Interaction Domain on Thioredoxin for Pseudomonas aeruginosa 5′-Adenylylsulfate Reductase*

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Jung-Sung Chung1, Valérie Nogueira-Mazon1, Jean-Marc Lancelin9, Sung-Kun Kim1,2, Masakazu Hirawasa1, Maggy Hologne3, Thomas Leustek4, and David B. Knaff3

From the 1Department of Chemistry and Biochemistry and 3Center for Biotechnology and Genomics, Texas Tech University, Lubbock, Texas 79409-1061, the 2Laboratoire de RMN et Spectrométrie de Masse Biomoléculaires, Université Claude Bernard, Lyon 1, UMR CNRS 5180 Sciences Analytiques, ESCPE, 69622 Villeurbanne, France, and the 4Biotechnology Center for Agriculture and the Environment, Department of Plant Biology and Pathology, Rutgers University, New Brunswick, New Jersey 08901-8520

NMR spectroscopy has been used to map the interaction domain on Escherichia coli thioredoxin for the thioredoxin-dependent 5′-adenylylsulfate reductase from Pseudomonas aeruginosa (PaAPR). Seventeen thioredoxin amino acids, all clustered around Cys-32 (the more surface-exposed of the two active-site cysteines), have been located at the PaAPR binding site. The center of the binding domain is dominated by nonpolar amino acids, with a smaller number of charged and polar amino acids located on the periphery of the site. Twelve of the amino acids detected by NMR have non-polar, hydrophobic side chains, including one aromatic amino acid (Trp-31). Four of the thioredoxin amino acids at the PaAPR binding site have polar side chains (Lys-36, Asp-61, Gln-62 and Arg-73), with three of the four having charged side chains. Site-directed mutagenesis experiments have shown that replacement of Lys-36, Asp-61, and Arg-73 and of the absolutely conserved Trp-31 significantly decreases the \( V_{\text{max}} \) for the PaAPR-catalyzed reduction of 5′-adenylylsulfate, with E. coli thioredoxin serving as the electron donor. The most dramatic effect was observed with the W31A variant, which showed no activity as a donor to PaAPR. Although the thiol of the active-site Cys-256 of PaAPR is the point of the initial nucleophilic attack by reduced thioredoxin, mutagenic replacement of Cys-256 by serine has no effect on thioredoxin binding to PaAPR.

Thioredoxins are ubiquitous redox-active proteins that catalyze thiol-disulfide exchange reactions (1). Thioredoxin has been implicated in diverse functions including the regulation of activity of enzymes and transcription factors (2, 3) and facilitation of protein folding (4) and as electron-donating substrates for redox enzymes (5–7). Thioredoxins contain an active site consisting of two cysteine residues separated by two intervening amino acids that undergo reversible redox reactions. The negative redox potentials of the disulfide/dithiol couples found at the active site in most thioredoxins would suggest that thioredoxin is capable of reducing almost all disulfide bonds in proteins. However, the reality is that thioredoxins are in many cases highly specific for their substrate (8, 9), suggesting that thioredoxin first interacts with its target protein before reducing the target. The interaction of thioredoxin with target proteins is poorly understood. Both electrostatic and hydrophobic interactions have been implicated in the binding (10).

One of the first enzymes to have been identified as a thioredoxin target is 3′-phosphoadenylylsulfate (PAPS)4 reductase, an enzyme necessary for sulfur assimilation and cysteine or methionine synthesis. In this process, sulfur is reduced from the +6 oxidation state found in sulfate to the −2 oxidation state found in sulfide. Ultimately, sulfide is incorporated into cysteine and methionine. PAPS reductase, the product of the cysH gene, carries out the 2-electron reduction of 3′-phosphoadenylylsulfate to yield sulfite and adenosine 3′,5′-bisphosphate (PAP). A structurally and catalytically distinct form of CysH also uses thioredoxin as the electron donor but uses 5′-adenylylsulfate (APS) as the substrate instead of PAPS. The key differences between these enzymes are the high sequence divergence (they share only ~25% identity; Fig. 1) and the fact that although PAPS reductase does not contain any prosthetic group, APS reductase contains a [4Fe-4S]2+ cluster.

Much is known about the structures of PAPS and APS reductases. Thus, these enzymes offer an ideal opportunity to define and compare their interactions with thioredoxin. The three-dimensional structures of Escherichia coli PAPS reductase (11) and of a disulfide-linked complex between the enzyme and thioredoxin (12) are known. This structural information allowed the catalytic mechanism to be determined. In the first step an absolutely conserved catalytic cysteine residue carries out a nucleophilic attack on PAPS, releasing PAP as the first product and producing a covalently modified form of CysH in which the sulfur of the catalytic cysteine. This is referred to as the S-sulfocysteine intermediate (E-Cys-S-SO₃⁻). Subsequently, the more N-terminal of the two active-site cysteine thios of

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1 Both authors contributed equally to this work.
2 Present address: Dept. of Chemistry and Biochemistry and Inst. of Biomedical Studies, Baylor University, Waco, TX 76798.
3 To whom correspondence should be addressed: Dept. of Chemistry and Biochemistry, Texas Tech University, 1 Circle Dr., Lubbock, TX 79409-1061. Tel.: 806-742-0288; Fax: 806-742-2025; E-mail: david.knaff@ttu.edu.

4 The abbreviations used are: PAPS, 3′-phosphoadenylylsulfate; APS, 5′-adenylylsulfate; DTT, dithiothreitol; HMQC, homonuclear single quantum correlation; HSQC, heteronuclear single quantum coherence; NOESY, nuclear Overhauser effect spectroscopy; NTR, NADPH:thioredoxin oxidoreductase; PAP, 3′,5′-bisphosphate; PaAPR, P. aeruginosa APS reductase.
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PaAPR MLPFATIPATERNSSAAQHQPSPMSQPFDSLPAASSLADKSPQDLKAAFEHFG
CysH ----------MSKLDMLNLNLKPKVDRILAETNAELEKLDSEGRWALDNLP

PaAPR DELWISFS-GAEDVVLVDMAWKLNRNVRKVFLDGPLPETYRFDQVREHYGI
CysH GEYVLSQSFQIAAASLVHLVNQTRPDPILVDTGYLFPEYRFDILTDKLKL

PaAPR AIDVLSPDPELEPLVKEGIFSLFYDGHGECGGIRKPELKLKLAGV--RAWA
CysH NLKVRATSEAAQEEARQKYADHPCNNKIEKYNINCKVLPMRKLENAQTFW

PaAPR TQRRDQSPGTRGQVAVELIDGAFSTPKKLYKPNPLSSMTSEEVGYIRMEL
CysH AGLRREGS--GSRNLPVLAQ-------RGQVGLPIISWTRNITYQLKHGL

PaAPR PYNLHERGYISIGCECPTRPVLPNQHEREGRWWEBAHRECGVLHAGNLISA
CysH KYHPILWDEGLSVGFTTHTRWEPQMAEETTFFG----LHRECGLHKG----

FIGURE 1. Comparison of PaAPR and of E. coli PAPS reductase sequences aligned with ClustalW2. Asterisks identify identical residues. Colors and periods denote high and low degrees of similarity, respectively. The conserved (P)APS reductase signature is boxed.

Wild-type PaAPR and its C256S variant were prepared and purified as described previously (17, 18). The gene encoding E. coli thioredoxin 1 (hereafter referred to simply as thioredoxin) was cloned by PCR into the region between the Ndel and BamHI restriction enzyme sites of the pET28b vector (Novagen) by PCR using 5'-AGCTTATACCA-TATGAGCGGTAAGAT-3' and 5'-ACGTCATATGGCCAGGATT-3' primers. The amplified DNA was cloned into the pET28b vector, the DNA sequence was confirmed by sequencing in the Biotechnology Core Facility at Texas Tech University. The E. coli thioredoxin was expressed and purified as described previously (17, 18). 15N labeling of E. coli thioredoxin was accomplished by growing E. coli cells harboring the expression plasmid in M9 minimal medium containing salts, 0.5% d-glucose, 10 mM MgCl2, 30 mg of thiamine, trace elements, a vitamin mixture, 50 µg of kanamycin, and 0.5 g of [15N]ammonium chloride (Cambridge Isotopes Laboratories, Inc., Andover, MA) per liter of culture. The 15N-labeled E. coli thioredoxin was purified using the same method used to purify the unlaemed thioredoxin. Site-specific mutation of E. coli thioredoxin was carried out using the QuikChange kit (Stratagene) according to the manufacturer’s instructions. The sequences of the primers used to create all of these thioredoxin variants are shown in Table 1 (sequences of the genes encoding these variants were confirmed by DNA sequencing in the Biotechnology Core Facility at Texas Tech University). These mutated thioredoxin variants were expressed and purified using the same method used for wild-type thioredoxin. All thioredoxin and PaAPR samples used in this study displayed a single Coomassie Blue-staining band after polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

The gene encoding E. coli NADPH:thioredoxin oxidoreductase (hereafter abbreviated NTR) was cloned into the pET28b vector between the Ndel and BamHI restriction enzyme sites by PCR using 5'-ACGTATATGGCCAGGATTACCA-CAG-3' as the forward primer and 5'-ACGTCATATGGCCAGGATTACCA-CAG-3' as the reverse primer and with E. coli genomic DNA as the template. After induction by isopropyl-β-thiogalactosidase, E. coli BL21(DE3) cells containing the NTR-pET28b vector were harvested and broken (NMR) spectroscopy of the non-covalent complex formed between PaAPR and 15N-labeled E. coli thioredoxin has been used to map the interaction domain on thioredoxin for PaAPR. The interaction domain was further explored using a series of site-specific E. coli thioredoxin variants that were designed based on the NMR results.

EXPERIMENTAL PROCEDURES

S. aureus thioredoxin carries out a nucleophilic attack of the sulfocysteine intermediate, releasing sulfite and forming a transient disulfide-bonded, covalent protein/protein intermediate between the attacking cysteine of thioredoxin and the active-site cysteine of PAPS reductase. Finally, the second active site cysteine of thioredoxin (the “resolving cysteine”) carries out the final nucleophilic attack producing oxidized thioredoxin and regenerating the original state of PAPS reductase. For the intermediate (12). A similar catalytic mechanism has been proposed for bacterial APS reductases with the difference that the initial reaction is with APS rather than PAPS (12–16). The x-ray studies of the PAPS reductase/thioredoxin complex, a mono-cysteine variant of the E. coli thioredoxin was accomplished by growing E. coli cells harboring the expression plasmid in M9 minimal medium containing salts, 0.5% d-glucose, 10 mM MgCl2, 30 mg of thiamine, trace elements, a vitamin mixture, 50 µg of kanamycin, and 0.5 g of [15N]ammonium chloride (Cambridge Isotopes Laboratories, Inc., Andover, MA) per liter of culture. The 15N-labeled E. coli thioredoxin was purified using the same method used to purify the unlabeled thioredoxin. Site-specific mutation of E. coli thioredoxin was carried out using the QuikChange kit (Stratagene) according to the manufacturer’s instructions. The sequences of the primers used to create all of these thioredoxin variants are shown in Table 1 (sequences of the genes encoding these variants were confirmed by DNA sequencing in the Biotechnology Core Facility at Texas Tech University). These mutated thioredoxin variants were expressed and purified using the same method used for wild-type thioredoxin. All thioredoxin and PaAPR samples used in this study displayed a single Coomassie Blue-staining band after polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

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TABLE 1
Sequences of oligonucleotide primers used in site-directed mutagenesis PCR amplification

| Primer       | Sequence 5′–3′                          | Mutated codon |
|--------------|-----------------------------------------|---------------|
| W31A For     | GAT TCC TGG GCA GAG GCG                 | TGG → GCG    |
| W31A Rev     | TTT GCA CGG ACC GCA                    |               |
| W31F For     | GAT TCC TGG GCA GAG GGG                | TGG → TTT    |
| W31F Rev     | TTT GCA CGG ACC GCA                    |               |
| K36E For     | TGG TGC GCT TCG GGA                    | AAA → GAA    |
| K36E Rev     | AAT CGG GGC GAT CTG                     | AAA → AGA    |
| K36R For     | TGG TGC GCT TCG AGA                    | AAA → AGA    |
| K36R Rev     | AAT CGG GGC GAT CTG                     | AAA → AGA    |
| D61N For     | GCA AAA CTG AAT ACC                    | GAT → AAT    |
| D61N Rev     | AGT GCC AGG GTT TGG                    |               |
| D61K For     | GCA AAA CTG AAT ACC                    | GAT → AAT    |
| D61K Rev     | AGT GCC AGG GTT TGG                    |               |
| R73E For     | CCG AAA TAT GTC AAT                    | CGT → GAA    |
| R73E Rev     | CAG AGT GAT GAG GAC                    |               |
| R73K For     | CCG AAA TAT GTC AAT                    | CGT → GAA    |
| R73K Rev     | CAG AGT GAT GAG GAC                    |               |
| K100E For    | TCT AAA GGT CAG TGG                    | AAA → GAA    |
| K100E Rev    | AGC GTC GAG AAT CTC                    | AAA → GAA    |
| D104K For    | TTT AAA GAG TCC CTC                    | GAC → AAA    |
| D104K Rev    | TTA CCA CGG GTT ACC TGG                |               |

by a French press, and the extract was centrifuged. The supernatant was filtered through a 0.45-μm pore-size membrane and applied to a Ni²⁺ affinity column (Hi-Trap Chelating HP, obtained from Amersham Biosciences) incorporated into a BioCAD perfusion chromatography system (PerSeptive Biosciences). SDS-PAGE analysis showed a single Coomassie staining band.

Absorbance spectra in the visible and ultraviolet regions were measured at 0.5-nm spectral resolution using a Shimadzu Model 2401PC spectrophotometer. Dissociation constants (K_d) for the complex between PaAPR and thioredoxin were measured using the spectral perturbation method described previously (17). Oxidation-reduction titrations were carried out using mixtures of oxidized and reduced dithiothreitol (DTT) for redox equilibration at defined ambient potentials followed by labeling of reduced thioredoxin with the fluorescent thiol modifier monobromobimane as described previously (19, 20).

The activity of the NADPH-dependent E. coli NTR was measured by following the reaction of reduced thioredoxin with 5,5′-dithio-bis(2-nitrobenzoic acid), as described by Krause and Holmgren (21). Rates were calculated from changes in absorbance at 412 nm using an extinction coefficient of 13.6 mM⁻¹ cm⁻¹, and the data were fitted to the Michaelis-Menten Equation using Microsoft Excel. The enzymatic activity of PaAPR with wild-type and site-specific variants of E. coli thioredoxin (with the exception of the R73E variant; see below) was measured by coupling the PaAPR-catalyzed oxidation of thioredoxin to the reduction of thioredoxin by NADPH catalyzed by E. coli NTR. The 1-ml reaction contained 100 mM Tris-HCl buffer (pH 8.5), 1 mM EDTA, 100 mM sodium sulfate, 100 μM APS, 5 μM E. coli NTR, 300 mM β-NADPH, 5 μM PaAPR, and E. coli thioredoxin (either wild type or a site-specific variant) at concentrations of 0.5, 1, 2.5, 5, 7.5, 10, or 12.5 μM. The oxidation of NADPH was monitored by following absorbance decreases at 340 nm. Rates were calculated from the amount of NADPH oxidized after 1 min using an extinction coefficient of 6.22 mM⁻¹ cm⁻¹. Data were fitted to the Michaelis-Menten equation using Microsoft Excel.

In the case of the R73E variant of thioredoxin, which showed no activity as an electron acceptor in the NTR-catalyzed reaction, PaAPR enzymatic activity was assayed essentially as described by Bick et al. (22). The 100-μl reaction mixture contained 5 μmol of Tris-HCl buffer (pH 8.0), 5 nmol of [³⁵S]APS (2500 Bq nmol⁻¹), and 10 ng of PaAPR. The concentrations of the reduced forms of wild-type thioredoxin and its R73E were varied up to a maximum of 50 μM, and the reaction was allowed to proceed for 20 min at 30 °C. A reaction lacking thioredoxin served as the blank. Thioredoxin was reduced by incubation for 60 min on ice with 5 mM freshly prepared reduced DTT in 50 mM sodium phosphate buffer (pH 8.0). DTT was removed using a Millipore Biomax-5 centrifugal filter device centrifuged at 12,000 × g at 4 °C. After ultrafiltration, the concentration of residual DTT was estimated to be ~0.02 mM, a concentration of DTT that was shown to produce negligible rates of APS reduction by PaAPR in the absence of thioredoxin. The data were fitted to the Michaelis-Menten equation by nonlinear regression using GraphPad Prism 4.03.

All NMR samples were prepared in 10 mM phosphate buffer (pH 7.5) containing 100 mM Na₂SO₄, 10% D₂O, and 0.02% NaN₃. The pH was adjusted by adding small amounts of 1 M NaOH or 1 M HCl to the sample. For all experiments, ¹³C- and ¹⁵N-thioredoxin was present at a concentration of 75 μM, and the concentration of PaAPR was varied from 0 to 195 μM for the wild-type protein and from 0 to 407 μM for the C256S variant of PaAPR.

NMR experiments were performed at 20 °C using a Varian INOVA Unity 600 spectrometer fitted with a normal triple resonance (HCN) probe and a z-field gradient coil. ¹H, ¹³C Sofast-HMQC (23) data were acquired with 512 complex points and a spectral width of 10,000 Hz in F₂ and 64 complex points, 2200 Hz in F₁ (¹⁵N). The recycle delay was 0.1 s, and the number of transients used was 256. All spectra were processed using NMRPIPE scripts (24) and were analyzed with NMRVIEW software (25). The HN resonance assignments for thioredoxin used in this work were obtained from the BioMagRes data bank, accession numbers 62 and 1812 (26–28). This assignment was made at 30 °C in 20 mM phosphate buffer (pH 5.7) containing...
150 mM NaCl. Because of the instability of PaAPR under these experimental conditions, the interaction between PaAPR and thioredoxin was studied at 20 °C in 10 mM phosphate buffer (pH 7.5) containing 100 mM Na₂SO₄. To check thioredoxin HN resonance assignments under these experimental conditions, a ¹⁵N NOESY-HSQC spectrum was recorded with 512 complex points in F₃ (¹H), 96 complex points in F₁ (¹H), 38 complex points in F₂ (¹⁵N), and a mixing time of 150 ms.

Mapping of the binding interface was achieved in a series of NMR titration experiments in which ¹H,¹⁵N Sofast-HMQC spectra of ¹⁵N-labeled thioredoxin (75 μM) were recorded as a function of increasing amounts of unlabeled wild-type PaAPR (from 15 to 195 μM). The titration included eight different PaAPR/thioredoxin ratios ranging from 0:1.0 to 2.6:1.0. ¹H,¹⁵N Sofast-HMQC spectra were recorded at each titration point. A similar titration was performed using ¹⁵N-labeled thioredoxin (75 μM) and the unlabeled C256S variant of PaAPR (at concentrations ranging from 0 to 407 μM). The perturbations observed in the thioredoxin spectrum during titration were quantified by calculating the weighted average ∆AV of ¹H and ¹⁵N chemical shifts given by the equation

\[ \Delta_{AV} = \left( \frac{\Delta \delta_{H}^2 + \Delta \delta_{N}^2}{25} \right)^{1/2} \]  

(Eq. 1)

where \( \Delta \delta \) is the chemical shift variation between free thioredoxin and thioredoxin bound to PaAPR.

RESULTS

Our earlier work on the interaction of PaAPR with thioredoxin demonstrated that the two proteins could form a non-covalent complex (17) with a \( K_d \) of ∼40 μM (with the exact value depending on the specific thioredoxin used and on the buffer composition). This non-covalent complex appears to function as the precursor to the disulfide-linked, covalent 1:1 complex formed between the enzyme and thioredoxin as a transient reaction intermediate, with Cys-256 supplying the enzyme site for transient covalent attachment of the thioredoxin (17). Given that the \( K_d \) for the non-covalent complex is in the range appropriate for studying protein/protein interactions by NMR spectroscopy (26), an attempt to map the site on thioredoxin involved in binding PaAPR using NMR seemed feasible. Although thioredoxin from \( P. aeruginosa \) was not available, \( E. coli \) thioredoxin can serve as an efficient electron donor to the thioredoxin-dependent PaAPR, and previous work in our laboratory had already characterized a number of aspects of the interaction of this thioredoxin with PaAPR (17). Moreover, \( P. aeruginosa \) PAO1, the strain from which PaAPR was cloned, contains a thioredoxin that is 86% conserved when compared with \( E. coli \) thioredoxin. For these reasons and also because a complete NMR assignment already exists in the literature for \( E. coli \) thioredoxin (27, 28), \( E. coli \) thioredoxin was chosen for this study.

Fig. 2 shows the effects of titrating ¹⁵N-labeled, wild-type \( E. coli \) thioredoxin with wild-type PaAPR on the HMQC spectrum and identifies the \( E. coli \) thioredoxin amino acid residues experiencing the greatest changes as a result of the addition of
PaAPR. Fig. 3 displays these results as a histogram in which the $^1$H and $^{15}$N chemical shift variations are plotted against thioredoxin sequence position. The data of Fig. 3 were then used to construct a map (Fig. 4) of the binding domain for PaAPR on thioredoxin. Before describing the details of this interaction domain, it should be mentioned that an area of $\sim 800$ Å$^2$ on the surface of E. coli thioredoxin that is exposed to the solvent in the free protein becomes buried as a result of complex formation with PaAPR. The size of this buried area is consistent with buried surface areas reported for other protein/protein complexes with $K_p$ values of $\sim 40$ μM (29).

Fig. 4 shows a map of the interaction domain on E. coli thioredoxin for PaAPR in which the amino acids experiencing the largest shift in NMR characteristics are color-coded on the structure of the E. coli protein. The interaction domain on E. coli thioredoxin for PaAPR involves a central hydrophobic core that includes residues Trp-31, Gly-33, Met-37, Ala-39, Ile-41, Leu-42, Ile-60, Ile-72, Ile-75, Gly-92, Ala-93, and Leu-94, all of which surround the more N-terminal cysteine of the catalytic site, Cys-32 (the other catalytic site cysteine, Cys-36, is buried in the interior of the protein and is not surface-accessible). On the periphery of this hydrophobic central domain lies one polar, uncharged amino acid (Gln-62) and three charged amino acids (Lys-35, Asp-61, and Arg-73) that are also involved in binding PaAPR. Data essentially identical to that shown in Figs. 2 and 3 for the effect of wild-type PaAPR on the NMR spectrum of E. coli thioredoxin were obtained with the C256S variant of PaAPR (data not shown).

To verify the involvement of several thioredoxin residues identified by NMR mapping of the interaction with PaAPR, a series of site-directed variants of E. coli thioredoxin were generated, and the effects of these amino acid replacements on binding and kinetic parameters with wild-type PaAPR were investigated. However, because the reduced thioredoxins used as possible electron-donating substrates for PaAPR in this study were generated by NADPH reduction of the oxidized thioredoxins in a reaction catalyzed by E. coli NTR (see “Experimental Procedures”), it was important to first make sure that the thioredoxin variants used were in fact reduced at reasonable rates by NTR. If that were not the case, then a lack of NADPH oxidation could not be interpreted as an inability of a thioredoxin variant to serve as a donor to PaAPR. Although earlier studies had compared the steady-state kinetic parameters of several of the thioredoxin variants used in this study to those observed with wild-type E. coli thioredoxin in the NTR-catalyzed reaction (see below), not all of the variants we wished to test had been characterized in terms of their ability to interact with NTR, and thus, these variants had to be tested as part of this study. NTR turnover numbers equal to or similar to those observed in this study had previously been reported for wild-type E. coli thioredoxin (21) and for its W31A (21), W31F (21), D61N (30), and K36E (31) variants. In the case of the two tryptophan replacement variants, small increases in the NTR $K_m$ values for thioredoxin were reported when compared with the $1.0$ μM value observed for wild-type thioredoxin ($K_m = 2.0$ μM for W31A and $K_m = 1.9$ μM for W31F (21)). In a separate study K36E was reported to have a $K_m$ of $6.7$ μM compared with a value of $2.0$ μM measured for wild-type thioredoxin (29). In the case of the D61N variant, it had been reported that the NTR turnover number was 90% that observed with wild-type E. coli thioredoxin and that the D61N variant actually had a lower $K_m$ value (0.4 μM) than did wild-type (0.9 μM). Fig. 5 shows plots of NTR activity, measured at a saturating NADPH concentration, as a function of thioredoxin concentration for wild-type E. coli thioredoxin and for each of six different site-specific variants. The data were obtained for all of the variants used in this study that had not been previously characterized in the literature. Five of the six variants tested (i.e. W31A, W31F, K36R, D61K, and R73K) had rate versus concentration profiles reasonably similar to that observed for wild-type E. coli thioredoxin, but no activity could be detected with R73E, even at the highest concentrations of this thioredoxin variant that were tested. All of the thioredoxins that showed detectable activity with NTR (i.e. wild-type thioredoxin and seven of the eight variants tested) exhibited Michaelis-Menten kinetics, and their $V_{max}$ and $K_m$ values are summarized in Table 2. The $K_m$ values obtained in this study for wild-type thioredoxin and for variants that had
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**TABLE 2**

| Thioredoxin variant | Relative \( V_{\text{max}} \) | \( K_m \) |
|---------------------|------------------|--------|
| Wild type           | 100              | 1.1    |
| W31A                | 95               | 1.5    |
| W31F                | 93               | 1.5    |
| K36E                | 83               | 2.2    |
| K36R                | 113              | 1.0    |
| D61N                | 100              | 1.0    |
| D61K                | 58               | 0.5    |
| R73E                | <0.3             |        |
| R73K                | 75               | 1.3    |

\(^{a}100\%\) corresponds to a rate 1.5 \( \mu \)mol of 2-nitro-5-thiobenzoate/min/mg of NTR.

**TABLE 3**

| Variant  | Relative \( V_{\text{max}} \) | \( K_m \) | \( K_m^* \) | \( E_m^* \) |
|----------|------------------|--------|-----------|---------|
| Wild type| 100              | 10     | 16        | −285    |
| W31A     | <0.7             | 25     | −299      |         |
| W31F     | 20               | 10     | −299      |         |
| K36E     | 36.7             |        | 20        | −270    |
| K36R     | 80               | 12     | 20−293    |         |
| D61N     | 50               | 10     | 20−280    |         |
| D61K     | 50               | 10     | 20−291    |         |
| R73E     | NA               | NA     | −267      |         |
| R73K     | 100              | 20     | 10−293    |         |
| K100E    | 80               | 14     | 3−295     |         |
| D104K    | 100              | 14     | 10−285    |         |

\(^{a}100\%\) corresponds to a rate 3 \( \mu \)mol of thioredoxin oxidized/min/mg of PaAPR.

Previously unpublished values.

Data obtained in this fashion showed that the R73E variant is able to reduce PaAPR to the catalytic site of PaAPR although much less efficiently than wild-type thioredoxin. The \( K_m \) for R73E was increased by 7.7-fold compared with wild-type thioredoxin in the PaAPR assay, and the \( V_{\text{max}} \) at 50 \( \mu \)M thioredoxin was decreased by 4.5-fold. This 4.5-fold decrease in \( V_{\text{max}} \) is only an estimate, as saturation of the PaAPR reaction with R73E was not achieved at 50 \( \mu \)M (the highest concentration tested).

A D61K variant, in which the negative charge on aspartate was replaced by a positive charge on lysine, exhibited steady-state parameters very similar to those observed for D61N (Table 3), in which the negative charge was simply eliminated by replacement with the uncharged asparagine instead of undergoing the more drastic charge reversal found in D61K. Control experiments in which conservative replacement of one positively charged residue by another, i.e. those involving the R73K and K36R variants, produced little or no effect on steady-state kinetic parameters (Table 3). Although the conservative replacement of one aromatic amino acid by another in W31F produced an 80% decrease in \( V_{\text{max}} \), the effect was less drastic than seen with W31A, which was completely inactive (see Fig. 6 and Table 3). Variants involving charge reversal substitutions at two positions on the “back side” of thioredoxin (i.e. the side opposite to that containing the binding domain for PaAPR identified by NMR spectroscopy), namely K100E and D104K, exhibited steady-state kinetic parameters identical to or only slightly different from those exhibited by wild-type thioredoxin (Table 3). These results are consistent with the observation that no changes in the NMR parameters of these two residues occur during complex formation with PaAPR. \( V_{\text{max}} \) and \( K_m \) kinetic parameters for all of the site-specific thioredoxin variants used in this study are summarized in Table 3 (all of the thioredoxins that showed detectable activity exhibited Michaelis-Menten kinetics). As no activity could be detected with W31A, no \( K_m \) value is shown for this variant. The \( V_{\text{max}} \) and \( K_m \) values in Table 3 were all obtained using the NADPH/NTR-coupled assay system, and therefore, the values for the R73E variant, which were...
obtained under different conditions using the direct, radioactive assay, have not been included.

As can also be seen in Table 3, all of the thioredoxin variants used in this study had oxidation-reduction midpoint potential ($E_m$) values that were identical to, within the ±10-mV experimental uncertainties of the measurements, or very similar to that of wild-type *E. coli* thioredoxin. Thus, the differences in steady-state kinetic parameters observed for the PaAPR-catalyzed reaction with several of the variants tested cannot be attributed to any significant change in the thermodynamic driving force for the reaction.

Table 3 also summarizes the $K_d$ values for the binding of wild-type PaAPR to wild-type *E. coli* thioredoxin and eight of the site-specific thioredoxin variants used in this study, all measured using our previously described spectral perturbation protocol (17). The difference spectra measured for all of the PaAPR-thioredoxin complexes in this study were essentially identical to those reported previously (17), with the exception of R73E, for which no spectral perturbations could be detected, even at high thioredoxin concentrations. With the exception of the K36R variant, plots of the Δ$A$ at 550 minus 395 nm versus thioredoxin concentration (17) gave good fits for the hyperbolic curve expected for a single binding isotherm (for reasons not yet understood, the data for K36R gave a relatively poor fit to the theoretical curve, and thus, no $K_d$ is reported for this thioredoxin variant). The replacement of the positively charged back-side Lys-100 by a negatively charged glutamate increased the affinity of the two proteins for one another by a factor slightly greater than 5. The data shown in Table 3 were obtained using a high ionic strength buffer designed to minimize the effects of electrostatic interactions and maximize the effects of hydrophobic interactions, but similar results were obtained when the binding experiments were carried out using a low ionic strength buffer.

**DISCUSSION**

Seventeen amino acids on *E. coli* thioredoxin undergo changes in their NMR parameters as a consequence of interaction with PaAPR. Although these changes could result in principle either from a direct involvement in binding to PaAPR or as an indirect consequence of conformational changes resulting from binding, the fact that these 17 amino acids form a continuous, coherent array on the surface of thioredoxin suggests that the amino acids identified by NMR spectroscopy are indeed present in the binding domain of this bacterial thioredoxin for PaAPR. The fact that site-specific mutagenic replacement of several *E. coli* thioredoxin amino acids identified by NMR spectroscopy as being located at the binding domain for PaAPR produce significant effects on the ability of thioredoxin to interact productively with PaAPR provides additional support for the validity of the NMR mapping.

The binding domain is centered on Cys-32, the active-site cysteine that makes the initial nucleophilic attack on Cys-256 of PaAPR (17). The observation that complex formation between a C256S variant of PaAPR and $^{15}$N-labeled thioredoxin produces the same perturbations in the NMR spectrum of thioredoxin that are seen with wild-type PaAPR indicates that although Cys-256 thiol of PaAPR is the target for the initial nucleophilic attack by a cysteine from reduced thioredoxin (17), the thiol of this cysteine is not needed for successful docking with thioredoxin. Twelve of the thioredoxin amino acids identified at the interaction domain with PaAPR, i.e. Trp-31, Gly-33, Met-37, Ala-39, Ile-41, Leu-42, Ile-60, Ile-72, Ile-75, Gly-92, Ala-93, and Leu-94, have non-polar side chains. One, Gln-62, has a polar but uncharged side chain, and the others, Lys-36, Asp-61, and Arg-73, have charged side chains. Proline 34, which is located in close proximity to Cys-32 and is surrounded by amino acids that are part of the PaAPR binding domain, cannot be observed in the two-dimensional NMR spectra utilized in this study because it lacks an amide hydrogen. However, the close proximity of Pro-34 to Cys-32 and the fact that Pro-34 is surrounded by other amino acids identified by NMR as involved in binding PaAPR suggest that Pro-34 is also part of this hydrophobic core of the PaAPR binding domain.

The binding domain can be described as consisting of a non-polar, hydrophobic core surrounded by a smaller number of polar and charged amino acids. In this study we have chosen to focus our mutagenesis efforts on Trp-31, Lys-36, Asp-61, and Arg-73. Both Trp-31 and Lys-36 are highly conserved in thioredoxins and are part of the “classical” WCGPCK active-site motif (32–34). Earlier studies had indicated that replacement of this conserved tryptophan residue decreased but did not eliminate the ability of *E. coli* thioredoxin and other thioredoxins to function in a wide variety of reactions (20, 21, 35, 36). However, the observation (see Table 3 and Fig. 6) that replacement of Trp-31 by the non-aromatic amino acid alanine completely eliminates the ability of *E. coli* thioredoxin to function as an electron donor for the PaAPR-catalyzed reduction of APS represents a much more drastic loss of activity. For one example, see the more modest effect of replacing Trp-31 by alanine on NTR activity shown in Fig. 5 and Table 2. The fact that the $E_m$ value for W31A and its $K_d$ for binding to PaAPR are both quite similar to wild-type *E. coli* thioredoxin (Table 2) suggests that the complete absence of activity observed for this variant arises neither from a change in the thermodynamic driving force for the PaAPR-catalyzed reaction nor from binding affinity for PaAPR per se. Furthermore, the observation that W31F retains at least 20% of the activity of wild-type thioredoxin, measured at saturating thioredoxin concentration, in the PaAPR-catalyzed reaction (Table 2 and Fig. 6) points to the requirement for an aromatic amino acid at this position.

An earlier study of the K36E variant of *E. coli* thioredoxin, a variant that has a significantly different structure in the active-site region than that of wild-type thioredoxin (37), indicated that the major effect of this charge reversal was to increase the $K_m$ for several target enzymes (31, 35, 38) but that similar effects were noted for the corresponding K36E variant of human thioredoxin (39), led to the proposal that this residue plays an important role in recognition of target enzymes (31). However, in the case of interaction with PaAPR, replacing the positively charged Lys-36 with a negatively charged glutamate produces no significant change in either $K_m$ or in $K_d$, but does result in a very large decrease in $V_{max}$.

Asp-61 is also well conserved, and its side-chain carboxyl group forms a structurally crucial hydrogen bond with the...
indole ring of Trp-31 in *E. coli* thioredoxin (40) (this hydrogen bond is conserved in the structure of thioredoxin h1 from the green alga *C. reinhardtii* (41)), although it is absent in the dimeric form of reduced human thioredoxin (42)). Thus, it is perhaps not surprising that even a relatively conservative replacement of this aspartate by asparagine produces a significant decrease in $V_{\text{max}}$. It should be noted that replacement of this aspartate by asparagine enhances the ability of *E. coli* thioredoxin to activate a plant fructose-1,6-bisphosphatase, possibly by eliminating an unfavorable electrostatic interaction (30).

With the possible exception of Arg-73, none of the “front side” amino acid replacements studied had a large effect on the $K_d$ side amino acid replacements studied had a large effect on the (30). Possibly by eliminating an unfavorable electrostatic interaction of this aspartate by asparagine enhances the ability of *E. coli* thioredoxin to activate a plant fructose-1,6-bisphosphatase, possibly by eliminating an unfavorable electrostatic interaction (30).

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