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Application of a Single-Plasmid Vector for Mutagenesis and High-Level Expression of Thioredoxin Reductase and Its Use to Examine Flavin Cofactor Incorporation

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Thioredoxin reductase from *Escherichia coli* is a dimeric enzyme containing one FAD and one redox-active disulfide per monomer and catalyzes the transfer of electrons from NADPH to thioredoxin, which subsequently performs several important cellular functions. To overcome problems with site-directed mutagenesis and low expression, the thioredoxin reductase gene was adapted for use in the plasmid vector pSL350 (Brosius, J., *Methods Enzymol.* 216, 469–483, 1992), which is designed both for protein expression and for production of single-stranded template DNA for mutagenesis, and examined expression of wild-type thioredoxin reductase under different growth conditions. In the absence of IPTG inducer, expression of thioredoxin reductase in saturated cultures accounts for 19% of the soluble protein, and with 1 mM IPTG expression increases to 61%. Some of the thioredoxin reductase is expressed as apoenzyme with the amount of apoenzyme increasing at higher IPTG concentrations, accounting for as high as 68% of the total thioredoxin reductase expressed. The apoenzyme in cell extracts is activated rapidly by addition of FAD, indicating correct folding of the enzyme in the absence of cofactor. Purification of wild-type thioredoxin reductase from the new system yielded 189 mg of enzyme from a 300-ml uninduced culture. The new plasmid was also used to generate an N155Y mutant which is purified and partially characterized.

The thioredoxin/thioredoxin reductase system of *Escherichia coli* consists of two protein components and serves to transfer reducing equivalents from NADPH to several important cellular processes. Thioredoxin is a small (M_r = 11,700) protein which contains one redox-active disulfide and plays a role in ribonucleotide reduction (1), in protein folding (2), and for bacteriophage assembly (3). Thioredoxin reductase is one of a class of redox-active disulfide-containing flavoenzymes which include lipoamide dehydrogenase, glutathione reductase, and mercuric ion reductase (4). It is a homodimer with one FAD and one redox-active disulfide per 35,000-kDa monomer. The flow of electrons is from NADPH to the FAD, then from the reduced FAD to the active-site disulfide, and from the dithiol to the disulfide of thioredoxin.

The crystal structure of thioredoxin reductase reveals that each monomer consists of two globular domains connected by a double-stranded β sheet. One domain contains the bound FAD while the other domain has the NADPH binding site and the active-site disulfide. A large conformational change has been proposed whereby the two domains rotate 66° relative to each other (5). This rotation and counterrotation allows for two conformational states, one with the NADPH close to the FAD resulting in flavin reduction (termed the FR form) and one with the redox-active disulfide close to the FAD where the flavin is oxidized (termed the FO form). There is kinetic evidence that in solution there is an equilibrium of the FO and FR conformations (6), but in the packed crystal the FO is favored.

In order to better understand the mechanism of thioredoxin reductase, site-directed mutagenesis has been used to alter specific residues and then express, purify, and characterize the mutated enzymes. The method developed by Ekstein (7) for oligonucleotide-directed mutagenesis was employed for these studies (8,9). The single-stranded template for the mutagenesis reactions consisted of the thioredoxin reductase gene which had been cloned into a bacteriophage f1 vector (10). However, the production of bacteriophage that resulted from the mutagenesis reaction was often very low. Indeed, for several cases repeated attempts to isolate some mutants were entirely unsuccessful. In those cases where mutants could be obtained there were often problems in getting expression of the mutated enzyme in *E. coli*.

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The plasmid system developed by Brosius (11) avoids many of the problems described above. Vector pSL350 was designed for maximal expression of genes cloned into it, and contains optimized signals for transcription, translation, and plasmid stability, a "superlinker" region containing 64 potential restriction sites for cloning, as well as a single-stranded replication origin for production of single-stranded DNA which can be used as a template for mutagenesis reactions. This paper reports the successful application of this single vector system to construct a new plasmid, pTrR301, which allows high-level expression of thioredoxin reductase as well as simplified mutagenesis procedures. The new expression system is used to examine the ability of *E. coli* to synthesize and incorporate FAD cofactor into thioredoxin reductase. The relevance of these findings to other flavoproteins expressed in *E. coli* is also discussed. Wild-type thioredoxin reductase is purified from cells harboring plasmid pTrR301, and the spectral and kinetic characteristics are compared with those from enzyme purified from native *E. coli.* Finally, the new system is used to create a new mutant, N155Y, which is purified and partially characterized.

MATERIALS AND METHODS

Strains and plasmids. The plasmid vector was pSL350 (11) and is diagrammed in Fig. 1. The starting material used in the following construction was bacteriophage f1R366 which contains the thioredoxin reductase gene on a 3.1-kb *Eco*RI fragment of *E. coli* DNA cloned into an f1 vector (10). *Escherichia coli* strain XL1-Blue (12) was used for growth of plasmid and single-stranded DNA. Strain A326 (13), which contains an insertion in the genomic thioredoxin reductase gene (*trx*B$^-$) and has the *lac*I^q locus was used for expression studies.

Single-stranded DNA was purified from f1R366 following standard procedures (14). The single-stranded form of pTrR301 DNA was prepared by using M13K07 helper phage (Gibco-BRL, Gaithersberg, MD) according to the directions of the manufacturer. Unless otherwise noted, standard molecular biological methods were used (15). All sequencing was performed at the University of Michigan Biomedical Research Core Facility using automated procedures.

Construction of plasmid pTrR301. A unique *Nco*I restriction site was introduced into f1R366 at the first base upstream of the start ATG codon of the thioredoxin reductase gene by using the oligonucleotide 5′-TGGTCGTGCCCATGGGATCCCCATA-3′ which made a T → C conversion at the underlined C. Mutagenesis reactions were performed with the Oligonucleotide-Directed Mutagenesis Kit, Version 2.1 (Amersham, Arlington Heights, IL), according to the manufacturer's instructions. Individual plaques resulting from the reactions were picked and grown, and the presence of the new restriction site was checked by digesting the double-stranded RF[1] DNA with *Nco*I.

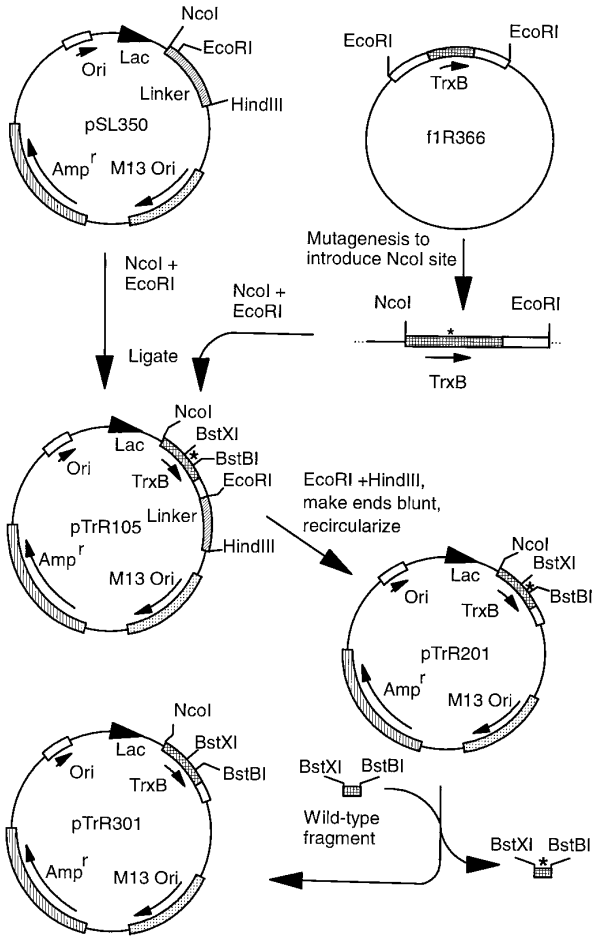

FIG. 1. Construction of plasmid pTrR301. The thioredoxin reductase gene (*trx*B) was mutagenized to introduce an *Nco*I site and then cloned into vector pSL350. The asterisk indicates an unintentional mutation at Glu 70 that resulted in a Glu to Asp conversion. This was corrected by removing most of the linker region of pTrR105, recircularizing, and then replacing a *Bst*XI–*Bst*BI containing the mutation with a corresponding wild-type fragment.

The selected mutant was digested with *Nco*I and *Eco*RI to remove the thioredoxin reductase gene. Vector pSL350 was digested with the same enzymes. The fragments were separated on a 1.5% (w/v) low-melting-point agarose gel (Gibco-BRL). The desired bands were cut from the gel and purified using a modification of the standard Qiaprep spin plasmid purification kit (Qiagen, Chatsworth, CA) using the buffers supplied by the manufacturer. Buffer PB was added to each gel slice to a final volume of 0.7 ml and heated in a 50°C

[1] Abbreviations used: IPTG, isopropyl-β-D-thiogalactopyranoside; RF, double-stranded replicative form circular DNA; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

water bath until the gel dissolved. After cooling to room temperature, the sample was applied to a Qiaprep spin column and centrifuged for 30 s, and washed with 0.5 ml of PB buffer followed by 0.7 ml of PE buffer. The DNA was eluted with 40 μl of 1 mM Tris, 0.1 mM EDTA, pH 8.0. After ligating the appropriate fragments, the resulting plasmid, pTrR105 (Fig. 1), was sequenced across the entire thioredoxin reductase gene. Analysis of the sequence revealed that, in addition to the desired *Nco*I site creation, there was a second unintentional A → C mutation at residue 70, resulting in a glutamate to aspartate substitution. This mutation was corrected by a two-step procedure: (i) interfering restriction sites in the superlinker were removed by digestion with *Eco*RI and *Hin*dIII, making the ends blunt with T4 DNA polymerase, and ligating to recircularize which yielded plasmid pTrR201, and (ii) the 614-bp *Bst*XI–*Bst*BI fragment containing the E70D mutation was excised and replaced with the corresponding wild-type fragment. This final construct, pTrR301, was verified by sequencing the entire thioredoxin reductase gene.

Time–course and induction studies of thioredoxin reductase expression. Cultures of A326(pTrR301) (20-ml cultures in 125-ml flasks) were grown at 37°C with rapid shaking in 2× YT medium (15) supplemented with 50 mM Na/K phosphate, pH 7, 20 mM glucose, 100 μg/ml ampicillin, 25 μg/ml kanamycin. Aliquots of cultures were chilled on ice, centrifuged, and frozen at −20°C. IPTG induction was measured by growing cultures as above to saturation for 18 h containing the indicated amount of inducer. The cells were thawed, resuspended in 50 mM Na/K phosphate, 0.15 mM EDTA, pH 7.6, disrupted by sonication, and centrifuged at 10,000*g* for 5 min. The supernatant was assayed directly, or after adding FAD to give a final concentration of 20 μM. This assay is similar to a previously published procedure (9) and is measured at 25°C in a buffer of 50 mM Na/K phosphate, 0.15 mM EDTA, pH 7.6, 200 μM 5,5′-dithiobis-(2-nitrobenzoic acid), 20 μM NADPH, and 20 μM *E. coli* thioredoxin. The units of activity were determined by measuring the increase in 412 nm absorbance and dividing this by two times the extinction coefficient of thionitrobenzoic anion (13,600 M^{-1}) to give μmol NADPH min^{-1}. The fraction of apoenzyme present in each sample was calculated from the increase in activity upon addition of excess FAD assuming that all of the increase is due to activation of apoenzyme. Total protein was assayed by using the Bio-Rad protein assay reagent using bovine serum albumin as the standard. The percentage of the total protein consisting of thioredoxin reductase was calculated from the specific activity of uninduced or induced cell extracts and dividing by the specific activity of purified enzyme. Expression was also analyzed by SDS–PAGE using a 12% acrylamide (29.2:0.8, acrylamide:bisacrylamide) gel prepared according to standard methods (15).

N155Y mutagenesis. Mutagenesis was performed on the single-stranded form of pTrR301 by using the oligonucleotide 5′-CTTCAACCGCGGTATACCCGCCGCCG-3′. The first underlined A is a T → A conversion, which changes asparagine-155 to a tyrosine, and the underlined C is a silent mutation at a glycine 154 that creates an *Acc*I restriction site that was used to screen potential mutants. The reactions were performed using the Amersham kit as described above except that individual colonies resulting from the mutagenesis were picked and grown overnight at 37°C with rapid shaking in 2 ml of 2× YT medium supplemented with 100 μg/ml ampicillin. Plasmid DNA was purified and analyzed for the presence of the new restriction by digestion with *Acc*I. Three candidate colonies were tested, and all three had the new restriction site. One isolate, designated pTrR344, was then sequenced across the entire thioredoxin reductase gene to verify the desired changes.

Purification of wild-type and N155Y thioredoxin reductases. Cells used for purification of enzyme were grown without IPTG induction since they yield the greatest fraction of holoenzyme, tend to have higher total protein content than induced cultures, and give more than adequate yields. The protocol used here is an extensively modified procedure of a previously published method (9). Frozen cells of A326 containing either plasmid pTrR301 or pTrR344 were streaked on 2× YT agar plates supplemented with 100 μg/ml ampicillin and 25 μg/ml kanamycin and incubated at 37°C for 16 h. A single colony was used to inoculate 300 ml of 2× YT medium supplemented with 50 mM phosphate, pH 7, 20 mM glucose, 100 μg/ml ampicillin, and 25 μg/ml kanamycin and grown for 20–24 h in a 2.8-liter flask at 37°C with vigorous shaking. The cultures were then chilled on ice and centrifuged at 10,000*g* at 4°C for 10 min and the cell pellets frozen at −20°C. The cells were thawed and resuspended in 2 vol of 10 mM phosphate buffer, 0.3 mM EDTA, pH 7.6 (buffer A), containing 0.5 mM phenylmethylsulfonyl fluoride and 1 μmol of FAD (Sigma, St. Louis, MO). The cells were disrupted by sonication and streptomycin sulfate was added to a final concentration of 2% (w/v). The crude preparation was centrifuged at 27,000*g* for 30 min at 4°C and the supernatant was saved. Ammonium sulfate was added (0.56 g/ml of supernatant) and dissolved completely, and the resulting suspension centrifuged at 12,000*g* for 10 min at 4°C. The yellow pellet was dissolved in 5–10 ml of buffer A and dialyzed overnight in 1 liter of buffer A with several changes. The preparation was then filtered through a 0.45-μm syringe filter.

The enzyme was then purified in a single-column procedure by using a 2-cm diameter × 19 cm long 2′,5′-

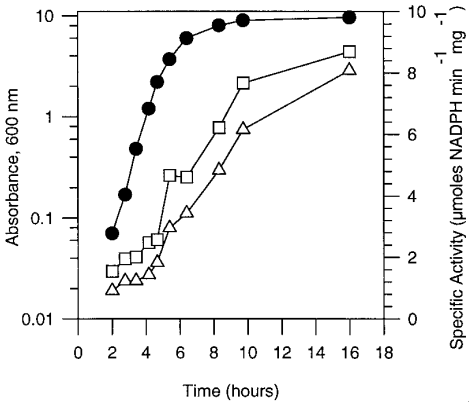

FIG. 2. Expression of thioredoxin reductase by pTrR301 in *E. coli* A326. Cell density of a culture grown without IPTG was followed by absorbance at 600 nm (●), and the specific activity of cell extracts at time points during growth was assayed directly (△), or with 20 μM FAD added to the assay mix (□).

ADP Sepharose 4B column (Pharmacia Biotech, Piscataway, NJ) which had been equilibrated with buffer A. After loading the preparation, the column was washed with buffer A until the 280-nm absorbance began to decrease. The enzyme was eluted by using a 124-ml 0 to 1 M NaCl gradient in buffer A. Fractions were monitored by absorbance and those with the lowest 280 nm/456 nm ratios were pooled and precipitated by adding 0.56 g/ml of ammonium sulfate. The precipitate was stored at 4°C. Extinction coefficients were determined by using SDS to resolve the FAD from the enzyme as previously described (9). Fluorescence spectra were recorded on a Perkin–Elmer MPF-44B at 25°C and corrected for variations in lamp intensity and photomultiplier sensitivity.

RESULTS AND DISCUSSION

Our work on the catalytic mechanism and conformational changes of thioredoxin reductase depends heavily on the ability to make and express site-directed mutants of the enzyme. Earlier studies had used a two-step procedure: first mutate the thioredoxin reductase gene on single-stranded bacteriophage DNA, and then clone a mutated DNA fragment from the bacteriophage and place it in a separate expression plasmid (8,9). It was found, however, that some mutations, particularly at the active site, yielded either very low or no mutant bacteriophage resulting from the mutagenesis reactions. In other cases, mutant phage could be isolated, but the yield of RF DNA was so low it was very difficult to purify enough mutated restriction enzyme fragment to substitute with the corresponding fragment in the expression plasmid. One plausible explanation for these problems lies in the fact that thioredoxin reductase plays an important role in bacteriophage assembly (3), indeed, *E. coli* having mutations in their thioredoxin reductase gene were identified by their greatly decreased bacteriophage yield. Cells that are expressing mutated thioredoxin reductase could be partially or completely inhibited in bacteriophage production.

A plasmid vector such as the pSL350 system circumvented these problems. It has an M13 origin of replication which allowed production of wild-type single-stranded DNA which serves as template for oligonucleotide-directed mutagenesis reactions. This is the only step that depends on the production of single-stranded DNA: there have been no problems obtaining sufficient quantities of wild-type single-stranded DNA for mutagenesis reactions. The resulting product of the muta-

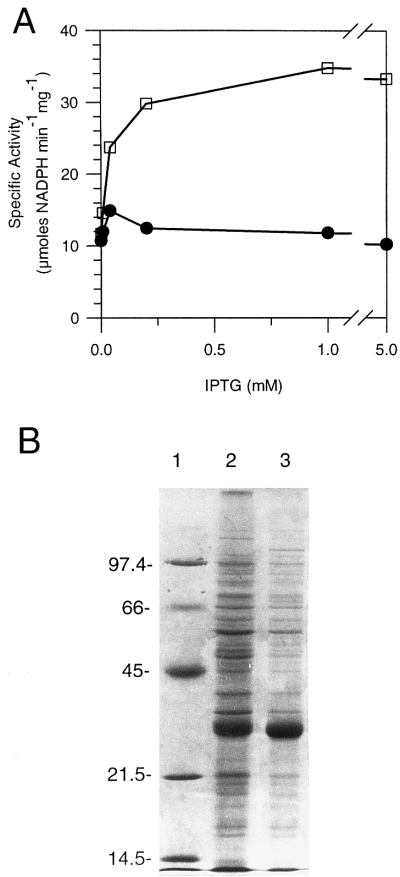

FIG. 3. Effect of IPTG concentration on thioredoxin reductase expression from pTrR301 in *E. coli* A326. (A) Saturated cultures grown in the indicated concentrations of IPTG were assayed directly (●), or after supplementing the crude extract with 20 μM FAD (□). For comparative purposes, the specific activity of purified thioredoxin reductase is 57 μmol NADPH min^{-1} mg^{-1}. The increase in activity after adding FAD is attributed to the amount of apoenzyme present in the extract. (B) SDS–PAGE analysis of total soluble proteins in sonicated cells. Lane 1, molecular weight standards; lane 2, a sample of cell extracts assayed in (A) grown without IPTG containing enzymatic activity (in excess FAD) equivalent to 2 μg of thioredoxin reductase; lane 3, a sample of cell extracts assayed in (A) grown in 5 mM IPTG containing 2 μg of thioredoxin reductase.

TABLE 1

Purification of Wild-Type and N155Y Thioredoxin Reductase

Fraction	Total protein (mg)[a]	Total units (U)[b]	Specific activity (U/mg)	Yield (%)
Wild type				
Sonicated cells	1028	14,879	14.5	100
Streptomycin sulfate	713	12,921	18.1	86.9
After ammonium sulfate concentration and dialysis	510	11,250	22.1	75.6
2′–5′ ADP column	189	10,757	57.0	72.3
N155Y				
Sonicated cells	760	730	0.96	100
Streptomycin sulfate	435	723	1.66	99.0
After ammonium sulfate concentration and dialysis	460	571	1.24	78.2
2′–5′ ADP column	50.6	504	10.0	69.0

[a] From 300 ml of culture.
[b] Activity assayed at 20 μM thioredoxin and 20 μM NADPH.

genesis reactions is double-stranded plasmid DNA which is transformed into *E. coli*, allowing the isolation and screening of mutant bacteriophage to be avoided. The pSL350 vector also has sequences for inducible transcription from an upstream *lac* promoter, efficient translation with a T7 gene 10 enhancer and optimized ribosome binding region, and greater plasmid stability, all of which contribute to high levels of expression of wild-type and mutated thioredoxin reductase.

A typical growth curve of uninduced *E. coli* A326(pTrR301) is shown in Fig. 2. The specific activities of cell extracts at each time point were assayed directly, and then assayed again after adding excess FAD and incubating for 15 min on ice. The level of active enzyme in the cells increased rapidly during exponential growth and continued to rise slightly after the culture was in stationary phase. Even after the cells had reached stationary phase, the specific activity of cell extracts assayed without added FAD increased 31% between 10 and 16 h, indicating that some enzyme and FAD synthesis was occurring in the absence of cell growth.

Addition of IPTG as an inducer increases thioredoxin reductase expression with a maximum effect at $\geqslant 1$ mM (Fig. 3A). The reason for the high expression of thioredoxin reductase with no IPTG is unclear, but may be due to the high copy number of pTrR301 which may titrate out the *lac* repressor in the cells, leaving some of the plasmid unrepressed. Alternatively, for unknown reasons, the binding of the *lac* repressor to pTrR301 may be weak. This was unexpected since *E. coli* strain A326 overproduces the *lac* repressor (*lac*I^q) but there apparently was not enough repressor to completely turn off expression. The high expression of thioredoxin reductase in the absence of IPTG may explain one important observation: pTrR301 fails to transform into any *E. coli* strain that lacks the *lac*I^q marker. Apparently the background expression of thioredoxin reductase inhibits some process in the early stages of the transformation. After the pTrR301 becomes established in the cell, high expression was not inhibitory to the maintenance of the plasmid: thioredoxin reductase levels were as high as 19% of the soluble protein in uninduced cells and 61% of the soluble protein in cultures grown in 1 mM IPTG (calculated from the FAD-supplemented specific activities from Fig. 3A). Thus, 1 liter of this induced culture contained about 1.5 g of soluble protein, of which 900 mg is enzyme.

A fraction of the thioredoxin reductase expressed in *E. coli* cells was apoenzyme. This is evident in Fig. 2 where addition of FAD to uninduced cell extracts resulted in increased specific activities, and in Fig. 3A in which cultures grown in increasing concentrations of IPTG showed larger amounts of activation upon addition of FAD to extracts. This probably reflects the fact that the enzyme was being produced faster and in greater quantities than the bacteria can manufacture FAD cofactor. Thus, in our situation, there seems to be an upper limit of FAD production in *E. coli* of about 10 μmol of FAD per liter of culture (16 g of wet cell weight). The fraction of apoenzyme in cultures was as high as 68.4% for cells grown in 5 mM IPTG. Figure 3B shows samples of sonicated extracts of cells grown without IPTG (lane 2), or in 5 mM IPTG (lane 3). Thioredoxin reductase is clearly the major protein component in the soluble extracts. Addition of 100 μM riboflavin to the growth medium did not increase the amount of active enzyme in these induced cells (data not shown), which may indicate that the steps in conversion of FAD from riboflavin are limiting FAD production in strain A326.

TABLE 2

Comparison of Parameters of Wild-Type Thioredoxin Reductase Purified from pTrR301 Expression with Previously Published Values

Parameter	Values from literature[a]	Values from this study
Turnover number (μmol NADPH min^{-1}/μmol enzyme)	2000	2475 ± 223
$K_{m,\ NADPH}$ (μM)	1.2	4.6 ± 1.0
$K_{m,\ thioredoxin}$ (μM)	2.8	1.7 ± 0.4
Spectral max. (nm)	271,380,456	271,380,456
Extinction coefficient ($M^{-1}\ cm^{-1}$)	11,300	11,360 ± 140
Absorbance ratio (271 nm/456 nm)	5.8	5.7
Absorbance ratio (380 nm/456 nm)	1.03	1.02

[a] References (19) and (20).

These results allow some conclusions to be made on the sequence of events in thioredoxin reductase synthesis and cofactor insertion: the enzyme is able to fold in the necessary structure prior to FAD incorporation since the apoenzyme that is formed in the cell is quickly activated by addition of exogenous FAD to sonicated cells. This finding agrees with *in vitro* reconstitution studies where denatured and refolded thioredoxin reductase apoenzyme is fully reactivated by addition of FAD (16). Our results are similar to those for *Bacillus* sp. recombinant sarcosine oxidase expressed in *E. coli*, where a large increase in activity was observed upon addition of FAD to cell extracts (17). In addition, Oster *et al.* reported large yields of the flavoprotein domain of cytochrome $P450_{BM-3}$ which contained stoichiometric amounts of cofactor (18). Their yield of holoprotein (3 μmol/30 g wet cell weight after purification losses) were still well below the upper limit of cofactor synthesis we have defined here (about 10 μmol/16 g wet cell weight). Strain A326, which is derived from K38, was the only *E. coli* strain tested for thioredoxin reductase expression since it is the only known strain that is both *trx*B^- and *lac*I^q. It is conceivable that other strains may provide higher FAD levels. Clearly, the capacity of *E. coli* to synthesize flavin cofactor needs to be considered when expressing flavoproteins at the very high levels described here. In cases where flavoproteins cannot be reactivated by direct addition of cofactor to cell extracts or purified away from the apoprotein, expression levels may have to be moderated in order to obtain protein with the correct stoichiometry of flavin.

Wild-type thioredoxin reductase expressed from 7.3 g of A326(pTrR301) was purified in a single column protocol and is summarized in Table 1. Assay of the sonicated cells shows that that the enzyme constituted 25% of the total protein. The final yield was 189 mg from a 300-ml culture and the enzyme showed a single band on an SDS–PAGE (data not shown). The kinetic and spectral characteristics of the purified enzyme are summarized in Table 2 along with the corresponding values that were published for the nonrecombinant enzyme that was purified from the native organism (19,20). The higher turnover number probably reflects that thioredoxin reductase expressed from pTrR301 is obtained in higher purity than was possible before.

The utility of the new plasmid system for site-directed mutagenesis was demonstrated by replacing asparagine-155 with a tyrosine. This mutant is a test of a novel strategy which introduces a tyrosine in a region that is expected to move relative to the FAD during the domain rotation conformational changes which have been proposed for this enzyme (5). Tyrosines in close proximity to FAD in proteins are known to quench flavin fluorescence (21,22). If the rotation model is correct, then the tyrosine-155 region should be close to the FAD in the FR form and far from the FAD in the FO form. In this way, it may be possible to observe changes in the FR/FO equilibrium under different conditions by measuring changes in the fluorescence of thioredoxin reductase mutants which have engineered tyrosines in near the FAD.

The mutagenesis to create N155Y was successful and the purification from 6.5 g of uninduced cells is summarized in Table 1. Expression of N155Y was lower than

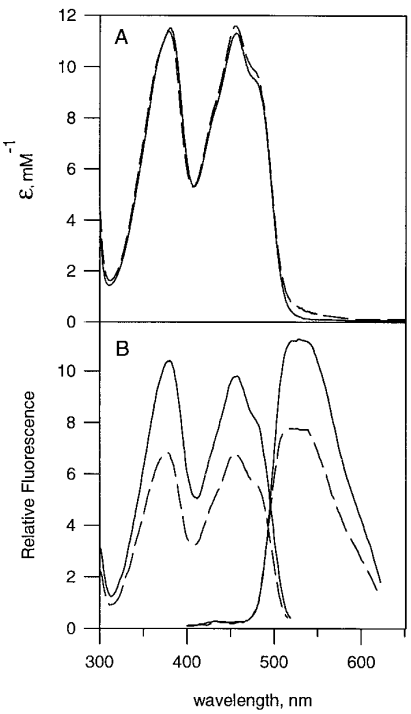

FIG. 4. Absorbance and fluorescence spectra of wild-type (—) and N155Y (---) thioredoxin reductases. (A) Absorbance spectra, (B) fluorescence excitation (emission at 540 nm, left spectra), and emission (excitation at 380 nm, right spectra) of 10 μM enzymes.

for wild-type, constituting only 9.6% of the total protein. The final enzyme had 17.5% the specific activity of wild-type thioredoxin reductase, but this is not surprising since asparagine-155 is near the NADPH binding site. The absorbance and fluorescence spectra are shown in Fig. 4. The extinction coefficient of the N155Y mutant was determined to be 11,620 ± 50 M^{-1} cm^{-1} at 456 nm. The fluorescence at 456 nm excitation and 540 nm emission was 69% that of wild-type, indicating that tyrosine-155 is quenching the flavin fluorescence. Although it is not possible to measure the exact ratios of the FR and FO conformations from these preliminary data, it is clear that the tyrosine mutagenesis and fluorescence quenching strategy may hold promise as a method to detect domain movement in thioredoxin reductase.

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