Kinetics of Electron Transfer from Thioredoxin Reductase to Thioredoxin†

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ABSTRACT: The reduction of *Escherichia coli* thioredoxin by thioredoxin reductase was studied by stopped-flow spectrophotometry. The reaction showed no dependence on thioredoxin concentration, indicating that complex formation was rapid and occurred during the dead time of the instrument. The k_{obs} for the reaction of approximately 20 s^{-1} probably reflects the rate of electron transfer from thioredoxin reductase to thioredoxin and agrees with the k_{cat} observed by steady-state kinetics. The reaction rate was unaffected by increasing the ionic strength, suggesting a lack of electrostatic stabilization in the interaction of the two proteins. A mutant thioredoxin in which a positively charged lysine in the active-site region was changed to a glutamic acid residue resulted in an electrostatic destabilization. Thioredoxin K36E was still a substrate for the reductase, but binding was impaired so that the rate could be measured by stopped-flow techniques as reflected by a dependence on protein concentration. Raising the ionic strength in this reaction served to shield the negative charge and increased the rate of binding to the reductase.

Thioredoxin reductase is a flavoprotein disulfide oxidoreductase which catalyzes the reduction of a cystine disulfide in thioredoxin. Thioredoxin, in turn, can reduce disulfide bonds in other proteins and serves as a reducing agent in reactions such as ribonucleotide reductase (Gleason & Holmgren, 1988). The basic mechanism of the thioredoxin reductase reaction resembles that found in the family of flavoproteins containing a redox-active disulfide and includes enzymes such as glutathione reductase and lipoamide dehydrogenase [see Ghisla and Massey (1989) for a review]. However, comparison of primary structures of *Escherichia coli* thioredoxin reductase with those of the other flavoproteins shows relatively little sequence identity except in the regions of cofactor binding (Russel & Model, 1988). Although all these proteins contain a pair of redox-active cysteines at the active site, these are separated by two residues in thioredoxin reductase while the other proteins have four residues between the cysteines. Both of the sulfhydryl groups in reduced thioredoxin reductase can be alkylated by iodoacetate, whereas in glutathione reductase and lipoamide dehydrogenase only the sulfhydryl which binds the substrate will react. Presumably, the active site of thioredoxin reductase is more "open" than that of enzymes such as glutathione reductase since, unlike the latter, it must interact with another protein (O'Donnell & Williams, 1985).

The catalytic sequence of these reductases is readily divided into two half-reactions. The first half-reaction is a reduction of the flavin by a pyridine nucleotide and electron transfer to the active-site cystine disulfide. The second step involves binding and electron transfer to the substrate. A number of studies of the first half-reaction in glutathione reductase and lipoamide dehydrogenase have been done using both steady-state and stopped-flow techniques [see, for example, Huber and Brandt (1980) and Massey et al. (1960)]. In contrast, the second half-reaction has not been subject to a transient kinetic analysis. Presumably, it is a simple disulfide exchange. In glutathione reductase, the protonation of the thiolate anion of the product may be the overall rate-limiting step (Wong et al., 1988). In thioredoxin reductase, however, this reaction is of special interest since it involves a precise protein–protein interaction as well as electron transfer. Recent advances in bacterial cloning techniques have allowed us to overproduce both thioredoxin and thioredoxin reductase from *Escherichia coli*. We report here the first rapid kinetic study of the interaction of thioredoxin with reduced thioredoxin reductase.

Materials and Methods

Dithiothreitol, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), bovine serum albumin, catalase, and NADPH were from Sigma Chemical Co., St. Louis, MO. Xanthine oxidase was a gift from Dr. D. E. Edmondson. *Escherichia coli* thioredoxin reductase was obtained from an overproducing strain containing the *trx*B gene in a pUC vector by a procedure modified from that described by Russel and Model (1988). The enzyme was purified on 2′,5′-ADP–Sepharose and hexylamine–agarose columns (Pigiet & Conley, 1977). Thioredoxin K36E was obtained by random in vitro mutagenesis (Myers et al., 1985). The mutant gene was used to transform *E. coli* strain JF521 which lacks any enzymatically or immunologically detectable thioredoxin. Characterization of the mutant protein has been described (Gleason et al., 1990). Wild-type *E. coli* thioredoxin was purified from an overproducing strain containing the thioredoxin gene on a pUC-type plasmid. Purification was by chromatography on DEAE-Sepharose and Sephadex-G50 (Lim et al., 1986). Yeast thioredoxin and thioredoxin reductase were purified by using slight modifications of the published procedures (Porque et al., 1970).

For steady-state kinetics, thioredoxin-catalyzed reduction of DTNB was monitored in the presence of *E. coli* thioredoxin reductase and NADPH [method 4 in Luthman and Holmgren (1982)]. Assay mixtures contained 20 mM potassium phos-

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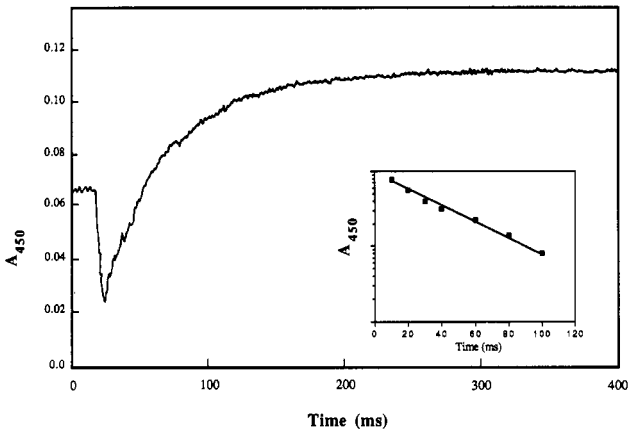

FIGURE 1: Reduction of thioredoxin by reduced thioredoxin reductase as observed by stopped-flow spectrophotometry. The final reaction mixture contained 50 μM *E. coli* thioredoxin and 5 μM *E. coli* thioredoxin reductase which had been previously reduced by NADPH. Additional details of the procedure are described under Materials and Methods. The inset shows a semilogarithmic plot of the data.

phate buffer, pH 7.0, 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. Ionic strength was varied by adding NaCl to the reaction mixture. Thioredoxins were added in a concentration range from 0.05 to 5 μM. Thioredoxin concentrations were estimated from the absorbance at 280 nm (Kallis & Holmgren, 1980). The reaction was initiated by adding *E. coli* thioredoxin reductase at a concentration (13 nmol) determined by the flavin absorbance at 460 nm (Thelander, 1967). The increase in A_{412} was monitored at 25 °C in a Hewlett-Packard 8450A spectrophotometer. The kinetic parameters were obtained from a least-squares analysis of Lineweaver–Burk plots.

Rapid-reaction kinetics were done in a Kinetics Instrument Co. stopped-flow spectrophotometer at 23 °C. The enzyme syringe contained 20 mM potassium phosphate buffer, pH 7.0, 250 μM xanthine, 100 units/mL catalase, 0.1 μM xanthine oxidase, and 10 μM thioredoxin reductase. Samples were deaerated for 2 h under nitrogen prior to the experiments. Thioredoxin reductase was then reduced by addition of 1 equiv of NADPH per FAD. The extent of reduction was determined by monitoring the flavin spectrum. Enzyme spectra were similar to those previously described for the two-electron-reduced reductase [see Williams (1976), Figure 4, p 99]. The substrate syringe contained 20 mM potassium phosphate buffer, pH 7.0, and oxidized thioredoxin in a concentration range of 25–200 μM. To increase the ionic strength, NaCl was added to both solutions. The final concentration of reductase in the mixing chamber was 5 μM. The reaction was monitored by observing the oxidation of the reductase flavin at 450 nm. Data were analyzed either by hand-fitting the experimental curves or by using the program SI-FIT (Olis Co.) on an IBM-compatible XT computer. Equivalent results were obtained by using both procedures.

Results

Electron transfer from reduced *E. coli* thioredoxin reductase to thioredoxin is readily observed in a stopped-flow experiment as shown in Figure 1. Within experimental error, the oxidation of the reductase follows single-exponential kinetics (Figure 1, inset). The reaction rate, as determined from analysis of stopped-flow data measured at various thioredoxin concentrations, was not dependent on substrate concentration as illustrated in Table I. The reaction kinetics were also unaffected by a 6-fold increase in the ionic strength; the k_{obs} value of approximately 20 s^{-1} was unchanged upon addition

Table I: Observed Rate Constants for Reduction of *E. coli* Thioredoxin by Reduced Thioredoxin Reductase[a]

[thioredoxin] (μM)	k_{obs} (s^{-1})	k_{obs} (s^{-1}) + 200 mM NaCl
12.5	19.8	19.2
25.0	22.3	20.0
50.0	19.2	22.5
100.0	19.0	ND[b]

[a] Details of the stopped-flow experiments are described under Materials and Methods. All reactions were performed in 20 mM potassium phosphate buffer, pH 7.0. [b] ND, not determined.

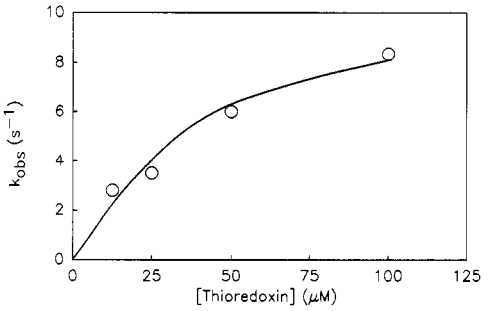

FIGURE 2: Dependence of reaction rate on concentration of mutant thioredoxin K36E. Open circles correspond to data points obtained by stopped-flow analysis. The curve was obtained by a nonlinear, least-squares procedure as described in the text.

of 200 mM NaCl (Table I). Preliminary experiments using a single concentration of yeast thioredoxin and yeast thioredoxin reductase in 20 mM phosphate buffer, pH 7.0, yielded a k_{obs} of 13 s^{-1}, suggesting that a similar reaction rate also occurs with other thioredoxin systems.

Lys-36 lies next to the active-site disulfide, Cys-35 and Cys-32, in thioredoxin. Changing this residue to a negatively charged glutamic acid might be expected to drastically alter the ability of the mutant thioredoxin to bind to the reductase. In contrast to the wild-type protein, the mutant thioredoxin K36E does exhibit a concentration dependence upon reaction with the reductase as determined in the stopped-flow instrument (see Figure 2). The nonlinear dependence on thioredoxin concentration implies a reaction mechanism which consists of at least two steps. From a computer analysis of the data using an exact solution of the differential equations describing the mechanism given in eq 1 [see Simondsen et al. (1982)], the

$$T_{ox} + \text{reductase}_{red} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} T_{ox}\text{–reductase}_{red} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} T_{red}\text{–reductase}_{ox} \quad (1)$$

following rate constants were calculated: $k_1 = 3.6 \times 10^5$ M^{-1} s^{-1}; $k_2 = 9.6$ s^{-1}; $k_3 = 11.0$ s^{-1}. The k_1 and k_2 values must be considered as minimal, although the ratio k_1/k_2 and k_3 are well determined by this analysis [see Simondsen et al. (1982)]. The back-reaction (k_4) is not considered since the equilibrium is unfavorable (Moore et al., 1964).

Steady-state kinetic analysis of the reduction of thioredoxin by thioredoxin reductase was determined under the usual conditions in which a catalytic amount of the enzyme was used. A typical Lineweaver–Burk plot for the wild-type thioredoxin as substrate is shown in Figure 3. The k_{cat} of 23 s^{-1} for this reaction agrees within experimental error with the k_{obs} from the stopped-flow experiments. Raising the ionic strength to 200 mM lowers the K_m but has no effect on the turnover number which is also consistent with the transient kinetics. At low ionic strength, the mutant thioredoxin K36E binds less tightly to the reductase and also exhibits a lower V_{max} (Figure 3). A k_{cat} of 11 s^{-1} using the mutant protein is in excellent agreement with the k_3 value found in the rapid-reaction analysis. In contrast to the wild-type thioredoxin, the mutant

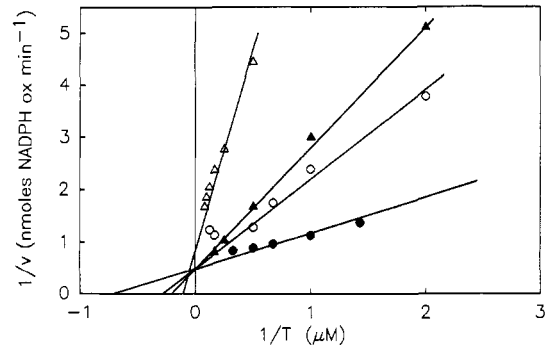

FIGURE 3: Lineweaver–Burk plots of the reduction of wild-type and mutant K36E *E. coli* thioredoxin by NADPH catalyzed by thioredoxin reductase. The open circles correspond to reduction in 20 mM potassium phosphate buffer, pH 7.0, and 13 nmol of thioredoxin reductase. Reduction is apparently inhibited at high concentrations of thioredoxin as seen in the scatter of the data near the origin. These data points were given less weight in the least-squares analysis. The closed circles correspond to reduction under the above conditions with the addition of 200 mM NaCl. Open triangles correspond to reduction of mutant thioredoxin K36E in 20 mM phosphate buffer catalyzed by 13 nmol of thioredoxin reductase. Closed triangles correspond to reduction of mutant thioredoxin K36E in the presence of 200 mM NaCl. Initial velocity (v) was calculated as nanomoles of NADPH oxidized per minute per reaction (Luthman & Holmgren, 1982). Reciprocals are shown as $(1/v) \times 10$.

Table II: Steady-State Rate Constants for Reduction of *E. coli* Thioredoxin by NADPH, Catalyzed by Thioredoxin Reductase[a]

thioredoxin	addition	K_m (μM)	k_{cat} (s^{-1})
wild type	none	3.0	23
wild type	200 mM NaCl	1.1	23
K36E	none	5.6	11
K36E	200 mM NaCl	3.3	23

[a] Activity was determined by monitoring the reduction of DTNB at 412 nm. Details of the assay procedure are given under Materials and Methods.

protein interaction with the reductase is affected by ionic strength. Addition of salt to the reaction mixture increases the relative rate to a maximum at approximately 150 mM NaCl as shown in Figure 4. This result could be caused by nonproductive complex formation at low ionic strength. Similar effects have been noted with cytochrome *c* peroxidase (Hazzard et al., 1988). Increasing ionic strength has little effect on the reduction of the wild-type protein except for a decline in the steady-state rate at high salt concentration, which is also observed with the mutant protein. The cause of this decline is unclear at present. The steady-state kinetic constants for both thioredoxins are summarized in Table II. At 200 mM NaCl, thioredoxin K36E is similar to the wild-type protein in its interaction with thioredoxin reductase.

Discussion

As expressed in eq 1, the reduction of thioredoxin by reduced thioredoxin reductase is at least a two-step reaction, involving initial complex formation between the proteins and subsequent electron transfer. This is clearly shown by the stopped-flow experiments with the mutant thioredoxin K36E. Since no dependence of reaction rate on substrate concentration was observed with the wild-type thioredoxin, it can be assumed that the complex formation step is rapid and occurs in the dead time of the stopped-flow instrument. The k_{obs} value in these experiments thus corresponds to an electron-transfer reaction occurring within a transient complex of the reductase and thioredoxin, which most likely represents the rate-limiting step in the reaction. As previously described, reduced thioredoxin reductase is a mixture of species (Williams, 1976). Under the

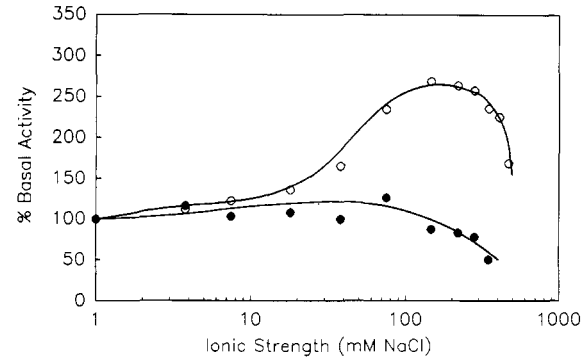

FIGURE 4: Semilogarithmic plot of the dependence of reaction rate on ionic strength. The reduction of thioredoxin by NADPH catalyzed by thioredoxin reductase was monitored at 412 nm as described under Materials and Methods. The final concentration of reductase was 25 nM. The ionic strength of the reaction mix was increased by adding NaCl. Closed circles show the reaction rate in the presence of 0.2 μM wild-type thioredoxin. Basal activity (100%) was 2.5 nmol of NADPH oxidized per minute per reaction. Open circles correspond to the reaction rate observed with 2 μM mutant thioredoxin K36E. Basal activity (100%) was 3.7 nmol of NADPH oxidized per minute per reaction.

conditions used in these experiments, most of the enzyme should be present in the $FADH_2$–S_2 form. However, previous work by O'Donnell and Williams (1983) indicates that at least 30% of the enzyme is in the FAD–$(SH)_2$ form. The reappearance of oxidized flavin is monitored in these experiments and not the actual formation of reduced thioredoxin. It is possible that an intramolecular electron transfer within the reductase may be rate limiting in the reduction of thioredoxin. Electron transfer could proceed from the dithiol form of the enzyme to the disulfide of thioredoxin, or, alternatively, both forms of the reductase could be active. If these different pathways exist, the rate constants must be comparable to one another since we did not observe biphasic kinetics (see Figure 1).

The agreement between the transient kinetic measurements and the steady-state k_{cat} value indicates that the same reaction step is rate-determining in both experiments. Since there is no apparent ionic strength effect on the electron-transfer rate, it would appear that electrostatic interactions between the two proteins are not significant in the electron-transfer step. The fact that the K_m does change on increasing the ionic strength without a corresponding change in k_{cat} (Table II) implies that the complex formation is inhibited somewhat at low ionic strength, consistent with a small repulsive electrostatic interaction which is masked at high ionic strengths.

Lys-36 lies next to the active-site disulfide in thioredoxin, and it has been suggested that this residue stabilizes the thiolate anion at Cys-32 in reduced thioredoxin (Kallis & Holmgren, 1980). Lys-36 is on the surface of the oxidized protein and partially blocks access to the disulfide (Katti et al., 1990). Mutation of this residue to a negatively charged glutamic acid does not impair the ability of thioredoxin to serve as a reducing agent at physiological pH nor does it seem to have a major impact on the structure of the protein; thus, thioredoxin K36E is still a substrate for thioredoxin reductase and a reducing agent for ribonucleotide reductase, but K_m values are higher than for the wild-type protein (Table II; Gleason et al., 1990). However, substitution with a negatively charged carboxyl group apparently disrupts thioredoxin–thioredoxin reductase interactions to a small extent. This is corroborated by our stopped-flow data in which a clear dependence of reaction rate on substrate concentration is seen (Figure 2). The calculated k_1 for this interaction is 3.6×10^5

M^{-1} s^{-1}, and the stopped-flow results imply that interaction with the wild-type thioredoxin is considerably more rapid (greater than 10^7 M^{-1} s^{-1}). Since the rate constant for electron transfer (k_3) is approximately half that of the reaction with wild-type thioredoxin, it appears that the mutation has only a small effect on this process.

Unlike the reaction with wild-type thioredoxin, reduction of the mutant is enhanced by ionic strength up to 200 mM NaCl. Increased salt would be expected to shield the negative charge on the glutamic acid and facilitate binding to the reductase. As seen from Table II, K_m for K36E is lower at 200 mM NaCl, and the k_{cat} value, i.e., the electron-transfer rate constant, is the same as with wild-type thioredoxin. This is consistent with a decrease in the small inhibitory electrostatic influence of the negative carboxyl ion on this process.

The data clearly show that rapid reaction kinetics can be used to study electron transfer between thioredoxin and reductase. Combined with site-directed mutagenesis of residues which are believed to be in the protein interaction site, additional information can thus be obtained on the forces which optimize this interaction.

Registry No. Thioredoxin reductase, 9074-14-0; lysine, 56-87-1; glutamic acid, 56-86-0.

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cDNA Cloning of Porcine Brain Prolyl Endopeptidase and Identification of the Active-Site Seryl Residue†

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ABSTRACT: Prolyl endopeptidase is a cytoplasmic serine protease. The enzyme was purified from porcine kidney, and oligonucleotides based on peptide sequences from this protein were used to isolate a cDNA clone from a porcine brain library. This clone contained the complete coding sequence of prolyl endopeptidase and encoded a polypeptide with a molecular mass of 80 751 Da. The deduced amino acid sequence of prolyl endopeptidase showed no sequence homology with other known serine proteases. [^{3}H]Diisopropyl fluorophosphate was used to identify the active-site serine of prolyl endopeptidase. One labeled peptide was isolated and sequenced. The sequence surrounding the active-site serine was Asn-Gly-Gly-Ser-Asn-Gly-Gly. This sequence is different from the active-site sequences of other known serine proteases. This difference and the lack of overall homology with the known families of serine proteases suggest that prolyl endopeptidase represents a new type of serine protease.

Prolyl endopeptidase (PE)[1] is a cytoplasmic protease (Dresdner et al., 1982; Green & Shaw, 1983) that is ubiquitously distributed among mammalian tissues (Yoshimoto et al., 1979; Kato et al., 1980). It is one of several proteases that are specific for proline [reviewed in Walter et al. (1980)]. However, in contrast to other proline-specific proteases that act as aminopeptidases, carboxypeptidases, or dipeptidases, PE cleaves peptide bonds on the C-terminal side of prolyl residues within peptides that are up to approximately 30 amino acids long (Walter & Yoshimoto, 1978; Taylor & Dixon, 1980;

† The nucleic acid sequence in this paper has been submitted to GenBank under Accession Number J05311.

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[1] Abbreviations: Ac, acetyl; CH_2Cl, chloromethane; DFP, diisopropyl fluorophosphate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PE, prolyl endopeptidase; pNA, *p*-nitroanilide; PTH, phenylthiohydantoin; TLCK, tosyllysine chloromethyl ketone; TPCK, tosylphenylalanine chloromethyl ketone; Z, benzyloxycarbonyl.

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