Characterization of *Escherichia coli* Thioredoxins with Altered Active Site Residues†

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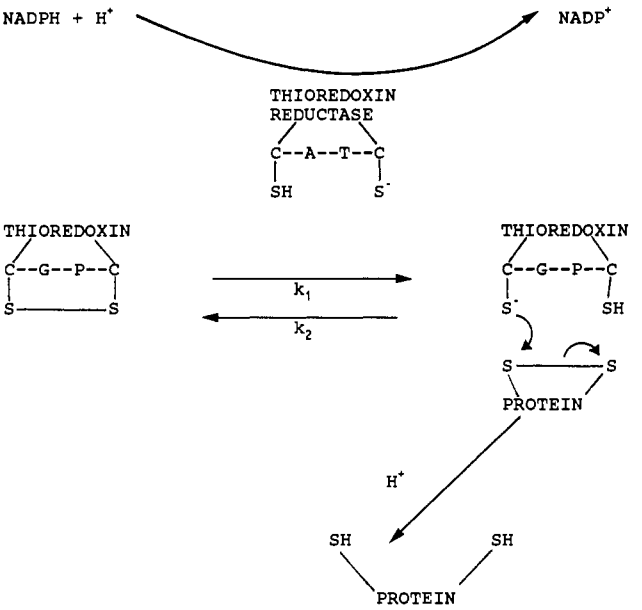
ABSTRACT: *Escherichia coli* thioredoxin is a small disulfide-containing redox protein with the active site sequence Cys-Gly-Pro-Cys-Lys. Mutations were made in this region of the thioredoxin gene and the mutant proteins expressed in *E. coli* strains lacking thioredoxin. Mutant proteins with a 17-membered or 11-membered disulfide ring were inactive in vivo. However, purified thioredoxin with the active site sequence Cys-Gly-Arg-Pro-Cys-Lys is still able to serve as a substrate for thioredoxin reductase and a reducing agent in the ribonucleotide reductase reaction, although with greatly reduced catalytic efficiency. A smaller disulfide ring, with the active site sequence Cys-Ala-Cys, does not turn over at a sufficient rate to be an effective reducing agent. Strain in the small ring favors the formation of intermolecular disulfide bonds. Alteration of the invariant proline to a serine has little effect on redox activity. The function of this residue may be in maintaining the stability of the active site region rather than participation in redox activity or protein–protein interactions. Mutation of the positively charged lysine in the active site to a glutamate residue raises the K_m values with interacting enzymes. Although it has been proposed that the positive residue at position 36 is conserved to maintain the thiolate anion on Cys-32 (Kallis & Holmgren, 1985), the presence of the negative charge at this position does not alter the pH dependence of activity or fluorescence behavior. The lysine is most likely conserved to facilitate thioredoxin–protein interactions.

Thioredoxin is a small (M_r approximately 12000), heat-stable, electron-transfer protein containing an active site cysteine disulfide/dithiol. The protein can be reduced to the dithiol by NADPH in a reaction catalyzed by the flavoprotein, thioredoxin reductase. Reduced thioredoxin participates in a number of redox reactions, acting as a reducing agent for ribonucleotide reductase (Laurent et al., 1964) and methionine sulfoxide reductase (Porque et al., 1970; Ejiri et al., 1980). Thioredoxin can also serve as a general protein disulfide reductase as demonstrated in a model reaction with insulin (Holmgren, 1979; see Scheme I). Thioredoxin, as a protein disulfide reductase, has been shown to reduce disulfide bonds in enzymes such as spinach chloroplast fructose bisphosphatase and NADP-dependent malate dehydrogenase (Clancey & Gilbert, 1987; Scheibe et al., 1986). The subsequent conformational changes in the target protein lead to enhancement of enzymatic activity.

Thioredoxin has been studied most extensively in *Escherichia coli*. In addition to its redox functions, the bacterial thioredoxin is also required for replication of some viruses. The T7 phage requires thioredoxin for growth. The viral gene 5 protein forms a 1:1 association with the host thioredoxin, and the resulting complex is a highly efficient DNA polymerase (Tabor et al., 1987). *E. coli* thioredoxin also plays some unknown role in the assembly of filamentous viruses M13 and f1 (Russel & Model, 1985; Lim et al., 1985b). Investigation of mutant *E. coli* strains which produce nonfunctional thioredoxins (cysteines changed to serines) indicates that although the reduced conformation of the protein is required for viral growth, redox function is not necessary (Huber et al., 1986; Russel & Model, 1986).

The primary structures of 10 bacterial-type thioredoxins have been determined [see Gleason and Holmgren (1988) for

Scheme I: Oxidation–Reduction Cycle of Thioredoxin[a]



[a] Both thiols in the active center of reduced thioredoxin reductase have pK_a's of approximately 7 (O'Donnell & Williams, 1985). Kallis and Holmgren (1980) determined the pK_a values for the cysteines in reduced thioredoxin. That of cysteine-32 is 6.7. k_1 is the rate constant for the forward reaction (reduction), and k_2, the rate constant for oxidation. Although the reduced form of thioredoxin is relevant in vivo, thioredoxin reductase can catalyze both reactions. Reduced thioredoxin is also slowly reoxidized in the presence of oxygen.

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a review]. All show approximately 50% amino acid identity to the *E. coli* protein. The major invariant feature is the 14-membered active site disulfide ring which is composed of residues Cys_{32}-Gly-Pro-Cys_{35}. Although studies of model peptides show considerable steric strain in a small disulfide loop (Hardy et al., 1971), it is nevertheless found in all thioredoxins and a number of related proteins such as glutaredoxin (Holmgren, 1985), thioredoxin reductase (O'Donnell & Williams, 1985), and protein disulfide isomerase (Edman et al., 1985).

We have produced two mutants of *E. coli* thioredoxin in which the size of the active site disulfide ring has been altered. In one of these proteins, designated thioredoxin-R,[1] an arginine residue has been inserted between the glycine and proline residues producing an expanded 17-membered disulfide ring. In the other mutant protein, designated thioredoxin-CAC, the active site is Cys_{32}-Ala-Cys_{35}, with an 11-membered disulfide ring. Both proteins were expressed in *E. coli* cells lacking thioredoxin. The mutant proteins were purified and characterized. Although the thioredoxin with the larger disulfide ring retained some redox function in vitro, the thioredoxin with the smaller ring did not.

In addition, we have characterized a mutant thioredoxin with the proline residue in the disulfide ring changed to a serine. This mutant thioredoxin, P34S, had been investigated previously (Russel & Model, 1986) and reported to have activity similar to wild-type thioredoxin. We have further characterized this protein and find that a proline residue at this position is not essential to maintaining redox activity or efficient interaction with other proteins.

Another constant feature of the active site region of thioredoxins is the presence of a positively charged residue (lysine or arginine) at position 36. It has been proposed that this residue is required to stabilize the thiolate anion of cysteine-32 in reduced thioredoxin. The presence of the positively charged residue may facilitate lowering the pK_a of the sulfhydryl group to 6.7, thus providing a reactive anion species at physiological pH (Kallis & Holmgren, 1980). We have replaced this residue with a glutamic acid. Thioredoxin K36E is still a good reducing agent at pH 7–8, indicating that the pK_a of the cysteine is not directly affected by the charge on residue 36.

Materials and Methods

Materials. *E. coli* DNA polymerase I Klenow fragment, T4 DNA ligase, all restriction enzymes, and molecular weight standards were purchased from Bethesda Research Labs Inc., Gaithersburg, MD, or Boehringer-Mannheim Biochemicals, Indianapolis, IN. These enzymes were used as specified by the manufacturer. Agarose, 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal), and isopropyl β-thiogalactoside (IPTG) were also from Bethesda Research Labs. Dithiothreitol, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), NADPH, bovine insulin, acrylamide, molecular weight standards, high-purity guanidine hydrochloride, and anti-sheep IgG alkaline phosphatase conjugate were from Sigma Chemical Co., St. Louis, MO. Nitrocellulose paper for blotting and sodium dodecyl sulfate (SDS) were obtained from Bio-Rad Laboratories, Richmond, CA. [5-^{3}H]CDP was purchased from New England Nuclear Boston, MA. Unlabeled nucleotides were obtained from Pharmacia/P-L Laboratories, Milwaukee, WI.

E. coli thioredoxin reductase was obtained from an overproducing strain containing the *trx*B gene in a pUC vector (J. A. Fuchs, unpublished results). The enzyme was purified on 2′,5′-ADP–Sepharose and hexylamine–agarose columns (Pigiet & Conley, 1977). A homogeneous preparation of *E. coli* ribonucleotide reductase was a generous gift of B.-M. Sjöberg, University of Stockholm. Polyclonal antibody to *E. coli* thioredoxin raised in sheep was a gift of A. Holmgren, Karolinska Institute.

Bacterial Strains and Phages. Thioredoxin-deficient (*trx*A) strains of *E. coli* JF510 and BH2012 were used as recipients for transformation (Lim et al., 1986). Strain JF510 is the *ilv* C::Tn5 *trx*A7004 derivative of 71-18 (Heidecker et al., 1980). Strain JF521 is an *ilv*C$^+$ *met*E *srl*::Tn10 *rec*A derivative of JF510. Strain BH2012 is *E. coli* K12 F$^-$ *ara*D139? *gal*U *hsr str*A *met*E46 *arg*H1 *trx*A7004 *ilv*C::Tn5. Strain BH5262, which is *E. coli* K12F *ara*D139? *gal*U *gal*K *hsr rps*L *arg*H1 *trx*A7004 *gsh*A *srl*::Tn10, was used for complementation tests. BH5262 is not able to grow on minimal medium unless supplemented with glutathione. Derivatives of BH5262 that are either Gsh$^+$ or *trx*A$^+$ do not require glutathione for growth (Haller and Fuchs, unpublished results). None of the above strains produce thioredoxin which can be detected by enzyme assays or immunoassays. Strain JF510 was also used to test the ability of filamentous bacteriophages M13 and f1 to form plaques. *E. coli* BH2012 was used to determine infection by bacteriophage T7. All strains were grown in Luria–Bertani medium (Miller, 1972) supplemented with glucose or in Davis–Mingioli minimal medium (Davis & Mingioli, 1950) supplemented with required components. Ampicillin was added when necessary at final concentrations of 50 or 30 μg/mL to enriched or minimal medium, respectively. Plasmids pUC13, pUC18, and pTK1 were used as cloning vectors. pTK1 is pTL14 (Sung et al., 1987) with the 1.3-kb M1u1 fragment containing the kanamycin resistance gene from pBS8 (Spratt et al., 1986) inserted into the *Sca*I site. Phages M13mp2, obtained from J. Messing, Rutgers University, and wild-type f1, obtained from M. Russel, Rockefeller University, were used for tests of phage replication.

Construction of Active Site Mutations. Construction of thioredoxins with altered disulfide ring size was facilitated by an *Ava*II restriction site between the codons for glycine-33 and proline-34 in the thioredoxin gene. After cleavage of the gene with the restriction enzyme, the single-stranded ends were either filled in by DNA polymerase Klenow fragment or digested with S1 nuclease. The resulting fragments were ligated with T4 DNA ligase. To generate the mutant with the arginine insert, plasmid pCJF8 (Lim et al., 1985a), containing the wild-type *E. coli* thioredoxin gene, was partially digested with *Ava*II and the ends were filled in with DNA polymerase (Klenow fragment) and then ligated and used to transform strain BH2012. Plasmids with the correct *Ava*II site filled in were identified by electrophoresis of an *Ava*II digest. Difficulty was encountered in generating the deletion mutant by S1 nuclease following partial *Ava*II digestion; therefore, a vector, pUC8-CM (A. Das, unpublished work), that contains no *Ava*II site was used. The *Eco*RI–*Hin*dIII fragment from pCJF8 was transferred into pUC8-CM which has the gene encoding β-lactamase replaced by a gene encoding chloramphenicol resistance, and thus the vector lacks the two *Ava*II sites located in the gene for β-lactamase. The *Ava*II site within *trx*A was digested with nuclease S1 and blunt-end-ligated, and

[1] Abbreviations: thioredoxin-R, mutant thioredoxin with an arginine residue inserted in the disulfide ring, i.e., C_{32}-G_{33}-R-P_{34}-C_{35}; thioredoxin-CAC, mutant thioredoxin with an 11-membered disulfide ring, i.e., C_{32}-A-C_{35}; thioredoxin K36E, mutant thioredoxin with a glutamic acid residue substituted for lysine-36; thioredoxin P34S, mutant thioredoxin with a serine residue substituted for proline-34; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside; IPTG, isopropyl β-thiogalactoside.

the resulting plasmid was used to transform strain JF510. Plasmids that lacked an *Ava*II site were sequenced by the dideoxy chain-termination method (Sanger et al., 1977) modified for use with [^{35}S]dATP. The *Eco*RI–*Hin*dIII fragment was transferred into plasmid pTK1 to generate pTK*trx*CAC, which was used for expression of the mutant thioredoxin-CAC.

Thioredoxin K36E was obtained by random in vitro mutagenesis (Myers et al., 1985). Single-stranded plasmid from pUC119 *trx*A-100 was mutagenized as described and made double stranded by AMV reverse transcriptase. The thioredoxin gene was excised from the plasmid with *Eco*RI and *Hin*dIII, gel purified, and ligated into pUC119 which had been cleaved with *Eco*RI and *Hin*dIII. This plasmid was used to transform strain JF521. Thioredoxin K36E was identified as a clone with decreased ability to use methionine sulfoxide as a source of methionine at 30 °C. The mutant was identified as thioredoxin K36E by DNA sequencing as described above. The mutant thioredoxin allele was transferred as an *Eco*RI–*Hin*dIII fragment to a pTK-1 vector for protein production.

Thioredoxin P34S was cloned from chromosomal DNA of strain A-95 [kindly supplied by M. Russel, Rockefeller University; see Russel and Model (1986)]. Chromosomal DNA was digested with *Kpn*I and *Pst*I, and a fragment of approximately 2.4 kb was eluted from a gel. This fragment was ligated into M13mp18 RF which had been digested with the same enzymes. JF521 was transformed and grown overnight at 30 °C. Phage produced were plaque purified on JF521, and DNA was sequenced as described to confirm the identity of the gene encoding thioredoxin P34S. The *Hin*cII–*Hpa*II insert containing the *trx*A gene was transferred into pTK-1 for protein production.

Enzyme Assays. Three methods were used to detect and quantitate thioredoxin activity.

Method 1. Thioredoxin-catalyzed reduction of insulin by dithiothreitol was monitored as a turbidity increase at 650 nm (Holmgren, 1979). This method was used to detect thioredoxin activity in crude extracts of *E. coli*. Homogeneous thioredoxin from *E. coli* was used as a standard. This method was also applied when estimating the ability of chemically reduced thioredoxins to function as protein disulfide reductases.

Method 2. In the purification of thioredoxin from extracts of *E. coli*, thioredoxin activity was determined by monitoring insulin reduction in the presence of *E. coli* thioredoxin reductase and NADPH [method 4 in Luthman and Holmgren (1982)].

Method 3. In the final steps of purification and for kinetic analysis, thioredoxin-catalyzed reduction of DTNB was monitored in the presence of *E. coli* thioredoxin reductase and NADPH [method 1 in Luthman and Holmgren (1982)].

Ribonucleotide reductase activity was determined by measuring the production of [^{3}H]dCDP. The reaction mixture contained 80 mM HEPES buffer, pH 7.6; 60 μg of bovine serum albumin; 25 mM $MgCl_2$; 3 mM ATP; 1 mM dithiothreitol; 1.5 mM [^{3}H]CDP (approximately 10000 cpm/nmol); and various concentrations of thioredoxin (0.5–50 μM) in a final volume of 0.05 mL. The reaction was initiated by addition of 2.5 μg of *E. coli* ribonucleotide reductase and incubated at 37 °C for 15 min. The reaction was stopped by boiling for 2 min. Deoxynucleotides were separated from ribonucleotides by chromatography on PEI–cellulose plates (Brinkmann Instruments, Inc., Westbury, NY) as previously described (Gleason & Holmgren, 1981). Labeled deoxynucleotide was eluted from the plates with 0.7 M $MgCl_2$ and added to PCS scintillation cocktail (Amersham Corp., Arlington Heights, IL). Radioactivity was determined in a Searle Mark IV scintillation spectrometer. All results were corrected for background generation of dCDP due to dithiothreitol alone.

Purification of Mutant Thioredoxins. E. coli cells harboring mutant thioredoxin genes were grown on enriched medium to an OD_{660} of 0.2, and IPTG (0.1 mM final concentration) was added to the medium. Growth was continued to stationary phase. Cells were harvested by centrifugation and stored at −20 °C. Cells were disrupted by sonication. Thioredoxin was purified by chromatography on DEAE-Sepharose. Protein was eluted from the column with a gradient of 0–0.3 M NaCl in Tris-HCl buffer, pH 7.5, containing 1 mM EDTA. Thioredoxin activity in the eluant was monitored by method 2 or 3. Active fractions were pooled and concentrated by ultrafiltration using an Amicon YM-5 filter. The thioredoxins with the arginine insert in the active site disulfide ring (thioredoxin-R), P34S, and K36E were purified to homogeneity by chromatography on Sephadex G-75. In contrast, the thioredoxin with the smaller disulfide ring (thioredoxin-CAC) eluted from the size-exclusion column with the high molecular weight proteins, suggesting aggregation. This thioredoxin was purified by FPLC using a mono-Q column. Fractions were eluted from this column with a gradient of NaCl (0–0.5 M) in 50 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA. Thioredoxin concentration was estimated from the absorbance at 280 and 310 nm (Kallis & Holmgren, 1980). Purity of the fractions was determined by polyacrylamide gel electrophoresis. Native gels containing 15% acrylamide were run at pH 8.9 and stained with Coomassie blue (Davis, 1964). When comparing mobilities of oxidized vs reduced thioredoxins, gels were preelectrophoresed to eliminate excess persulfate. Molecular weights of thioredoxins were estimated by electrophoresis on Tricine–SDS–polyacrylamide gels. The procedure was modified from that described by Schägger and vonJagow (1987) for determination of molecular weights of proteins with masses less than 20 kDa.

Immunoblotting. The basic procedure for detecting thioredoxin by immunoblotting was adapted from that described by Tsang (Tsang et al., 1983). Proteins were separated on native 15% polyacrylamide slab gels and transferred to nitrocellulose. After transfer, the paper was incubated with sheep polyclonal antibodies to *E. coli* thioredoxin. The reactive bands were visualized by alkaline phosphatase staining (Blake et al., 1984).

Kinetic Studies. Kinetic measurements of the reaction of thioredoxins with *E. coli* thioredoxin reductase were carried out according to method 3 by using a Model 8450A Hewlett-Packard spectrophotometer at 25 °C. The kinetic parameters were obtained from a least-squares analysis of Lineweaver–Burk plots. *E. coli* thioredoxin reductase concentration was estimated from the flavin A_{460} (Thelander, 1967). Ribonucleotide reductase activity was determined by CDP reduction as described above, and data were analyzed as for thioredoxin reductase.

Fluorescence Measurements. Fluorescence spectra were recorded with a Perkin-Elmer MPF-44A spectrometer. Measurements were made in a 1-cm path length cell in a total volume of 1.0 mL. The exciting wavelength was 280 nm, and emission was recorded from 290 to 400 nm at 30 °C. *E. coli* thioredoxin was used as a standard. Thioredoxins were dissolved in the following buffers (50 mM) for determination of pH-dependent fluorescence emission: sodium acetate, pH 5.0; potassium phosphate, pH 5.5–7.5; Tris-HCl, pH 8.0–9.0; ethanolamine hydrochloride, pH 9.5–10.5. Spectra were not corrected for monochromator and detector responses.

Table I: Phage Growth on *E. coli* Cells Harboring Cloned Thioredoxin Genes[a]

phage	thioredoxin				
	wild type	T-R	T-CAC	P34S	K36E
T7	+	+	+	+	+
T7tas1[c]	+	+	+	+	+
M13mp2	+	+[b]	+[b]	ND	+
f1	+	+[b]	ND	+[d]	ND

[a](+) Growth; (−) no growth; ND, not determined. [b]Wild-type phage formed very tiny, unclear plaques. [c]Tas = thioredoxin altered specificity. This is a mutant virus that will replicate in the presence of a variety of heterologous thioredoxins (Lim et al., 1986). [d]Previously described by Russel and Model (1986).

Modeling. Models of the active site of wild-type thioredoxin and mutant proteins with altered ring size were provided by Hans Eklund, Swedish Agricultural University, Uppsala, Sweden. The FRODO system of programs was used (Jones & Thirup, 1986).

RESULTS

Biological Characterization of Mutant Thioredoxins in E. coli. Thioredoxin-deficient mutant strains exhibit reduced colony size when plated on enriched medium. It had previously been shown that the large colony size was restored in transformants containing plasmids coding for *E. coli* thioredoxin (Lim et al., 1986). However, plasmids coding for thioredoxin-R or thioredoxin-CAC were not able to restore normal growth of the mutant strains. Neither was effective in enhancing the growth rate in a *gsh*A *trx*A double mutant which requires glutathione for growth. *E. coli* BH2012 (*trx*A *met*E) is unable to use methionine sulfoxide to satisfy its methionine requirement since thioredoxin is an essential cofactor for the methionine sulfoxide reductase. These mutant thioredoxins were unable to function effectively in this reaction in vivo. In contrast, *E. coli* strains producing mutant thioredoxins, P34S and K36E, had the wild-type phenotype at 37 °C, which indicated that these two mutant proteins were efficient reducing agents in vivo.

E. coli BH2012 will not support the growth of T7 bacteriophage because the host thioredoxin is required for efficient processivity by the viral DNA polymerase (Tabor et al., 1987). This same strain containing the plasmid coding for any of the mutant thioredoxins will support phage replication under conditions where a high copy number plasmid was used. No attempt was made to further quantitate this effect.

E. coli thioredoxin is also required for filamentous phage assembly. Strain JF510, containing plasmids encoding any of the mutant thioredoxins, was able to support growth of these viruses. When plasmids containing thioredoxins with altered ring size were inserted, small unclear plaques were produced in these organisms compared to a strain containing the wild-type thioredoxin, indicating diminished efficiency of these thioredoxins. The activity of thioredoxin P34S in viral replication has been more thoroughly characterized by Russel and Model (1986). Our results are summarized in Table I.

Isolation and Characterization of Mutant Thioredoxins. Altered thioredoxins were purified to greater than 95% homogeneity by chromatography on DEAE-Sepharose and Sephadex G-50 or G-75 and, for thioredoxin-CAC, by FPLC on a Mono-Q ion-exchange column. Thioredoxin-R and thioredoxins P34S and K36E were obtained in high yields. Thioredoxin-CAC exhibited aggregation and poor resolution on size-exclusion chromatography. Purity was estimated by polyacrylamide gel electrophoresis. As seen in Figures 1 and 2, both mutant proteins with altered ring size and thioredoxin

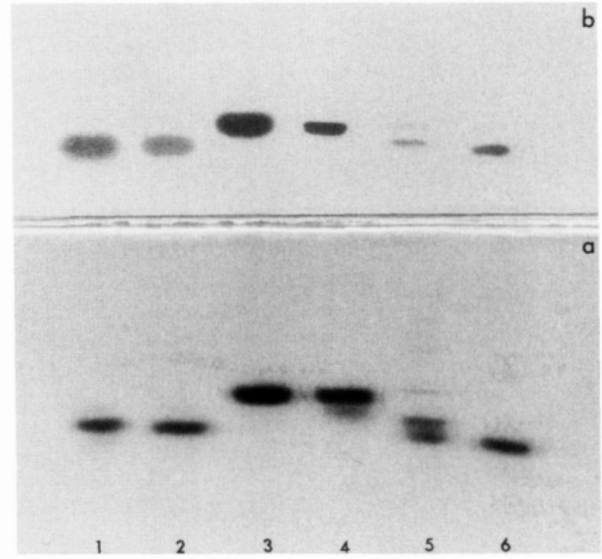

FIGURE 1: (a) Separation of thioredoxins with altered ring size by native polyacrylamide gel electrophoresis. Protein was loaded onto a native 15% polyacrylamide gel and run at pH 8.6. Lane 1, 4 μg of oxidized *E. coli* thioredoxin; lane 2, 4 μg of reduced *E. coli* thioredoxin; lane 3, 7 μg of oxidized thioredoxin-R; lane 4, 7 μg of reduced thioredoxin-R; lane 5, 5 μg of oxidized thioredoxin-CAC; lane 6, 5 μg of reduced thioredoxin-CAC. Proteins were reduced with 0.2 M dithiothreitol before electrophoresis. (b) Western immunoblot analysis of thioredoxins. Proteins (1–2 μg per lane) were separated on native 15% polyacrylamide gels as described above. The proteins were then transferred to nitrocellulose paper and incubated with polyclonal antibodies to *E. coli* thioredoxin. Reactive conjugates were visualized by alkaline phosphatase staining. Lanes correspond to those on the gel as described above.

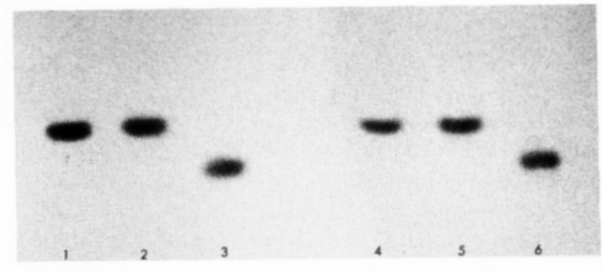

FIGURE 2: Separation of thioredoxins P34S and K36E by native polyacrylamide gel electrophoresis. Proteins were reduced and run as described in Figure 1. Lane 1, 4 μg of oxidized *E. coli* thioredoxin; lane 2, 4 μg of oxidized P34S; lane 3, 3 μg of oxidized K36E; lane 4, reduced *E. coli* thioredoxin; lane 5 reduced P34S; lane 6, reduced K36E.

K36E exhibited altered mobility compared to *E. coli* thioredoxin.

Oxidized thioredoxin-CAC exhibits one major and two or three minor bands on native gels (lane 5, Figure 1). However, as seen in the corresponding Western blot, these bands also react with antibody to *E coli* thioredoxin. They are apparently disulfide-bonded aggregates which form when the protein is oxidized. Reduced thioredoxin exhibits only one band as does *E. coli* thioredoxin. The presence of higher molecular weight aggregates of thioredoxin-CAC was confirmed by electrophoresis in the presence of SDS. As seen in Figure 3, lane 4, the oxidized form of thioredoxin-CAC separates into three bands on denaturing gels. The major band is at a molecular weight of approximately 28 000, indicating that most of the protein forms disulfide-bonded dimers. Presumably these are linked by two disulfide bonds since the oxidized protein does not react with DTNB. A slight band at the top of the gel may be a small amount of higher molecular weight aggregates. In the presence of 6 M guanidine hydrochloride, approximately

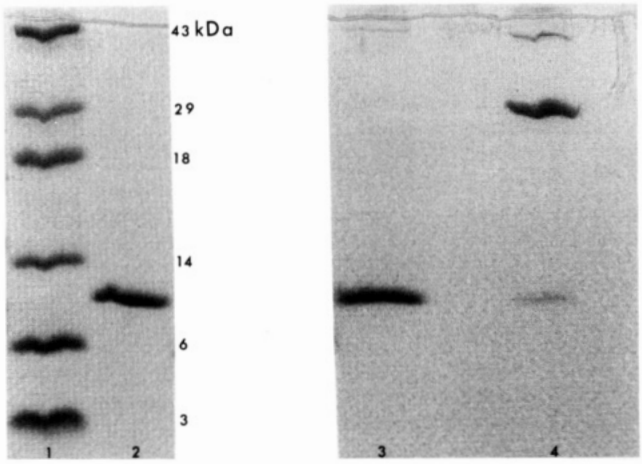

FIGURE 3: Molecular weight determination of thioredoxin-CAC on SDS-polyacrylamide gel electrophoresis. Polyacrylamide (15%) gels containing SDS were prepared with Tris-HCl buffer, pH 8.5. Gels were run for 18 h in a buffer containing 0.1 M Tris-HCl, 0.1 M Tricine, and 0.15% SDS to facilitate separation of low molecular weight proteins. Lane 1, molecular weight markers from Bethesda Research Labs; lane 2, *E. coli* thioredoxin, reduced with dithiothreitol; lane 3, thioredoxin-CAC; lane 4, oxidized thioredoxin-CAC.

Table II: Ability of Thioredoxins To Serve as Substrates for *E. coli* Thioredoxin Reductase[a]

thioredoxin	K_m (μM)	k_{cat} (min^{-1})	catalytic efficiency (k_{cat}/K_m) (μM^{-1} min^{-1})
wild type	2.0	1365	683
thioredoxin-R	125	620	5
thioredoxin-CAC	125	650	5
P34S	2.0	1360	680
K36E	6.7	1360	203

[a] Assay mixtures contained 100 mM Tris-HCl, pH 8.0; 2 mM EDTA; 50 μg of BSA; 0.5 mg of DTNB; and 0.24 mM NADPH in a final volume of 0.5 mL. Thioredoxins were added in a concentration range from 0.05 to 100 μM. The reaction was initiated by adding *E. coli* thioredoxin reductase at a concentration of 42 nM as determined by the flavin absorbance at A_{460} (Thelander, 1967). The increase in A_{412} was monitored at 25 °C, and activities were calculated by method 1 in Luthman and Holmgren (1982).

0.5% of the protein will react with DTNB, suggesting the presence of buried sulfhydryls in the aggregated protein (data not shown). In contrast, one band at a molecular weight of 12000 is seen when thioredoxin-CAC is reduced (Figure 3, lane 3).

Enzymatic Activity. All the mutant thioredoxins are substrates for *E. coli* thioredoxin reductase and are reduced by NADPH via the flavoprotein. As noted in Table II, the K_m values for the thioredoxins with altered ring size are considerably higher than for *E. coli* thioredoxin. In contrast, thioredoxin P34S binds as well as the wild-type protein, while thioredoxin K36E has a higher K_m, most likely due to the addition of a negative charge near the interaction site. Turnover numbers (k_{cat}'s) of mutant thioredoxins with altered ring size were approximately half that of wild-type protein.

The binding of thioredoxin-R to the reductase was pH dependent. The data in Table III illustrate the pH dependence of the K_m values for the different thioredoxins. In contrast to *E. coli* thioredoxin and the other mutant proteins, thioredoxin-R is a better substrate at pH 9 than at pH 7. Turnover numbers did not vary significantly with pH.

The ability of thioredoxins to function as general protein disulfide reductases was determined by monitoring precipitation of reduced insulin in the presence of dithiothreitol and

Table III: Effect of pH on the K_m Values of *E. coli* Thioredoxin Reductase with Mutant Thioredoxins[a]

thioredoxin	K_m (μM) determined at pH 7.0	8.0	9.0
wild type	1.3	2.0	6.7
thioredoxin-R	286	125	83
thioredoxin-CAC	67	125	250
P34S	1.0	2.0	5.0
K36E	4.4	6.7	25

[a] Activities were determined as described in the footnotes to Table II. Turnover numbers (k_{cat}'s) did not vary significantly with pH, except for slightly lower values at pH 7 and 9 when thioredoxin P34S was used as a substrate.

Table IV: Ability of Altered Thioredoxins To Serve as Reducing Agents for *E. coli* Ribonucleotide Diphosphate Reductase[a]

thioredoxin	K_m (μM)	k_{cat} (min^{-1})	catalytic efficiency (k_{cat}/K_m) (μM^{-1} min^{-1})
wild type	3.3	246	75
thioredoxin-R	5.8	66	11
thioredoxin-CAC		not active[b]	
P34S	1.7	238	140
K36E	4.4	240	55

[a] CDP reductase activity was determined by monitoring the formation of dCDP in the presence of 1 mM dithiothreitol at pH 7.6. Details of the assay procedure are given under Materials and Methods. [b] Activity after addition of 60 μM thioredoxin-CAC did not exceed the background due to addition of dithiothreitol alone, 0.02 nmol of dCDP generated/min per reaction.

thioredoxin (method 1 under Materials and Methods). Approximately 0.35 nmol of *E. coli* thioredoxin catalyzes precipitation of 167 μM insulin after incubation for 15 min. The same reaction requires 6.80 nmol of thioredoxin-R. Thioredoxin-CAC, at the same concentration, is not effective within 30 min but will catalyze precipitation at 15 nmol per reaction (data not shown). In contrast, P34S and K36E act at levels comparable to those of wild-type thioredoxin.

Thioredoxin-R and thioredoxins P34S and K36E can also serve as reducing agents in the ribonucleotide reductase reaction. Both K_m and k_{cat} are altered for thioredoxin-R so that the mutant protein is approximately 15% as efficient as the wild-type thioredoxin (see Table IV). The mutant protein, P34S, exhibits a higher catalytic efficiency due to a lower K_m and presumably a tighter binding to the reductase. Thioredoxin K36E is a somewhat less efficient reducing agent, again due to a diminished ability to bind to the interacting protein. Thioredoxin-CAC, at the highest concentration tested (60 μM), does not serve as a reducing agent under these same assay conditions. Reduction does not exceed that due to the addition of 1 mM dithiothreitol alone (0.02 nmol of dCDP produced/min per reaction).

Fluorescence Spectra. E. coli thioredoxin has a typical tryptophan-dominated fluorescence spectrum which shows a 3-fold increase in intensity on reduction (Holmgren, 1972). A similar type of behavior is seen with thioredoxin-R (Figure 4) and, also, P34S and K36E (data not shown). In contrast, oxidized thioredoxin-CAC exhibits a large broad emission maximum characteristic of reduced thioredoxin with no increase in intensity on reduction (see Figure 5). The relative quantum yields for oxidized, reduced, and denatured thioredoxins at pH 7.0 are seen in Table V. All thioredoxins exhibit a red shift in maximum on unfolding, but the shift is less for thioredoxin-CAC. It was previously shown that the increase in fluorescence intensity of *E. coli* thioredoxin is pH dependent. The intensity difference between oxidized and

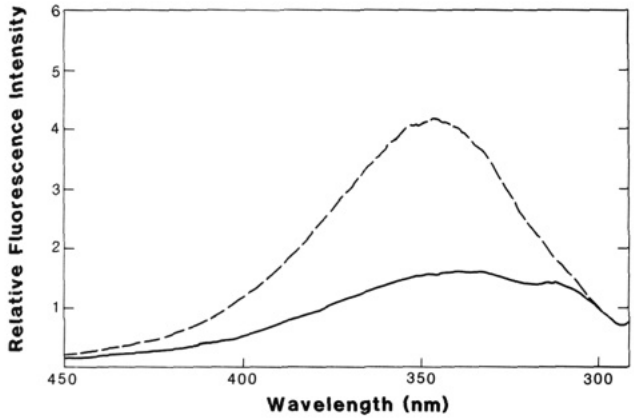

FIGURE 4: Fluorescence emission spectra of thioredoxin-R. Thioredoxin was dissolved in 50 mM phosphate buffer, pH 7.0, containing 1 mM EDTA. Spectra were recorded after excitation at 280 nm. (—) Oxidized thioredoxin-R; (---) reduced thioredoxin-R (1 mM dithiothreitol).

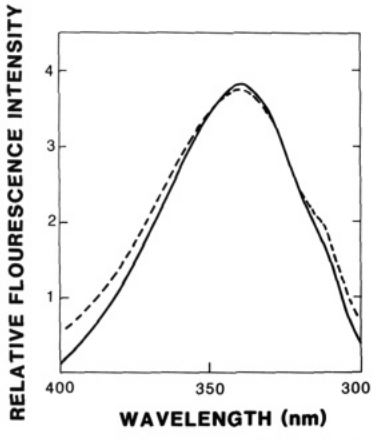

FIGURE 5: Fluorescence emission spectra of thioredoxin-CAC. Protein was dissolved in 50 mM phosphate buffer, pH 7.0, containing 1 mM EDTA. Spectra were recorded after excitation at 280 nm. (—) Oxidized thioredoxin-CAC; (---) reduced thioredoxin-CAC (1 mM dithiothreitol).

Table V: Fluorescence Properties of Altered Thioredoxins[a]

thioredoxin	quantum yields ($\times 10^3$)		
	oxidized	reduced	denatured
wild type	14 (345)	43 (345)	46 (360)
thioredoxin-R	11 (345)	25 (345)	43 (360)
thioredoxin-CAC	100 (340)	88 (340)	32 (345)
P34S	16 (345)	35 (345)	39 (360)
K36E	20 (345)	43 (345)	28 (360)

[a] The excitation wavelength was 280 nm. Spectra were run at pH 7.0 in 50 mM potassium phosphate buffer at 30 °C. Thioredoxins were reduced with 1 mM dithiothreitol. Thioredoxins were denatured in 6 M guanidine hydrochloride in the same buffer. The numbers in parentheses indicate the wavelength of maximum emission. Quantum yields were calculated based on Q of tryptophan in water equal to 0.144 (Wiget & Luisi, 1978).

reduced proteins is greatest at pH 5.5 (Holmgren, 1972). Titration of the thioredoxin-R mutant protein shows a dramatic shift in the intensity maximum of the reduced protein to pH 8.5 (see Figure 6). Similar to *E. coli* thioredoxin, P34S and K36E also exhibit maximum fluorescence emission at pH 5.5. There is no major difference in intensity between oxidized and reduced forms at any pH for thioredoxin-CAC (data not shown).

Molecular Modeling. Molecular modeling was done by using the FRODO system of programs which is based on residue geometry as determined by X-ray diffraction (Jones & Thirup, 1986). X-ray coordinates for oxidized thioredoxin to 1.7-Å resolution were used as input (Katti et al., 1990). The resulting protein models showed little change in the backbone structure after mutagenesis. Alterations were confined to the active site region. As seen in Figure 7, the smaller disulfide ring is readily accommodated without any major shift in the adjoining residues. Insertion of an extra residue into the disulfide loop does

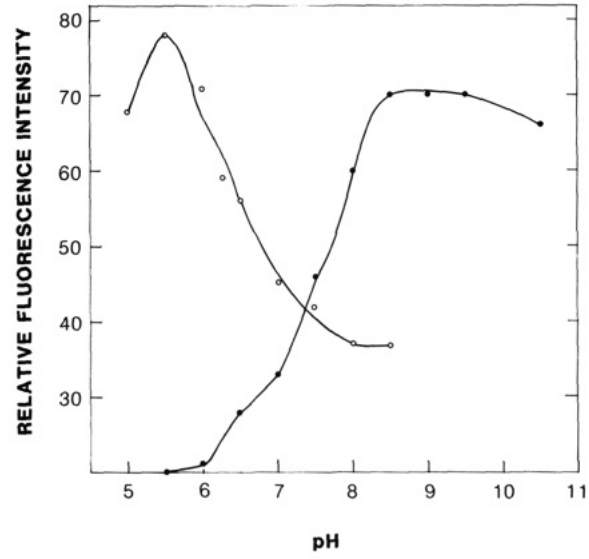

FIGURE 6: Effect of pH on fluorescence intensity of reduced thioredoxins. Thioredoxins were dissolved in buffers at different pH's and reduced with 1 mM dithiothreitol as described under Materials and Methods. Spectra were recorded after excitation at 280 nm. The relative intensity at 350 nm is plotted vs pH for *E. coli* thioredoxin (○) and thioredoxin-R (●).

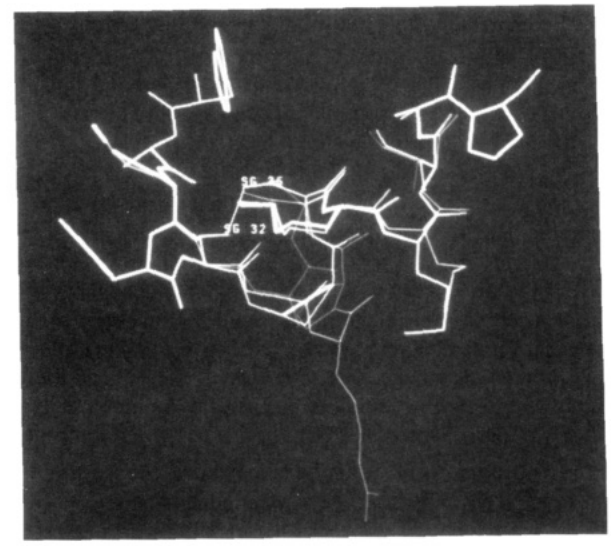

FIGURE 7: Model of the active site of *E. coli* thioredoxin and proteins with altered active site disulfide rings. The models were constructed by using the FRODO system of programs. The disulfide bridge between cysteine-32 and cysteine-35 is labeled in *E. coli* thioredoxin, which is shown in bold lines. The disulfide ring formed by the deletion mutant, thioredoxin-CAC, is seen as a thin line inside the larger, 14-membered ring. Other than the residues between the two cysteines, no other changes in the thioredoxin structure are required to accommodate the smaller 11-membered ring. The structure of the insertion mutant, thioredoxin-R, is also shown as a thin line, outside the normal disulfide ring. The side chain of the arginine residue protrudes from the otherwise flat surface of the disulfide ring. Although its exact position cannot be fixed, modeling indicates that this is the least disruptive conformation for the arginine residue. Shifts in residues in the α-2 helix following the active site are also noted. The N-terminus of the protein is on the left and progresses to the C-terminus at the right.

lead to a minor shift in position of some of the residues in the α-2 helix following the active site, especially residues lysine-36 and methionine-37. The large, guanido side chain of the arginine is shown projecting from the surface of the protein. Mutations at positions 34 and 36 caused no alteration in the backbone structure (data not shown).

Discussion

From a survey of disulfide bonds in proteins, Thornton (1981) reported that small rings are relatively rare. Other than thioredoxin and related proteins, a 14-membered ring is found only in deoxyribonuclease A and posterior pituitary peptide where these are structural features rather than redox active disulfides/dithiols. Investigation of naturally occurring disulfides as well as model studies with small peptides (Snyder, 1987) indicate that a 14-membered ring would be subject to considerable steric strain, while larger rings, having four to six residues between the cysteines, are favored. Since the two-residue separation between cysteines in thioredoxin and related proteins is so highly conserved, it can be argued that it is essential to maintaining the active site structure and redox function of these proteins.

As shown by X-ray diffraction (Holmgren et al., 1975), the active site disulfide of thioredoxin lies in a relatively exposed region on the surface of the protein. Mutations in this region would be expected to cause little change in the overall structure of the protein. Molecular modeling suggests that none of the mutations described here has a significant effect on tertiary structure. Minor shifts in the backbone are observed only to accommodate the thioredoxin with an expanded disulfide ring.

Polyclonal antibodies generally recognize epitopes having an antigenic sequence folded into the correct secondary structure (Benjamin et al., 1984). From an analysis of hybrid thioredoxins, we had previously shown that antibodies to thioredoxin recognize epitopes in the C-terminal half of the protein (Lim et al., 1988). The models showing that the native thioredoxin structure is preserved in the mutants is supported by these data, illustrating that all these thioredoxins react with antibody to *E. coli* thioredoxin (Figure 1).

The mutant thioredoxins will also support phage replication (Table I). While no attempt was made to determine the efficiency in our system, previous work with thioredoxin P34S showed that it was as effective as wild-type protein in supporting both f1 (Russel & Model, 1986) and T7 (Huber et al., 1986) replication at 37 °C. Since the viruses require a native, reduced thioredoxin conformation, the data on viral growth further substantiate that the overall structure of these mutant thioredoxins is conserved in vivo.

The thioredoxin with a larger disulfide ring, thioredoxin-R, retains some redox function. The altered protein is still a substrate for *E. coli* thioredoxin reductase, although a rather poor one (Table II). The difference is mainly in the K_m, suggesting a weak binding of thioredoxin-R to the reductase. However, turnover is about half as rapid as for the wild-type protein, which implies that the larger ring is accommodated and the disulfide can be reduced. The open nature of the thioredoxin reductase active site (O'Donnell & Williams, 1985) can apparently accommodate the larger mutant thioredoxin ring, including the positively charged arginine residue. Recent work with thioredoxin reductase mutants also indicates considerable flexibility in the binding of substrate to this enzyme since either thiol alone may function in reduction (Prongay et al., 1989). The overall catalytic efficiency of the reaction is very low, which explains the lack of enzymatic activity in vivo.

The pH dependence of thioredoxin-R interaction with thioredoxin reductase may be due to changes in the overall surface charge of the molecule as the pH is raised (see data in Table III). It has been suggested that the protein interaction surfaces of thioredoxin are relatively flat, hydrophobic areas around the residues glycine-33, proline-34, isoleucine-75, and proline-76 and the cluster valine-91, glycine-92, and alanine-93 (Eklund et al., 1984). However, lysine-36 and lysine-57 are both near the active site of thioredoxin, and the addition of another positive charge in this area may adversely affect binding to the reductase. As the pH is raised, one of the lysines may be neutralized. It has been reported that pK_a's of lysines can be as low as 6 when the residue is present in a cluster of positive charges as would be the case in this mutant protein (Cleland, 1977). The fact that only K_m but not k_{cat} values change as the pH is raised supports this argument.

Reduced thioredoxin-R will serve as a protein disulfide reductase. The mutant protein will catalyze reduction of insulin by dithiothreitol although significantly more of the mutant protein is required than wild-type thioredoxin. Thioredoxin-R will also function as a reducing agent in the ribonucleotide reductase reaction, but overall catalytic efficiency is low. It appears that the relatively poor reducing ability of thioredoxin-R can be attributed mainly to a disruption of protein–protein interactions rather than to any major defect in redox function caused by an enlarged disulfide ring.

This is in contrast to the thioredoxin-CAC, which is not an effective reducing agent. Strain in the 11-membered ring favors the formation of intermolecular disulfides rather than intramolecular bonding. Inspection of denaturing gels indicates that dimers are apparently the most common form of the purified oxidized protein (Figure 3). Thioredoxin-CAC is a poor substrate for *E. coli* thioredoxin reductase. It is not possible to distinguish whether only the residual monomeric form is reduced or the dimers also serve as substrates. Thioredoxin-CAC, like thioredoxin-R, will react with antibodies to *E. coli* thioredoxin, but the interaction is much weaker than with other thioredoxins (Figure 1b), especially in the case of oxidized thioredoxin-CAC. Staining intensity on the blots should be proportional to protein concentration and antibody interaction (Blake et al., 1984). However, binding of antibody to the dimers and higher molecular weight aggregates will be inhibited if access to epitopes in the C-terminal part of the proteins is hindered. Thioredoxin-CAC also exhibits altered mobility on native gels compared to *E. coli* thioredoxin. In the reduced form, thioredoxin-CAC apparently migrates as a monomer with an extra negative charge, most likely due to the thiolate anion. This altered mobility of the reduced form is not seen in wild-type *E. coli* thioredoxin, suggesting that there is a charge compensation in the native protein which does not occur in the mutant. The reduced form of the mutant protein is kinetically favored. The observation is supported by the fact that thioredoxin-CAC is almost nonfunctional as a protein disulfide reductase with insulin, even though the active site of the mutant protein retains a flat hydrophobic surface for interaction with other proteins. Thioredoxin-CAC fails to serve as a reducing agent in the ribonucleotide reductase reaction where constant redox turnover of a catalytic amount of thioredoxin is required. Both results can be explained a large decrease in the rate of reoxidation (k_2 in Scheme I) of the reduced protein due to ring strain. It can be concluded that disulfide-containing proteins with 11-membered rings would not function effectively in cellular redox reactions.

Mutation of the invariant proline-34 in the active site of thioredoxin appears to have little effect on redox activity. The mutant protein is a good substrate for thioredoxin reductase with a slightly lower K_m (see Table III) as compared to wild-type protein, which agrees with results for P34S reported by Russel and Model (1986). A similar result is seen in the ribonucleotide reductase reaction (Table IV). The fact that thioredoxin P34S can restore the wild-type phenotype in various *E. coli* mutants indicates that it functions as an effective reducing agent in vivo. Since the flat proline residue has been replaced by a short, hydrophilic hydroxyl group in P34S, it would appear that a strictly hydrophobic character at this site is not essential for protein–protein interactions, although similar mutations at the other proposed interaction sites, glycine-74 (Russel & Model, 1986) and glycine-92 (Holmgren et al., 1981), caused a marked decline in enzymatic activity. Proline-34 is conserved in all known thioredoxins but is replaced by a histidine in protein disulfide isomerase (Edman et al., 1985). Our results indicate that conservation of the proline at this site does not seem to be related to redox function but may be important in maintaining the structural stability of the active site region. This conclusion is supported by the data of Russel and Model (1986), who showed that thioredoxin P34S does not support f1 phage replication at 42 °C, although it is effective at lower temperatures. Proline-34 may also confer additional specificity which is not obvious from our investigation.

It has been proposed that the positively charged residue at position 36 in thioredoxin is important in stabilizing the thiolate anion of cysteine-32, thus making thioredoxin an efficient reducing agent at physiological pH (Kallis & Holmgren, 1980). Replacing Lysine-36 with a negatively charged glutamic acid has no effect on the overall structure of the protein. However, the negatively charged carboxylate might be expected to destabilize the thiolate anion. The mutant protein would then function poorly as a reducing agent at pH 7–8. However, thioredoxin K36E serves as a good hydrogen donor for ribonucleotide reductase (Table IV) and catalyzes the reduction of insulin as effectively as wild-type protein. K_m values for the mutant protein with both ribonucleotide reductase and thioredoxin reductase are higher than with *E. coli* thioredoxin, but turnovers are comparable. The data indicate that the charge on residue 36 is important for efficient thioredoxin-protein interactions, but it apparently does not affect the pK_a of the active site cysteine.

Two tryptophan residues, at positions 28 and 31, are also highly conserved in the active site region of prokaryotic thioredoxins. Tryptophan-28 is partially buried behind the disulfide, while tryptophan-31 is on the surface of the protein and can be seen in Figure 7. The static fluorescence behavior of these residues in *E. coli* thioredoxin has been previously characterized. The fluorescence emission, principally that of tryptophan-28, is quenched presumably by its proximity to the disulfide bridge. An approximately 3-fold increase in intensity is seen on reduction (Holmgren, 1972). From NMR data, it can also be concluded that the position of this tryptophan changes significantly on reduction (Dyson et al., 1989). The fluorescence can thus be used as an indicator of the state of the active site.

Thioredoxin-R and thioredoxins P34S and K36E all exhibit an increase in fluorescence intensity on reduction. The spectra for thioredoxin-R in Figure 4 are typical. The quantum yields in Table V increase 2–3-fold on reduction for all thioredoxins except thioredoxin-CAC. The tryptophan fluorescence in this protein is not quenched in the oxidized form. Because of dimer formation, tryptophan-28 may be further removed from the disulfides and, in addition, the aggregates may provide a more hydrophobic environment around both tryptophan residues. As a result, fluorescence intensity is high in the oxidized form and does not increase on reduction and reconstitution of monomeric protein (Figure 5 and Table V).

The fluorescence intensity of reduced *E. coli* thioredoxin is also dependent on pH. This pH dependency has been attributed to titration of the thiolate anion on cysteine-32 which has a pK_a of 6.7, although interaction with other residues such as Lys-36 may also contribute to quenching (Holmgren, 1972). A titration curve for *E. coli* thioredoxin is shown in Figure 6. Mutant proteins P34S and K36E show similar curves (data not shown). This corroborates the kinetic data which indicate that residue 36 has no influence on the pK_a of cysteine-32. Lysine-32 may contribute to fluorescence quenching as the quantum yield in thioredoxin K36E is higher in the oxidized form than for the wild-type protein. Fluorescence behavior, and presumably dimer formation, of thioredoxin-CAC is not influenced by pH. In thioredoxin-R, the intensity maximum has shifted to alkaline pH (Figure 6). Some other residue with a pK_a of approximately 8 is responsible for tryptophan quenching in this protein. The fluorescence data support the conclusions from kinetic analysis. In addition to the steric hindrance caused by addition of the large, protruding arginine side chain, the addition of another positive charge in the active site region of thioredoxin has a detrimental influence on the redox properties of the protein.

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Regulation of the Redox Potential of General Acyl-CoA Dehydrogenase by Substrate Binding†

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ABSTRACT: Significant thermodynamic changes have been observed for general acyl-CoA dehydrogenase (GAD) upon substrate binding. Spectroelectrochemical studies of GAD and several of its substrates have revealed that these substrates are essentially isopotential for chain lengths of C-4 to C-16 ($E^{\circ\prime}$ = −0.038 to −0.045 V vs SHE). When GAD is bound by these substrates, a dramatic shift in the midpoint potential of the enzyme is observed ($E^{\circ\prime}$ = −0.136 V for ligand-free GAD and −0.026 V for acyl-CoA-bound GAD), thus allowing a thermodynamically favorable transfer of electrons from substrate to enzyme. This contrasts with values reported elsewhere. From these data an isopotential scheme of electron delivery into the electron-transport chain is proposed.

Acyl-CoA dehydrogenases catalyze the oxidation of acyl-CoA[1] esters to their *trans*-2-enoyl CoA products with concomitant reduction of the active site flavin in the first step of the fatty acid oxidation cycle. This cycle is an important source of ATP in biological systems, supplying up to 90% of the ATP in cardiac muscle (Bremer & Osmundsen, 1984). Thus, an understanding of the electron-transferring properties of this class of enzymes is imperative to understanding their role in regulating this vital energy production pathway. While a body of information has been amassed concerning the physiological importance (Tanaka, 1979) and physical (Auer & Frerman, 1980; Thorpe et al., 1979; Ikeda et al., 1985),

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[1] Abbreviations: ACD, acyl-CoA dehydrogenases; BCD, butyryl-CoA dehydrogenase; CoA, coenzyme A; $\hat{E}$, conditional potential; E, measured potential; $E^{\circ\prime}$, midpoint potential; ETF, electron-transferring flavoprotein; FAD, flavin adenine dinucleotide; GAD, general acyl-CoA dehydrogenase; IDS, indigotin-5,5′-disulfonic acid; PYC, pyocyanin; FA-CoA, *trans*-β-2-furylacryloyl-CoA; FP-CoA, β-2-furylpropionyl-CoA; ITS, indigotrisulfonate.

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