The thioredoxin superfamily consists of enzymes that catalyze the reduction, formation, and isomerization of disulfide bonds and exert their activity through a redox active disulfide in a Cys-Xaa1-Xaa2-Cys motif. The individual members of the family differ strongly in their intrinsic redox potentials. However, the role of the different redox potentials for the in vivo function of these enzymes is essentially unknown. To address the question of in vivo importance of redox potential for the most reducing member of the enzyme family, thioredoxin, we have employed a set of active site variants of thioredoxin, which largely depends on the redox potential of lipoic acid by NADPH as a model reaction. We propose in vitro ratification assays. The same correlation could be established in vivo function of thioredoxin, as recycling of reduced thioredoxin through NADPH and thioredoxin reductase, providing a basis for the maintenance of the reducing redox status of the cytosol (5). In addition, thioredoxin catalyzes the NADPH-dependent reduction of a number of important metabolic enzymes such as ribonucleotide reductase, 3’-phosphoadenosine-5’-phosphosulfate reductase, and methionine-sulfoxide reductase, whose active sites become oxidized as part of their catalytic cycle (see Holmgren and Björnstedt (6)). In contrast, members with oxidizing redox properties like DsbA and DsbC are found in the periplasmic periplasm. These enzymes catalyze the formation and isomerization of disulfide bonds during folding of newly translocated proteins (7, 8). An exception to this correlation between cellular localization and the redox potential appears to be the periplasmically oriented thioredoxin-like proteins that are anchored to the inner bacterial membrane. An example is TlpA from Bradyrhizobium japonicum, which has a low redox potential (−259 mV) despite its periplasmic orientation and is required for cytochrome aa3 maturation (9, 10).

Thio-disulfide oxidoreductases with thioredoxin fold share a catalytic disulfide bond with the sequence Cys-Xaa1-Xaa2-Cys. An influence of the active site dipeptide Xaa1-Xaa2 on the redox properties of these enzymes has been demonstrated, which results from a modulation of the pKa value of the more N-terminal, nucleophilic active site cysteine (11–14). In DsbA, the extremely low pKa value (−3.5) of the nucleophilic cysteine stabilizes the reduced state of the active site making the protein an excellent disulfide bond donor (15). Replacement of the Xaa1-Xaa2 sequence of DsbA by that of thioredoxin decreased the redox potential by 90 mV making DsbA a 1000-fold better reductant in terms of equilibrium constants with substrate compounds (13). Conversely, a DsbA-like Xaa1-Xaa2 variant of thioredoxin became a 200-fold better oxidant (14). Despite these dramatic changes in redox potentials, the corresponding mutant disulfide oxidoreductases did not show phenotypic effects in bacteria under physiological conditions (10, 11, 16, 17). Furthermore, the most reducing member of the thioredoxin superfamily, thioredoxin, and variants thereof can, at least partially, substitute for the most oxidizing member DsbA when exported to the periplasm (18, 19). This raises the question whether redox potential is at all an important property of these proteins under physiological growth conditions. A certain correlation between the redox potentials of DsbA variants and their ability to complement a DsbA null phenotype could only be demonstrated for the recovery of resistance toward the reductant dithiothreitol (DTT)1 in the growth medium (11).

Thioredoxin and glutaredoxin-1 from E. coli serve as cyto-

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1 The abbreviations used are: DTT, dithiothreitol; PDI, protein-disulfide isomerase; TR, thioredoxin reductase; HPLC, high pressure liquid chromatography; uPA, urokinase-like plasminogen activator.
plasmic mutants of 3'-phosphoadenosine-5'-phosphosulfate reductase, an enzyme in the pathway of sulfate reduction. Absence of both trxA and grxA is accompanied by the inability to grow on sulfate minimal medium unless a source of reduced sulfur such as cysteine is provided (20). Thioredoxin is also the preferred redundant of methionine-sulfoxide reductase as shown by the inability of trxA mutants to use methionine sulfoxide as the sole source of methionine (21). Moreover, a pathway for periplasmic disulfide bond isomerization has recently been described (22) that funnels reducing equivalents from thioredoxin in the cytosol to the periplasmic disulfide isomerase DsbC via the membrane protein DsbD (23). To address the question whether the intrinsic redox potential of thiol-disulfide oxidoreductases is of any major importance in vivo, we have used these in vivo functions of thioredoxin to test a set of thioredoxin variants with increased redox potentials (ranging from 270 to 195 mV, cf. Table I) for complementing activity in E. coli thioredoxin null mutants. The selected mutant proteins are the three most oxidizing thioredoxin variants that have been reported so far and bear the Xaa3-Xaa9 dipeptide sequences of the following members of the thioredoxin family: glutaredoxin (active site: Cys-Pro-Tyr-Cys), DsbA (active site: Cys-Pro-His-Cys), and eukaryotic protein-disulfide isomerase (PDI; active site: Cys-Gly-His-Cys). The basis for the in vivo studies presented in this paper is the finding that these thioredoxin variants are all substrates of thioredoxin reductase (TR) and show a clear in vitro correlation between their redox potentials and efficiency of catalyzing the reduction of lipoc acid by NADPH as a model reaction.

**EXPERIMENTAL PROCEDURES**

**Materials**—DE52 and CM52 cellulose were from Whatman, the Superdex 200 HiLoad 26/60 column and the F101 columns were from Amersham Pharmacia Biotech, and 2'5'-ADP-agarose was from Sigma. Tryptone and yeast extract were from Difco, and isopropyl-D-thiogalactopyranoside was purchased from AGS (Heidelberg, Germany). l-arabinose, amino acids, DTT, oxidized DTT, dithionitrobenzoic acid, m-lipoic acid, methionine sulfoxide, and NADPH were from Sigma. Bovine insulin was obtained from Fluka (Buchs, Switzerland). All other chemicals were of analytical grade.

**Purification of Thioredoxin**—Thioredoxin wild type and the thioredoxin variants were purified from overproducing E. coli strains as described previously (14). The protein concentrations were measured by the specific absorbance at 280 nm (14).

**Construction of Thioredoxin Expression Plasmids with Arabinose Promoter**—Molecular cloning techniques were based on Sambrook et al. (24). Direct cloning of the thioredoxinencoding region was performed by the polymerase chain reaction using the following primers: trxA-SacI, 5'-AGG CGA GCT CCT

**Stoichiometric Reduction of Thioredoxin by DTT**—Reduced thioredoxin was prepared by incubation of the oxidized protein with a 1000-fold molar excess of DTT (50 mM) at pH 7.0 for 1 h and subsequent removal of DTT on a PD10 gel filtration column (Amersham Pharmacia Biotech). Assays with thioredoxin as stoichiometric reductant were carried out at 25 °C in 100 mM Tris/HCl, pH 8.0, 0.2 mM EDTA, and subsequent removal of DTT on a PD10 gel filtration column. The insulin reaction products were separated by reversed-phase HPLC at 55 °C on a Vydac 218TP54 column using a linear gradient from 30 to 60% acetonitrile in 0.1% trifluoroacetic acid. The decrease in the peak area of native insulin served to quantify the initial velocity of the reactions. Identical reactivities of the three disulfide bonds in insulin were assumed for evaluating the initial decrease in native insulin.

**Reduction of Insulin, Lipoic Acid, and Oxidized DTT by Thioredoxin**—Reduced thioredoxin was prepared by incubation of the oxidized protein with a 1000-fold molar excess of DTT (50 mM) at pH 7.0 for 1 h and subsequent removal of DTT on a PD10 gel filtration column (Amersham Pharmacia Biotech). Assays with thioredoxin as stoichiometric reductant were carried out at 25 °C in 100 mM Tris/HCl, pH 8.0, 0.2 mM EDTA, and subsequent removal of DTT on a PD10 gel filtration column. The insulin reaction products were separated by reversed-phase HPLC at 55 °C on a Vydac 218TP54 column using a linear gradient from 30 to 60% acetonitrile in 0.1% trifluoroacetic acid. The decrease in the peak area of native insulin served to quantify the initial velocity of the reactions. Identical reactivities of the three disulfide bonds in insulin were assumed for evaluating the initial decrease in native insulin.

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In Vivo Importance of the Redox Potential of Thioredoxin

Table I

| Thioredoxin variant (Xaa-Xaa dipeptide) | \( E^0 \) | \( K_M \) | \( k_{cat} \) | \( k_{cat}/K_M \) |
|----------------------------------------|----------|-----------|------------|----------------|
| Wild type (Gly-Pro)                    | -270     | 260 \( \mu M \) | 28.5 \( \pm 1.1 \) | \( 1.1 \times 10^7 \) |
| TR-type (Ala-Thr)                      | -222     | 520 \( \mu M \) | 33.9 \( \pm 1.8 \) | \( 6.5 \times 10^6 \) |
| PDI-type (Gly-His)                     | -221     | 130 \( \mu M \) | 22.5 \( \pm 1.4 \) | \( 1.6 \times 10^7 \) |
| DsbA-type (Pro-His)                    | -204     | 110 \( \mu M \) | 22.5 \( \pm 1.3 \) | \( 1.9 \times 10^7 \) |
| Grx-type (Pro-Tyr)                     | -195     | 125 \( \mu M \) | 24.1 \( \pm 0.6 \) | \( 1.9 \times 10^7 \) |
| DsbA wild type                         | -122     | NR        | NR         | NR             |

\( a \) From Ref. 13 and 14.

\( b \) The thioredoxin reductase assay was performed as described (31) using fixed concentrations of TR (33 nM), NADPH (200 \( \mu M \)), and dithio- nitrobenzoic acid (500 \( \mu M \)) and varying concentrations of thioredoxin or DsbA (0.04–40 \( \mu M \)). The uncatalyzed reaction was negligible under these conditions.

\( c \) No reactivity could be observed for DsbA.

![FIG. 1](https://example.com/figure1)

**FIG. 1.** Dependence on thioredoxin redox potential of thioredoxin-catalyzed reduction processes. A, reduction of insulin (130 \( \mu M \)) by DTT (1 mM) at pH 7.0 and 25 °C catalyzed by 1 \( \mu M \) thioredoxin wild type or its more oxidizing active site variants. The reactions were followed by an increase in optical density at 650 nm caused by precipitation of reduced insulin. \( \bullet \), thioredoxin wild type; \( \blacksquare \), glutaredoxin-type thioredoxin; \( \blacktriangle \), DsbA-type thioredoxin; \( \square \), PDI-type thioredoxin; \( \blacktriangleleft \), uncatalyzed reaction. B, dependence on thioredoxin redox potential of the initial rate of the reduction of lipoic acid (1 mM) by NADPH (0.5 mM) at pH 8.0 and 25 °C catalyzed by thioredoxin reductase (33 mM) and the different thioredoxin variants (20 \( \mu M \) each).

RESULTS

All Active Site Variants of Thioredoxin with Increased Redox Potential Are Substrates of TR—NADPH constitutes the common donor of reducing equivalents for all thioredoxin-dependent reduction processes in *E. coli*. Thus, as a prerequisite for an *in vivo* characterization of the active site variants of *E. coli* thioredoxin listed in Table I, it had to be proven that the variants are still efficient substrates of TR. For this purpose, recombinant TR from *E. coli* was overexpressed and purified. The substrate properties of the variants toward *E. coli* TR were then tested using the TR- and thioredoxin-catalyzed reduction of Ellman’s reagent (dithionitrobenzoic acid) by NADPH (31). In this assay, all thioredoxin variants showed, within a factor of two, the same substrate properties (\( k_{cat}/K_M \) values) as wild-type thioredoxin (Table I). Consequently, all variants principally share the potential of complementing thioredoxin deficiency *in vivo*. In contrast, DsbA wild type is not a substrate of TR (Table I).

In Vitro Model Reactions to Probe the Importance of Thioredoxin Redox Potential—To establish an in vitro model reaction that can probe the importance of thioredoxin redox potential in thioredoxin-catalyzed processes, we compared the different thioredoxin variants with respect to their ability to catalyze the reduction of insulin by DTT at pH 7.0. This reaction is a widely used standard assay for detecting thiol-disulfide oxidoreductase activity (36). Unexpectedly, this assay gave an inverse correlation between redox potential of the thioredoxin variants and the rate of insulin reduction, with the PDI-like, the glutaredoxin-like, and the DsbA-like variant being more efficient catalysts than wild-type thioredoxin (Fig. 1A). This result can however be rationalized by considering the fact that the rate-limiting step in the thioredoxin-catalyzed reduction of insulin by DTT is the re-reduction of thioredoxin by DTT (36) and that the more oxidizing variants, in accordance with their increased redox potentials, are indeed reduced 20–40 times faster by DTT than the wild type (Table II, values of \( k_2(DTT_{red}) \)). A decreased rate of insulin reduction by the variants, which one might expect from their increased redox potentials, is obviously not a rate-limiting factor for this model reaction.

In a search for a model reaction that directly probes the redox potential of the thioredoxin variants, we thus had to choose another reaction where the reductive force of the thioredoxin variants and not their own reduction is rate-limiting for the catalytic cycle. First, we used NADPH and TR for rapid and efficient recycling of reduced thioredoxin (cf. Table I). As thioredoxin appears to be mainly in the reduced state in *E. coli* cells (37), it is likely that reduction of thioredoxin by NADPH is also not rate-limiting for the catalytic cycle of thioredoxin *in vivo*. Second, we looked for an alternative disulfide substrate that is reduced slower by thioredoxin than insulin. We compared insulin with the disulfide substrates lipoic acid and oxidized DTT with respect to the rate constants of their reduction by the reduced thioredoxin variants at pH 8.0. Unexpectedly, the rate constants of insulin reduction, measured by the in-
In Vivo Importance of the Redox Potential of Thioredoxin

| Thioredoxin variant (Xaa-Xaa dipeptide) | Thioredoxin as stoichiometric oxidant or reductant | Thioredoxin as catalyst |
|----------------------------------------|-----------------------------------------------|------------------------|
|                                        | $E^+$                                         | $k_2$ (DTT red)$^a$     | $k_2$ (insulin)$^d$ | $k_2$ (lipoic acid)$^d$ | $v_i$ (NADPH)$^e$ | $v_i$ (native insulin)$^e$ | $v_i$ (lipoic acid, NADPH)$^e$ |
|                                        | $mV$                                         | $m^{-1} s^{-1}$         | $m^{-1} s^{-1}$     | $m^{-1} s^{-1}$     | $u s^{-1}$  | $u s^{-1}$  | $u s^{-1}$ |
| Wild type (Gly-Pro)                    | $-270$                                       | $1.1 \pm 0.3 \times 10^3$ | $8.8 \pm 1.0$     | $1.3 \pm 0.1 \times 10^3$ | $313 \pm 19$ | $5.7 \pm 0.7 \times 10^{-7}$ | $2.8 \pm 0.3 \times 10^{-7}$ | $4.3 \pm 0.6 \times 10^{-5}$ |
| PDII-type (Gly-His)                    | $-221$                                       | $2.4 \pm 0.6 \times 10^4$ | $5.8 \pm 2.2$     | $1.5 \pm 0.5 \times 10^3$ | $55.8 \pm 19$ | $6.4 \pm 0.2 \times 10^{-7}$ | $4.0 \pm 0.4 \times 10^{-7}$ | $2.4 \pm 0.2 \times 10^{-5}$ |
| DsbA-type (Pro-His)                    | $-204$                                       | $4.6 \pm 0.2 \times 10^6$ | $2.4 \pm 0.8$     | $1.3 \pm 0.1 \times 10^4$ | $11.6 \pm 3$  | $5.4 \pm 0.7 \times 10^{-7}$ | $3.9 \pm 0.4 \times 10^{-7}$ | $7.7 \pm 0.3 \times 10^{-6}$ |
| Grx-type (Pro-Tyr)                     | $-195$                                       | $4.7 \pm 0.9 \times 10^6$ | $3.3 \pm 1.9$     | $1.2 \pm 0.1 \times 10^4$ | $9.7 \pm 4$   | $4.7 \pm 0.8 \times 10^{-7}$ | $1.2 \pm 0.1 \times 10^{-7}$ | $6.0 \pm 0.1 \times 10^{-6}$ |

$^a$ Apparent second-order rate constant of the reduction of thioredoxin by DTT at pH 7.0 and 25 °C measured by the increase in thioredoxin fluorescence.

$^b$ Apparent second-order rate constant of the oxidation of thioredoxin by oxidized DTT at pH 8.0 and 25 °C measured by the decrease in thioredoxin fluorescence.

$^c$ Apparent second-order rate constant of the reduction of insulin by thioredoxin at pH 8.0 and 15 °C measured by the decrease in thioredoxin fluorescence.

$^d$ Apparent second-order rate constant of the reduction of lipoic acid by thioredoxin at pH 8.0 and 25 °C measured by the decrease in thioredoxin fluorescence.

$^e$ Initial velocity of NADPH consumption during thioredoxin-catalyzed reduction of insulin by NADPH at pH 8.0 and 25 °C, using concentrations of 500 μM NADPH, 33 nM thioredoxin reductase, 80 μM bovine insulin, and 20 μM thioredoxin. The reaction was followed by the decrease in NADPH absorbance at 340 nm.

$^f$ Initial velocity of the decrease in native insulin during its thioredoxin-catalyzed reduction by NADPH, as judged by HPLC analysis. Same conditions as in footnote e.

$^g$ Initial velocity of NADPH consumption during thioredoxin-catalyzed reduction of lipoic acid at pH 8.0 and 25 °C, using concentrations of 500 μM NADPH, 33 nM thioredoxin reductase, 1.0 mM lipoic acid, and 20 μM thioredoxin. The reaction was followed by the decrease in NADPH absorbance at 340 nm.

crease in thioredoxin fluorescence, were extremely high ($10^4$-$10^5$ M$^{-1}$ s$^{-1}$). In contrast, oxidized DTT and lipoic acid were reduced 2–4 orders of magnitude slower (Table II). In particular, the rate constants decreased with increasing redox potential of the thioredoxin variants. Therefore, both oxidized DTT and lipoic acid appeared to be suitable substrates for a thioredoxin-catalyzed model reaction probing the redox potential of thioredoxin. As the apparent rate constants for the reduction of lipoic acid by the thioredoxin variants were more sensitive toward the redox potentials of the variants compared with the reduction of oxidized DTT (Table II), we chose lipoic acid as a model substrate for reactions with thioredoxin as reduction catalyst. Indeed, when the thioredoxin-catalyzed reduction of lipoic acid by NADPH was measured for all the variants at saturation of TR (20 μM thioredoxin, cf. Table I), a good correlation between the initial velocities of lipoic acid reduction (measured by NADPH consumption) and the redox potentials of the variants was observed (Fig. 1B and Table II).

Conversely, the reaction with insulin as substrate under the same conditions was entirely independent of the redox potentials of the variants with respect to initial NADPH consumption (Table II). HPLC analysis of insulin reduction also yielded very similar initial velocities for the reaction (Table II). The difference between insulin and lipoic acid reduction can be explained by calculating the actual velocities of each step catalyzed by thioredoxin. We found that recycling of thioredoxin by NADPH was still slower than the reduction of insulin under the conditions of our assay. With concentrations of 20 μM thioredoxin, 33 nM for TR, and 80 μM for insulin and $k_{cat}$ values for thioredoxin reduction of 20–30 s$^{-1}$ (Table I), the calculated initial velocity of insulin reduction is at least ten times faster than recycling of reduced thioredoxin. Thus, insulin is such a good substrate of thioredoxin that at least 100-fold higher concentrations of TR would have been necessary to make insulin reduction the rate-limiting step.

In Vivo Characterization of Thioredoxin Variants with Different Redox Properties—Having established an in vitro assay for thioredoxin-catalyzed reduction of a disulfide substrate in which a clear correlation between redox potential and reactivity could be observed (Fig. 1B), we wanted to address the question whether the thioredoxin variants would show the same pattern of redox potential-dependent reactivity in vivo. The variants were cloned into the vector pBAD33 where protein expression is tightly regulated by the arabinose promoter (25). In the presence of the inducer arabinose (0.2% w/v) the expression levels in the trxA null mutant RI362 (23) were very similar for all thioredoxin variants as determined by Western blot analysis (Fig. 2).

Mutants of E. coli lacking thioredoxin have a number of readily assayed phenotypes. For instance, growth on minimal medium with sulfate as a sole source of sulfur requires either thioredoxin or glutaredoxin, because $trxA$, $grxA$ double mutants cannot reduce sulfate (20). When the plasmids encoding the thioredoxin variants were introduced into a $trxA$, $grxA$ double mutant, we found that they all complemented for growth on sulfate minimal medium, in contrast to the control plasmids lacking $trxA$ or encoding the inactive thioredoxin variant C35A that lacks the buried active site cysteine (Table III). The colony sizes correlated very well with the redox potentials of the complementing thioredoxin variants, with the most oxidizing glutaredoxin-like variant barely being able to sustain growth (Table III).

On minimal medium, growth in the presence of methionine sulfoxide should reflect the ability of the thioredoxin variants to complement a thioredoxin null mutant strain for the reduction of methionine sulfoxide to methionine in a methionine auxotroph strain (21). These experiments were carried out by introducing the set of thioredoxin expression plasmids to the $trxA$ null mutant RI362 (23). Again, the same redox potential-dependent order of complementation as that for sulfate reduction was observed for the growth on methionine sulfoxide as the sole methionine source (Table III).

Periplasmic disulfide bond isomerization is dependent on the periplasmic disulfide isomerase DsbC. As only the reduced form of DsbC is catalytically active as an isomerase, it is maintained in the reduced state by reducing equivalents from NAPDH in the cytosol, a process catalyzed by TR and thioredoxin in the cytoplasm, and by DsbD in the inner membrane of E. coli (22, 23). The evidence for this pathway is that disruption of any of the corresponding genes leads to the accumulation of oxidized DsbC and a severe defect in disulfide bond isomerization during folding of proteins with multiple disulfide bonds in...
In Vivo Importance of the Redox Potential of Thioredoxin

The present investigation is the first study on the influence of the intrinsic redox potential of a cytoplasmic member of the thioredoxin family on its function in vivo. Complementation of three phenotypes of trxA strains, i.e., deficiency in reduction of sulfate, methionine sulfoxide, and periplasmic disulfide bond isomerization, by a series of thioredoxin variants with different redox potentials was investigated. In all cases, the degree of trxA complementation strictly increased with decreasing redox potential. Thus, the low redox potential of thioredoxin is clearly a very important factor for its in vivo function.

In general, a change in redox potential of a thiol-disulfide oxidoreductase can be caused by altered rate constants of its own reduction and/or its own oxidation. We found that the in vivo results on the different thioredoxin variants could only be reproduced in vitro under conditions where recycling of reduced thioredoxin is not rate-limiting for catalysis. We propose that the main requirement for the low redox potential of thioredoxin is the fact that reduction of thioredoxin by NADPH is not rate-limiting for catalysis. We therefore conclude that recycling of reduced thioredoxin is not rate-limiting for catalysis. We propose that the main requirement for the low redox potential of thioredoxin is the fact that reduction of thioredoxin by NADPH is not rate-limiting for catalysis. We therefore conclude that recycling of reduced thioredoxin is not rate-limiting for catalysis. This view is supported by the insulin reduction assays (Fig. 1A). Here the reduction of thioredoxin by DTT is rate-limiting for catalysis so that an inverse correlation between the redox potential of the thioredoxin variants and their catalytic efficiency was obtained, again as predicted from the order of their redox potentials. Consequently, an in vitro

**Table III**

| Thioredoxin variant (Xaa-Xaa dipeptide) | $E_0$ | Sulfate reduction$^a$ | Methionine sulfoxide reduction$^b$ |
|---------------------------------------|-------|----------------------|-----------------------------------|
| Wildtype (Gly-Pro)                    | -270  | 2.3 ± 0.07           | 1.8 ± 0.25                        |
| PDI-type (Gly-His)                    | -221  | 1.27 ± 0.08          | 0.83 ± 0.15                       |
| DsbA-type (Pro-His)                   | -204  | 0.4 ± 0.07           | 0.55 ± 0.13                       |
| Grx-type (Pro-Tyr)                    | -195  | 0.12 ± 0.04          | 0.17 ± 0.03                       |
| C35A (Cys-Gly-Pro-Ala)                |       | No growth            | No growth                         |
| Vector without thioredoxin            |       | No growth            | No growth                         |

$^a$ The trxA, grxA strain FA47 (32) was transformed with the pBAD33 derivatives containing the genes of the corresponding thioredoxin variants and then grown at 37 °C on agar plates with M63 minimal medium (containing 10 g/liter ammonium sulfate as sole source of sulfur) in the presence of the inducer t-arabinose. The size of at least 5 colonies after 3 days of growth was measured for each complementation assay and averaged.

$^b$ The trxA, metE strain A313 (21) was transformed with the pBAD33 derivatives containing the genes of the corresponding thioredoxin variants and grown at 37 °C on agar plates with M63 minimal medium supplemented with the inducer t-arabinose and methionine sulfoxide as sole methionine source. Colony sizes were determined after 3 days of growth as in Footnote $^a$. The table shows the recovery of DsbC-dependent disulfide bond isomerization in the periplasm of the trxA strain RI363 by plasmid-encoded cytoplasmic expression of thioredoxin variants. A, thioredoxin-dependent disulfide bond isomerization in E. coli was assayed by the activity of plasmid-encoded mouse uPA in the periplasm, a protein with multiple disulfide bonds that requires DsbC and thioredoxin for efficient folding. Cell extracts were subjected to nonreducing SDS-polyacrylamide gel electrophoresis, and the gel was subsequently underlayed with agar containing plasminogen and casein. The dark zones result from proteolytic degradation of casein by activated plasmin, which is generated by functional uPA. uPA activity in the wild-type (WT) strain RI281 (23) cotransformed with the expression vector pBAD33 lacking trxA (lanes 1 and 2) was compared with the uPA activity in the trxA strain RI363 (23) cotransformed with pBAD33 derivatives for expression of the following thioredoxin variants: inactive thioredoxin C35A (active site: Cys-Gly-Pro-Ala) (lane 4), glutaredoxin-like thioredoxin (lane 5), DsbA-like thioredoxin (lane 6), PDI-like thioredoxin (lane 7), and thioredoxin wild type (lane 8). B, Western blot analysis of thioredoxin levels in the E. coli lysates from A.

**DISCUSSION**

The question of the importance of the redox potential of thioredoxin variants with increased redox potentials but not by wild-type thioredoxin (12), indicating that the oxidative force of this essential protein is crucial for its function in vivo. This view is supported by active site variants of yeast PDI that cause DTT sensitivity and a reduced rate of protein folding in the endoplasmic reticulum (38). In the periplasm of E. coli, active site variants of DsbA, which are more than 1000-fold weaker oxidants than the wild type, could still functionally replace DsbA under normal growth conditions (11, 16, 17). Efficient functional replacement of DsbA by thioredoxin could, however, only be achieved by secretion of the three most oxidizing thioredoxin variants that were used in the present study (18, 19). It thus appears that the redox potential of the final oxidant of polypeptides in the E. coli periplasm is also important and that a certain minimum of oxidative force must be retained for maintenance of disulfide bond formation in the periplasm.

In Vivo Properties of the Active Site Variants of Thioredoxin with Increased Redox Potentials

| Thioredoxin variant (Xaa-Xaa dipeptide) | $E_0$ | Sulfate reduction$^a$ | Methionine sulfoxide reduction$^b$ |
|---------------------------------------|-------|----------------------|-----------------------------------|
| Wildtype (Gly-Pro)                    | -270  | 2.3 ± 0.07           | 1.8 ± 0.25                        |
| PDI-type (Gly-His)                    | -221  | 1.27 ± 0.08          | 0.83 ± 0.15                       |
| DsbA-type (Pro-His)                   | -204  | 0.4 ± 0.07           | 0.55 ± 0.13                       |
| Grx-type (Pro-Tyr)                    | -195  | 0.12 ± 0.04          | 0.17 ± 0.03                       |
| C35A (Cys-Gly-Pro-Ala)                |       | No growth            | No growth                         |
| Vector without thioredoxin            |       | No growth            | No growth                         |

$^a$ The trxA, grxA strain FA47 (32) was transformed with the pBAD33 derivatives containing the genes of the corresponding thioredoxin variants and then grown at 37 °C on agar plates with M63 minimal medium (containing 10 g/liter ammonium sulfate as sole source of sulfur) in the presence of the inducer t-arabinose. The size of at least 5 colonies after 3 days of growth was measured for each complementation assay and averaged.

$^b$ The trxA, metE strain A313 (21) was transformed with the pBAD33 derivatives containing the genes of the corresponding thioredoxin variants and grown at 37 °C on agar plates with M63 minimal medium supplemented with the inducer t-arabinose and methionine sulfoxide as sole methionine source. Colony sizes were determined after 3 days of growth as in Footnote $^a$.
In Vivo Importance of the Redox Potential of Thioredoxin

assay for thiol-disulfide oxidoreductases that reflects the in vivo situation must be performed under conditions that guarantee that the rate-limiting catalytic step is the same as that in vivo. We believe that our newly established assay, making use of the catalyzed reduction of lipoic acid by NADPH, is a valuable tool to study the function of NADPH-dependent members of the thioredoxin family in vitro.

Another question that needs to be addressed in the context of our in vivo experiments is whether the intracellular redox potential in the cytoplasm determines a steady-state ratio of oxidized and reduced thioredoxin and thus possibly the available concentrations of reduced thioredoxin. One would indeed expect that the intracellular concentration of the reduced relative to the oxidized enzyme increases in the case of the more oxidizing thioredoxin variants compared with wild-type thioredoxin. However, it has been shown that wild-type thioredoxin is already mainly reduced in the cytoplasm (about 70% in freshly prepared cell extracts (37)). Therefore, assuming unchanged overall concentrations of thioredoxin, the concentration of reduced thioredoxin can only increase to a small extent in the case of the more oxidizing variants. Thus, the concentration of reduced thioredoxin cannot be affected significantly in the case of the more oxidizing variants. This further supports our conclusion that it is the rate by which substrates are reduced by thioredoxin that determines the in vivo function of this enzyme and that slower substrate reduction by the thioredoxin variants is a consequence of their increased redox potential. We have also demonstrated that the thioredoxin expression level in our in vivo complementation studies is practically the same as that in wild-type cells (Fig. 2B).

In the present study we have demonstrated the in vivo importance of redox potential for a single cytoplasmic member of the thioredoxin family. We would like to emphasize that the rate-limiting step in the catalytic cycle of a thiol-disulfide oxidoreductase is the main determinant to what extent and in which direction its intrinsic redox potential influences its in vivo function. On the other hand, the situation in vivo is more complex because there are often several enzymes that fulfill the same function but may recognize their substrates with different specificities. For instance, ribonucleotide reductase (NrdAB) has a lower K_m value for glutaredoxin 1 than for thioredoxin, but glutaredoxin 1 has a higher redox potential (39, 40). Similarly, only glutaredoxin 1 and the thiol-disulfide oxidoreductase NrdH are effective reductants of the second E. coli ribonucleotide reductase, NrdEF (41), whereas thioredoxin shows no reactivity despite the fact that it is the strongest reductant of the three enzymes. It will thus be the combined effect of redox potential, substrate specificity, and expression levels of related thiol-disulfide oxidoreductases that accounts for the different phenotypes that are observed for E. coli mutants lacking components of the thioredoxin- and glutaredoxin-dependent pathways. In the case of NADPH-dependent members of the thioredoxin family, the expression level of TR may determine the rate-limiting step of catalysis. Clearly, all of these factors have to be taken into account to get a complete view of the in vivo functions of each individual member of the thioredoxin family.

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