Supporting Methods

The pQE30 plasmid containing the insert of (His-tagged) wild-type DmTrxR-1 (National Center for Biotechnology Information accession no. AF301144) was prepared as described (1). The pSUABC plasmid was used as described in ref. 2.

DmTrxR mutants (see Table 1 for C-terminal sequences and functional groups at the C-terminal tetrapeptide) were prepared from the plasmid carrying wild-type insert using the same forward-primer (5'-CGC*GGATCC*GCGCCCGTGCAAGGTTCC; *Bam*HI-restriction site is in italics) in PCRs with the following reverse primers (C-terminal tetrapeptide of the resulting mutants is shown at the left, *Hin*dIII-restriction sites are in italics):

GCCG	5'-CGC*AAGCTT*TTAGCCGCAGCAGCCGGCC
SCCG	5'-CGC*AAGCTT*TTAGCCGCAGCAGCTGGCC
GCCS	5'-CGC*AAGCTT*TTAGCTGCAGCAGCCGGCC
GCCD	5'-CGC*AAGCTT*TTAGTCGCAGCAGCCGGCCGG
DCCG	5'-CGC*AAGCTT*TTAGCCGCAGCAGTCGGCCGG
DCCD	5'-CGC*AAGCTT*TTAGTCGCAGCAGTCGGCCGG
SCSS	5'-CGC*AAGCTT*TTAGCTGGAGCAGCTGGCCG
GCUG	5'–CGC*AAGCTT*GGCTGCATTAGGCTAACGATTGGTGCAGACCTGCA ACCGATTATTAGCCTCAGCAGCCGGCCGGCG
SCUG	5'–CGC*AAGCTT*AGCTGCATTAGGCTAACGATTGGTGCAGACCTGCA ACCGATTATTAGCCTCAGCAGCTGGCCGGCG
GCUS	5'–CGC*AAGCTT*GGCTGCATTAAGCTAACGATTGGTGCAGACCTGCA ACCGATTATTAGCTTCAGCAGCCGGCCGGCG
SCUS	5'–CGC*AAGCTT*AGCTGCATTAAGCTAACGATTGGTGCAGACCTGCA ACCGATTATTAGCTTCAGCAGCTGGCCGGCG

All primers were obtained from Metabion (Martinsried, Germany), and were purified by HPLC. Cloning enzymes were obtained from MBI-Fermentas (St. Leon-Rot, Germany).

Hot-start PCRs were conducted in 50 μl with template plasmid and 20 pmol of both primers with 160 μM dNTP in 2 mM $MgCl_2$/75 mM Tris•HCl/20 mM $(NH_4)_2SO_4$/0.01% Tween 20, pH 8.8, by using a 3-min 95°C denaturation step followed by the addition of Taq-DNA-polymerase (1 unit) and 25 cycles of 30 s at 95°C, 60 s at 60°C, and 60 s at 72°C. The reaction was concluded with a 10-min elongation step at 72°C.

The ≈1.5-kbp PCR products were purified, digested with *Bam*HI and *Hin*dIII, inserted into pQE30 vector plasmid (Qiagen, Hilden, Germany), and propagated in NovaBlue-competent cells (Novagen) in the presence of carbenicillin (50 μg/ml). Plasmid was purified from individual clones and inserts were verified by sequence analysis.

For expression of the selenium-containing mutants, pSUABC was cotransformed by using the TSS method (3), which was essentially as described for production of rat TrxR (2).

Protein Expression. Proteins were expressed and purified essentially as described for DmTrx-2 (4). Cultures for production of Cys-Sec mutants contained, in addition, 34 μg per ml chloramphenicol, 5 μM Na_2SeO_3, and 100 μg per ml L-cysteine. In all preparations, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 400 μM, 3 h before the harvest. Protein purity was confirmed by silver-stained SDS/PAGE.

Selenium Determination. Enzyme samples were dialyzed against 25 mM potassium phosphate, 500 μM EDTA, pH 7.4. Four different dilutions per sample were prepared in distilled water (total volume of 1 ml per sample). Dialysis buffer and a cysteine mutant enzyme sample (at a similar concentration as judged by FAD content) served as negative controls. Selenium concentration was determined at a commercial core facility (V. Muntean, Labor Seelig, Karlsruhe, Germany) by using graphite oven atomic absorption spectrometry. The concentration of the stock solution as calculated from the different dilutions did not vary significantly. Selenium concentration per protein-bound FAD,

reflecting the content of full-length protein, was ≈40% for most preparations (see Table 1 and Fig. 5).

Prematurely truncated TrxR species are completely inactive in thioredoxin reduction (2), and determinations of kinetic parameters were therefore judged not to be affected by the presence of truncated protein species in these enzyme preparations.

Metabolic Labeling. The selenocysteine and respective cysteine mutants were grown at 37°C in 3 ml of LB containing 50 μg/ml carbenicillin and 100 μg/ml L-cysteine. Chloramphenicol (34 μg/ml) was added to the cultures expressing selenocysteine mutants. When the optical density of the cultures reached 1.0 (λ = 600 nm), 1 ml of the cultures was transferred to a tube to which 1 μCi ^{75}Se isotope as 5 μM [^{75}Se]selenite (University of Missouri Research Reactor, Columbia) and 0.5 mM IPTG were added, and growth was continued for 16 h at 37°C. The cells were harvested by centrifugation, and were resuspended in 350 μl of 0.1% wt/vol SDS/10 mM Tris•HCl, pH 6.8/10% wt/vol glycerol/0.5% bromophenol blue/100 mM DTT, and boiled for 10 min. Debris was removed by centrifugation and 20 μl of the supernatant was subjected to SDS/PAGE. Proteins were stained with Coomassie blue and radioactivity was visualized by autoradiography using a PhosphorImager. Comparing labeling of the recombinant selenoprotein with the corresponding cysteine mutants revealed that the selenium incorporation was specific; the cysteine mutants completely lacked ^{75}Se-labeling (see Fig. 5).

1. Kanzok, S. M., Fechner, A., Bauer, H., Ulschmid, J. K., Müller, H. M., Botella-Munoz, J., Schneuwly, S., Schirmer, R. & Becker, K. (2001) *Science* **291,** 643–646.

2. Arnér, E. S. J., Sarioglu, H., Lottspeich, F., Holmgren, A. & Böck, A. (1999) *J. Mol. Biol.* **292,** 1003–1016.

3. Chung, C. T., Niemela, S. L. & Miller, R. H. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 2172–2175.

4. Bauer, H., Kanzok, S. M. & Schirmer, R. H. (2002) *J. Biol. Chem.* **277,** 17457–17463.