

G33D Mutant Thioredoxin Primarily Affects the Kinetics of Reaction with Thioredoxin Reductase. Probing the Structure of the Mutant Protein[†]

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ABSTRACT: *Escherichia coli* thioredoxin is a redox-active protein. A mutant protein with an aspartic acid substitution for the largely conserved glycine at position 33 (G33D) in the active site of thioredoxin has been generated to study the effects of a negatively charged residue in the active site of the protein. Despite the close proximity of the negative-charged Asp to the redox active cysteines, the effective concentration of the cysteines does not deviate significantly from that of the wild-type protein. The redox potential (E°) measured by the equilibrium between NADPH and the mutant thioredoxin is also close to that of the wild-type. Kinetic measurements of the reaction between thioredoxin and thioredoxin reductase show that G33D mutant and the wild-type proteins have identical k_{cat} values. However, the K_m for G33D mutant is approximately 10-fold higher than that for the wild-type protein. In vivo assay of the growth of *E. coli* strain carrying wild-type or G33D mutant thioredoxin on methionine sulfoxide indicates that the G33D mutant protein is a slower electron donor for methionine sulfoxide reductase. Structural stability of the oxidized protein is not altered by the G33D substitution, as illustrated by the same unfolding free energies studied by urea. The substitution does not show significant change of the near UV and far-UV circular dichroic (CD) and the fluorescence spectra for either the reduced or the oxidized protein. Therefore, the global structure of the G33D protein is not changed. However, the surface of the active site has been altered locally by G33D substitution, which accounts for the above kinetically poor behaviors. A model of G33D structure is constructed based on these studies.

Thioredoxin is a small protein of M_r approximately 12 000. It was initially isolated as a reducing agent for ribonucleotide reductase (1) and methionine sulfoxide reductase (2, 3). Subsequently, it was found in all living organisms from archaeobacteria to humans (4–6). The protein serves as a general protein disulfide reductase. It is an effective reductant of insulin in animal cells (7), and an activator of certain enzymes of carbon dioxide fixation, such as spinach chloroplast fructose biphosphatase and NADP-dependent malate dehydrogenase (8, 9) in higher plants. It is also involved in the activation of glucocorticoid receptor (10).

Escherichia coli thioredoxin has been studied most extensively. The three-dimensional structure of the oxidized form of the protein has been determined by X-ray crystallography (11) and refined to 1.68 Å (12). The NMR structures of the oxidized and the reduced thioredoxin have also been obtained in high resolution (13, 14). Thioredoxin consists of a central core of five symbol β strands enclosed by four α helices. The active site is composed of residues Cys32-Gly-Pro-Cys35. It is located at the amino end of $\alpha 2$ and after $\beta 2$ on the surface of the protein. Cys 32 is exposed to solvent, whereas Cys 35 is recessed and interacts with residues in other parts of the molecules.

The active-site sequence is largely conserved among thioredoxin of different species. Intriguingly, Harms et al.

(15) recently isolated thioredoxin from *Clostridium sticklandii* and found that it has a Glu at the position 33 (*E. coli* nomenclature) of the active-site consensus sequence. This thioredoxin does not react with thioredoxin reductase from *E. coli* using a complementation assay in which NADPH formation from dithiothreitol was determined (15). *C. sticklandii* thioredoxin varies from *E. coli* thioredoxin in several positions (15). Therefore, it is not clear whether the Gly \rightarrow Glu replacement is responsible for the incompatibility. Mutation of the consensus active site sequence in *E. coli* thioredoxin has been performed by a number of studies (16–19), but not with a negatively charged residue. To understand the effects of a negatively charged amino acid in the active site on the structure and function of the protein, we have changed Gly at position 33 of thioredoxin to Asp by site-directed mutagenesis. The redox potential, kinetic parameters of redox reactions, structural properties, and stability of the mutant thioredoxin were compared with those of the wild-type protein. The structural model of the mutant protein was then constructed on the basis of these results.

EXPERIMENTAL PROCEDURES

Materials. Dithiothreitol, NADP, NADPH, methionine sulfoxide, bovine serum albumin, and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB)¹ were purchased from Sigma. Restriction enzymes, T4 DNA ligase, and T4 polynucleotide kinase were obtained from New England Biolabs, and Promega. Isopropyl- β -D-thiogalactopyranoside (IPTG) was from Medford Laboratory Ltd. (England). Sodium dodecyl

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sulfate (SDS) was purchased from Merck. DNA sequencing was performed by a Perkin-Elmer 377 DNA auto sequencer using ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer). DEAE Sepharose Cl-6B, Sephadex G-50, and Sephadex G-100 were obtained from Pharmacia Biotech.

Bacterial Strains. *E. coli* strains A179 [HfrC(λ)-*trxA::kan*] and A179(pGP1-3) were generously provided by Dr. S. Tabor (Harvard Medical School). pGP1-3 provides a source of T7 RNA polymerase for expression of thioredoxin cloned in pET plasmids under control of T7 transcription. *E. coli* SK3981 was generously supplied by Dr. S. R. Kushner (University of Georgia). Dr. M. Russel (Rockefeller University) kindly provided *E. coli* A278/pPMR14 that carries a cloned *trxB* gene, A312 (*metE::Tn10*), and A313 (*trxA::kan metE::Tn10*) (20).

Construction of Mutant Plasmid pET/G33D. The G33D mutant was generated by sequential PCR reactions (polymerase chain reactions) using a plasmid which contains a thioredoxin gene (pET/Trx) as a template. For the first PCR reaction, T7 primer 5'-TAATACGACTCACTATAGGG-3' was used as 5' primer, as well as an oligonucleotide with the sequence 5'-TTTGCACGGATCGCACCCTCTGC-3' was used as 3' primer. The underline indicates the position of the base substitution. The PCR reaction was repeated 30 times by using a Perkin-Elmer GeneAmp PCR System 2400. The reaction product was used as 5' primer for the second PCR reaction. The 3' primer was 5'-CCAGAACCAGAT-CAGGCCAGGTTAGC-3'. The product of PCR reaction was treated with T4 kinase and digested by *Xba*I. The resulting DNA was ligated with the larger fragment of pET/Trx that had been digested with *Xba*I and *Msc*I to yield the G33D mutant plasmid (pET/G33D). The nucleotide sequence of the mutant gene was confirmed by DNA sequencing using a Perkin-Elmer 377 DNA autosequencer. pET/Trx and pET/G33D were then transformed into *E. coli* strain A179 (pGP1-3) to obtain A179 (pGP1-3, Trx) and A179 (pGP1-3, G33D), respectively.

Purification of Thioredoxin. Five hundred milliliters of A179 (pGP1-3, G33D) culture was grown to OD₆₀₀ of 0.8. IPTG was added to a final concentration of 0.4 mM. The culture was then grown at 37 °C for 10 min, switched to 42 °C for 20 min, and then returned to 37 °C for 3 h. The cells were harvested. The pellet was resuspended in 0.05 M Tris, 1 mM EDTA, pH 7.5 (buffer A), with 1 mM phenylmethanesulfonyl fluoride (PMSF), sonicated to obtain the crude extract. The crude extract was heated to 80 °C for 10 min and centrifuged at 29000g, 25 min, to obtain the supernatant. Ammonium sulfate was added to the supernatant until 77% saturation, and the precipitate was collected by centrifugation at 18400g for 100 min at 4 °C. The precipitate was dissolved in 15 mL of buffer A. Streptomycin sulfate was added to the solution to yield a final 0.7% (w/v). The solution was centrifuged at 18400g for 100 min at 4 °C. The supernatant was dialyzed against buffer A and applied to a DEAE column. Thioredoxin was eluted with a gradient of

NaCl from 0 to 0.4 M in buffer A. The elution was monitored by absorbance at 280 nm. The peaks eluted were checked by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and the fractions corresponding to thioredoxin were pooled and concentrated. The solution was then loaded onto a G-50 gel filtration column and eluted by buffer A. The elution was monitored at 280 nm, and peaks were checked by SDS-PAGE. The thioredoxin peak was collected, dialyzed, and lyophilized. Thioredoxin thus purified yielded a single band on SDS-PAGE. Wild-type thioredoxin was purified from one liter of stationary-phase culture of *E. coli* SK3981 following the above purification procedures without induction.

Purification of Thioredoxin Reductase. Thioredoxin reductase was purified from *E. coli* A278/pPMR14. The culture was grown in LB/ampicillin medium to mid-log phase. The bacteria were spun down and washed with 0.05 M Tris/HCl and 0.05 M NaCl, pH 7.4, and resuspended in 25 mL of 0.05 M Tris/HCl and 1 mM EDTA, pH 7.4. The cells were broken by French Press. Nucleic acids were precipitated in a solution of 1.4% streptomycin sulfate. After centrifugation, ammonium sulfate was added to the supernatant to 82% saturation, the precipitate was collected, redissolved and dialyzed against 0.05 M Tris/HCl, 1 mM EDTA, 0.06 M NaCl, pH 7.4, and applied to a DEAE column equilibrated with the same buffer. The column was developed with a linear gradient of 0.06 to 0.5 M NaCl in 0.05 M Tris and 1 mM EDTA, pH 7.4, and monitored by absorbance at 280 nm. Fractions containing the largest amount of thioredoxin reductase as checked by SDS-PAGE and DTNB assay were concentrated and applied to a Sephadex G100 column in 0.05 M Tris, 1 mM EDTA, and 0.06 M NaCl, pH 7.4. The elution was monitored by absorbance at 280 nm. Fractions containing the largest amount of thioredoxin reductase as checked by SDS-PAGE were concentrated and rechromatographed through the same column to remove the residual impurity. The purified protein appears 99% pure on SDS-PAGE.

Electrophoresis. SDS-PAGE analysis of reduced and denatured proteins was performed in 10–15% SDS-PAGE gel as described by Laemmli (21). Native gel electrophoresis for the wild-type and the mutant thioredoxin was run on 15% polyacrylamide gel under nondenaturing condition (22).

DTNB Assay. The procedure follows that of Moore et al. (23) and Slaby and Holmgren (24). The assay mixture contains 0.1 mg/mL of bovine serum albumin, 0.5 mM DTNB, 50–500 nM thioredoxin, 0.24 mM NADPH in a solution of 0.1 M Tris/HCl, and 2 mM EDTA, pH 7.5. The reaction was started by addition of thioredoxin reductase, and the absorbance was followed at 412 nm in a GBC 918 spectrophotometer with an extinction coefficient of $\epsilon_{412} = 13\,600\text{ M}^{-1}\text{ cm}^{-1}$ for thionitrobenzoate. Kinetics of the reaction was studied by using 0.1–5 μM wild-type or 0.2–50 μM G33D thioredoxin, 2–7 nM thioredoxin reductase, 0.24 mM NADPH, 0.1 mg/mL BSA, 0.5 mM DTNB in 0.1 M sodium phosphate buffer, 2 mM EDTA, pH 7.

Measurements of Redox Potential. The oxidized form of thioredoxin is converted to the reduced form in a reaction catalyzed by thioredoxin reductase according to the following reaction (25):



¹ Abbreviations: DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); IPTG, isopropyl- β -D-thiogalactopyranoside; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CD, circular dichroism; PMSF, phenylmethanesulfonyl fluoride; PCR, polymerase chain reaction; MetSO, methionine sulfoxide.

The reaction was performed by adding 10 nM thioredoxin reductase to 5–15 μM thioredoxin and 20–24 μM NADPH in 0.1 M sodium phosphate buffer and 2 mM EDTA, pH 7. The reduction of oxidized thioredoxin was monitored by the decrease in absorbance at 340 nm (23). The extinction coefficients for NADPH and NADP⁺ were $\epsilon_{340} = 6.2 \text{ mM}^{-1} \text{ cm}^{-1}$ and $\epsilon_{260} = 15.3 \text{ mM}^{-1} \text{ cm}^{-1}$, respectively (26, 17). After the equilibrium had been reached, 1.2 mM NADP⁺ was added to generate a new equilibrium. The redox potential ($E^{\circ'}$) was determined by the following equation:

$$E^{\circ'} = E_{\text{NADP}^+}^{\circ'} + \frac{RT}{nF} \ln \frac{[\text{Trx}_{\text{SH}}^{\text{SH}}][\text{NADP}^+]}{[\text{Trx}_{\text{S}}^{\text{S}}][\text{NADPH}]}$$

where a value of -0.315 V was used for the redox potential of NADP⁺ (27).

Measurements of Effective Concentration. For a protein with two cysteines that can form a disulfide bond, C_{eff} is that ratio of the pseudo equilibrium constant for the disulfide formation in the protein to that for intermolecular disulfide formation in a reference such as glutathione (28, 29):

$$P_{2\text{SH}} + \text{GSSG} \xrightleftharpoons{K_{\text{eq}}} P_{\text{SS}} + 2\text{GSH}$$

$$C_{\text{eff}} = \frac{[P_{\text{SS}}][\text{GSH}]^2}{[P_{2\text{SH}}][\text{GSSG}]} = K_{\text{eq}}$$

where $P_{2\text{SH}}$ refers to reduced protein, and P_{SS} refers to oxidized protein. The method for measuring C_{eff} has been described by Lin and Kim (29). Briefly, the protein (approximately 5 μM) is incubated with reduced (GSH) and oxidized (GSSG) glutathione in 0.1 M Tris, 0.2 M KCl, and 1 mM EDTA, pH 8.7. The concentrations of GSH and GSSG are at least 50-fold higher than that of the protein. The reaction is performed under nitrogen to prevent air oxidation. After equilibration (checked after 2 h of incubation), the reaction mixture is quenched with HCl to pH 2 and then loaded on to a C18 reversed-phase HPLC column. The reduced and oxidized forms of the protein are separated. The peaks are detected by absorbance at 229 nm, and quantitated by integration.

Assay of Thioredoxin Function in Vivo. *E. coli* A313 (20) containing pET(pGP1–3, G33D) or pET(pGP1–3, WT) were plated on the M9 minimal agar supplemented with 10 $\mu\text{g}/\text{mL}$ or 40 $\mu\text{g}/\text{mL}$ methionine sulfoxide (MetSO). Ten micrograms per milliliter of tetracycline, 10 $\mu\text{g}/\text{mL}$ of kanamycin, 20 $\mu\text{g}/\text{mL}$ of ampicillin, and 0.5 mM IPTG were supplemented when required. The plates were incubated at 37 °C, and the colony numbers were observed at different time intervals. The colony numbers obtained for these strains growing on minimal medium containing Met instead of MetSO were used as reference for evaluating plating efficiencies.

Circular Dichroism Measurements. The CD spectra of the wild-type and the mutant proteins were determined with a Jasco model J-715 circular dichroism spectropolarimeter at 25 °C. For far-UV CD, the measurements were done at a protein concentration of approximately $9 \times 10^{-6} \text{ M}$ using a 1 mm path-length quartz cell. For near-UV CD, the protein concentration was approximately $6 \times 10^{-5} \text{ M}$ in a 1 cm path-length cell. The buffer was 0.05 M sodium phosphate and

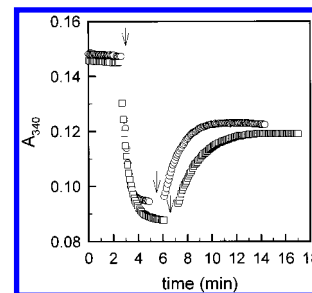


FIGURE 1: Thioredoxin reductase catalyzed reduction of wild-type and G33D thioredoxin. Reduction of disulfides in 8.6 μM wild-type thioredoxin (O) and 9.3 μM G33D (□) was started by addition of 15.7 nM *E. coli* thioredoxin reductase (indicated by the first arrow in the time course). When the reaction reached equilibrium, NADP⁺ was added (indicated by the following arrows) to a final concentration of 1177 μM . The formation of NADPH was followed from the increase at 340 nm. Redox potential was determined from at least three such experiments.

0.1 mM EDTA, pH 7.5. For the reduced protein, approximately $6 \times 10^{-5} \text{ M}$ protein was first reduced by about 10 times higher concentration of DTT. The solution was then diluted to the final protein concentration for measurements.

Fluorescence Measurements. Protein fluorescence was measured with a thermostated Hitachi F-4500 spectrofluorimeter at 25 °C. The excitation wavelength was at 280 nm. The band width was 5 nm for both excitation and emission. The samples contained approximately 7 μM concentrations of thioredoxin in 1 mL of 50 mM sodium phosphate, pH 7.5. Reduction of oxidized thioredoxin or G33D mutant protein was achieved by addition of 20-fold higher concentration of DTT at 0.4 mM concentration of protein, and then dilution to the final protein concentration for measurements. Solvent blanks were subtracted from the sample spectra. The spectra were normalized to $1 \times 10^{-5} \text{ M}$ of protein.

RESULTS

Mutagenesis and Purification of G33D Mutant Thioredoxin. The amino acid residue Gly33 in *E. coli* thioredoxin is largely conserved in homologous proteins from different species. To investigate the effects of a negative charge in the active site on the structure and function of thioredoxin, mutant thioredoxin with a Gly33 → Asp substitution was generated by site-directed mutagenesis. An oligonucleotide corresponding to the antisense strand except for one base mismatch was used as one of the primers in the PCR reaction for producing the Gly33 → Asp mutation. The mutant *trxA* was confirmed by the DNA sequence of the entire gene. The protein was expressed and purified by DEAE ion-exchange and G50 gel filtration chromatography. G33D thioredoxin appears as a single band at the same position as the wild-type thioredoxin on a SDS–PAGE gel. Electrophoresis of the G33D mutant protein on a native polyacrylamide gel results in a band running faster to the anode than the wild-type protein, demonstrating that the mutation causes a lowering of the isoelectric point of the protein.

Measurement of Redox Potential. The disulfide form of thioredoxin is a substrate for *E. coli* thioredoxin reductase. Reduction of the wild-type and the mutant thioredoxin by NADPH catalyzed by *E. coli* thioredoxin reductase was studied to obtain redox potential (Figure 1). The value of $E^{\circ'}$ is -0.267 V for the wild-type protein at pH 7, 25 °C, which is close to the reported value of -0.270 V (17). $E^{\circ'}$

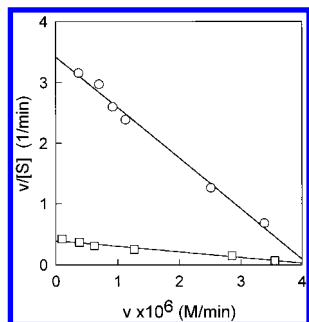


FIGURE 2: The kinetics of wild-type and G33D thioredoxin as substrates of thioredoxin reductase. Wild-type and mutant thioredoxin were assayed for their ability to serve as substrates of thioredoxin reductase as described in the Experimental Procedures. The assay mixture contained 3.6 nM of thioredoxin reductase and 0.1–52 μM wild-type or G33D mutant thioredoxin. (○) Wild-type thioredoxin; (□) G33D thioredoxin.

Table 1: Kinetic Parameters of the Thioredoxin Reductase Reaction with Thioredoxin at pH 7, 25 °C

Trx	k_{cat} (min^{-1})	K_{m} (μM)	$k_{\text{cat}}/K_{\text{m}}$ ($\mu\text{M}^{-1} \text{min}^{-1}$)
G33D	1300 ± 200	11.8 ± 1.5	110
wild-type	1190 ± 40	1.2 ± 0.1	992

for the G33D mutant is -0.264 V , suggesting that the redox potential does not vary significantly.

Effective Concentration of G33D Mutant. Effective concentration is a measure of the propensity for two cysteines to form a disulfide bond. It also reflects the folding of the protein, as a large C_{eff} will indicate the thiols held in good geometry and distance by the structure of the protein. The effective concentration (C_{eff}) of the cysteines in the active site of thioredoxin was measured using glutathione as a reference species. C_{eff} for the G33D mutant is 7 M, and that for the wild-type is 8 M, which is also close to the reported wild-type value (10 M) at pH 8.7, 25 °C (29). Therefore, it suggests that the redox property is not altered, and the folding of the protein is likely to be similar for the G33D mutant.

Kinetics of the Redox Reaction. The kinetics of the thioredoxin reductase catalyzed reduction of the G33D mutant and the wild-type were assessed (Figure 2). Replacement of Gly33 with Asp decreases the rate of the reaction at low substrate concentration. The value of k_{cat} of the G33D mutant is the same as the wild-type thioredoxin (Table 1). However, the K_{m} increases approximately 10 times due to the substitution. Therefore, the catalytic efficiency of thioredoxin reductase on the G33D mutant is reduced by approximately 10-folds compared to the wild-type.

In Vivo Assay of G33D Mutant as an Electron Donor. An in vivo assay for the biological activity of thioredoxin has been developed by Russel and Model (20). Growth of the methionine-requiring strain of *E. coli* on MetSO requires the reduction of methionine sulfoxide reductase by thioredoxin. Therefore, growth of *E. coli* strain A313 (*metE trxA*) transformed with pET/Trx or pET/G33D plasmid on minimal medium with MetSO was investigated. After incubation at 37 °C for approximately 20 h, the *trxA*⁺ strain obtained a plating efficiency of 1 on minimal plates supplemented with 10 or 40 $\mu\text{g}/\text{mL}$ of MetSO. However, G33D mutant still did not show colonies clear enough for counting on either concentration of MetSO. The G33D mutant strain reached

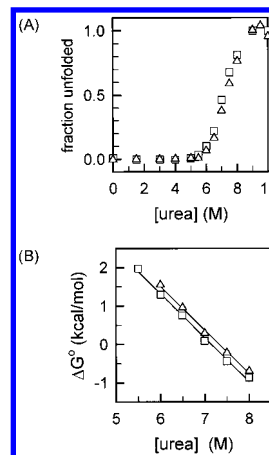


FIGURE 3: Unfolding of the G33D and the wild-type thioredoxin as a function of urea concentration. The mutant and the wild-type thioredoxin in the oxidized form were unfolded by urea in 0.05 M phosphate buffer, at pH 7.5 and 25 °C. (A) Fraction of unfolded proteins at different concentration of urea. (B) Unfolding free energy of the proteins. (□) wild-type thioredoxin; (△) G33D thioredoxin.

an efficiency of 1 after incubation for ~ 30 h on the 40 $\mu\text{g}/\text{mL}$ MetSO and ~ 36 h on the 10 $\mu\text{g}/\text{mL}$ plates. Therefore, Gly33 \rightarrow Asp replacement slows down the growth of bacteria on MetSO, but does not affect the final plating efficiency. In a control experiment, spreading the same amount of bacteria of strain A313 with no transformed plasmid on plates of either concentration of MetSO showed no colonies during the same period of incubation time.

Stability of G33D Mutant Thioredoxin. Thermodynamic stability of the G33D mutant and the wild-type thioredoxin was determined by unfolding of the protein in urea solution at pH 7.5 and 25 °C, assuming a two-state model of unfolding (30). The midpoint of unfolding occurs at 7.3 M urea for the G33D mutant, which is essentially identical to the 7.2 M for the wild-type protein (Figure 3). The free energy of stabilization of the oxidized wild-type protein, 8.2 kcal/mol, is similar to 8.7 kcal/mol reported by Kelley and Richards (31). The unfolding free energy and m value for the G33D protein are 8.3 kcal/mol and 1132 cal/mol M, respectively, which are in good agreement with those of the wild-type protein.

Structure of G33D Mutant Thioredoxin. To check the structure of G33D thioredoxin, CD and fluorescence spectra of the wild-type and the G33D thioredoxin at pH 7.5 were compared. In the far UV region, the CD spectra of both wild-type and the mutant oxidized thioredoxin display a minimum ellipticity of $-6400 \text{ deg cm}^2 \text{ dmol}^{-1}/\text{amino acid residue}$ at 219 nm (Figure 4). This value is similar to that reported by Reutiman et al. (32). The spectra of the reduced wild-type and G33D thioredoxin also do not show difference, although they shift a little toward higher ellipticity relative to the oxidized forms in the region between approximately 220 and 245 nm, which could indicate a small difference in the structure between the oxidized and the reduced forms (Figure 4). The near-UV CD spectra of the G33D thioredoxin are close to the wild-type spectra for either the oxidized or the reduced form, although the ellipticity of the reduced forms decreases around the 280 nm region (Figure 5). Comparison of the fluorescence spectra (Figure 6) of the wild-type and G33D thioredoxin demonstrates that both are characterized by an emission maximum of 341 nm using an excitation

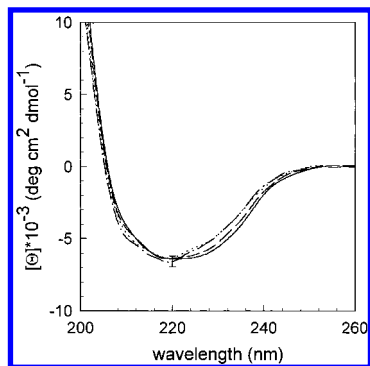


FIGURE 4: Far-UV CD spectra of the wild-type and the G33D mutant thioredoxin at 25 °C. (—) Oxidized G33D thioredoxin; (---) the oxidized wild-type thioredoxin; (···) the reduced G33D thioredoxin; (-·-) the reduced wild-type thioredoxin. $[\theta]$ is the molar ellipticity per residue ($\text{deg cm}^2 \text{dmol}^{-1}$). The bar indicates the range of values observed among the samples.

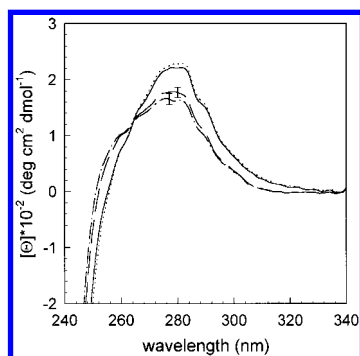


FIGURE 5: Near-UV CD spectra of the wild-type and the G33D mutant thioredoxin at 25 °C. (···) The oxidized G33D thioredoxin; (---) the oxidized wild-type thioredoxin; (—) the reduced G33D thioredoxin; (-·-) the reduced wild-type thioredoxin. $[\theta]$ is the molar ellipticity per residue ($\text{deg cm}^2 \text{dmol}^{-1}$). The bar indicates the range of values observed among the samples.

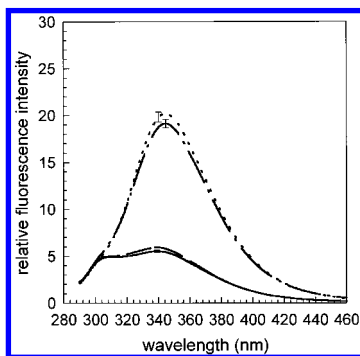


FIGURE 6: Fluorescence emission spectra of the wild-type and the G33D mutant thioredoxin at 25 °C. (—) Oxidized G33D thioredoxin; (---) the oxidized wild-type thioredoxin; (···) the reduced G33D thioredoxin; (-·-) the reduced wild-type thioredoxin. The bar indicates the range of values observed among different samples.

wavelength of 280 nm. Reduction of the disulfide bonds resulted in a 3.5-fold increase in fluorescence intensity for both the G33D mutant and the wild-type proteins. This increase in the wild-type thioredoxin has been attributed to the absence of the quenching of the tryptophan fluorescence by the disulfide bond (33, 34). The emission maximum of the reduced proteins also shifted to 345 nm (Figure 6). Therefore, the far-UV CD spectra demonstrate that the secondary structure of thioredoxin is not changed by the G33D substitution, while the near-UV CD and fluorescence

spectra suggest that the tertiary structure of the mutant still resembles that of the wild-type protein.

DISCUSSION

One of the probably most important physiological functions of thioredoxin is to participate in the cellular oxidation–reduction reactions. In this paper, we demonstrate that the catalytic efficiency of thioredoxin reductase-catalyzed reduction of thioredoxin by NADPH is reduced for the G33D mutant. The substitution also alters the efficiency of the protein as an electron donor for methionine sulfoxide reductase. We show that this decrease in the activity of thioredoxin is not attributed to a significant change of its redox potential as a result of the substitution in the active site. Cys32 in the active site was reported to have a pK_a slightly larger than 7 (35, 36). The effective concentration of cysteines was measured at pH 8.7, where the active-site Cys32 should be in an ionized state. That C_{eff} does not change by the G33D substitution suggests that the side chain of aspartic acid is positioned away from the Cys32. G33D mutant thioredoxin serves as a poor substrate for *E. coli* thioredoxin reductase. Its k_{cat} value is 1300 min^{-1} , which is close to the measured wild-type value of 1190 min^{-1} and the previous reported value of 1365 min^{-1} (19). The K_m of the measured wild-type thioredoxin, $1.2 \mu\text{M}$, is also in good agreement with the reported $2 \mu\text{M}$ (19). However, the K_m of the G33D mutant protein increases to $11.8 \mu\text{M}$. The reduced efficiency of thioredoxin reductase in catalyzing reduction of the mutant thioredoxin arises from a 10-fold increase of K_m value without altering k_{cat} , suggesting the side chain of aspartic acid points to the interaction surface between thioredoxin reductase and thioredoxin.

The Cys-X-X-Cys motif in the active site of thioredoxin has been found in other thiol/disulfide oxidoreductases. For instance, DsbA from the periplasm of *E. coli* possesses a catalytic disulfide of sequence Cys-Pro-His-Cys. A recent paper by Huber-Wunderlich and Glockshuber (37) showed that when the dipeptide sequence PH in the wild-type DsbA was changed to those of other oxidoreductases, the redox potentials of the variants decreased and shifted according to those of the natural enzymes. In the present study, the Gly33 to Asp replacement does not show significant effect on the redox potential. Whether only certain X-X sequences will affect the redox potential is currently being investigated.

In vivo assay of the ability of *Met⁻ E. coli* containing G33D mutant *trxA* to utilize methionine sulfoxide as the sole methionine source demonstrates that the mutation reduces the rate of growth. However, the final plating efficiency is the same as the strain carrying the wild-type thioredoxin. The observed in vivo results could suggest that the G33D substitution primarily affects the kinetics of interaction with methionine sulfoxide reductase, which would agree well with the above in vitro results. Another explanation for the reduced growth rate of the mutant would be that any possible difference in the oxidation state of the mutant and the wild-type thioredoxin in vivo because of the change of K_m for thioredoxin reductase affects the rate of utilization of methionine sulfoxide, but this effect is probably quite small.

CD and fluorescence spectra indicate that the structure of either oxidized or reduced thioredoxin is not altered by the G33D substitution. The tryptophan residues (residue 28 and

31) are in close proximity to the active site. No significant change of the fluorescence spectra between the wild-type and the G33D mutant particularly illustrates the conservation of the tertiary structure in the active-site region. Unfolding of the G33D mutant and the wild-type proteins as a function of urea manifests that the midpoint and free energy of unfolding do not vary, again suggesting that the overall structure of the protein is not changed by the G33D replacement. Therefore, the replacement of Gly33 with Asp primarily affects the kinetics of the cellular reactions that thioredoxin participates in, but not the redox potential, structure, and stability of the protein.

A model of the structure of the G33D mutant protein is therefore constructed based on the above investigation (available as Supporting Information). Alteration was confined to the position of residue 33 with little change in the backbone conformation. The structure of thioredoxin is such that placing an aspartic acid at position 33 will make a charged residue protrude from this surface to the accessible side of the disulfide bridge. This provides support for that position 33 of thioredoxin participates in a critical interaction with methionine sulfoxide reductase and thioredoxin reductase, and a mutation from Gly to Asp in residue 33 impedes the interaction.

The newly isolated thioredoxin from *C. sticklandii* deviates from the consensus active site sequence -CGPC- by a striking G → E substitution. This thioredoxin cannot react with thioredoxin reductase from *E. coli* (15). Our results reveal that thioredoxin with aspartic acid at position 33 reduces the enzymatic efficiency by approximately 10-folds mainly through an increase of the K_m . It therefore suggests that Glu at position 33 could substantially impair the interaction of *C. sticklandii* thioredoxin with *E. coli* thioredoxin reductase; however, combination with amino acid changes at other positions of *C. sticklandii* thioredoxin might further suppress the enzymatic reaction catalyzed by these two proteins. The complete sequence of *C. sticklandii* thioredoxin reductase is not available yet. Therefore, it is difficult to address the possible compensating amino acid residue(s) in that protein which would allow for accommodation of the negative charge at the active site of thioredoxin.

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SUPPORTING INFORMATION AVAILABLE

Structural model for G33D thioredoxin. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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