with the more flexible Gly in the preceding position. Position 15 is the first residue of the helix and has larger flexibility than position 16. A mutant with Pro in this position would not be expected to affect the redox potential to the same extent, which is consistent with our results.

In conclusion, our results show that the redox properties of T4 thioredoxin and its interactions with E. coli ribonucleotide reductase can be made to mimic those of the E. coli thioredoxin by mutating the residues between the two active site cysteines to the sequence found in the bacterial protein whereas these changes do not significantly affect other properties of T4 thioredoxin like its interaction with T4 ribonucleotide reductase and E. coli thioredoxin reductase. Generally a change in the second position investigated has a larger effect on activity than the changes in the first position.

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