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Characterization of *Escherichia coli* thioredoxin variants mimicking the active-sites of other thiol/disulfide oxidoreductases

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Abstract

Thiol/disulfide oxidoreductases like thioredoxin, glutaredoxin, DsbA, or protein disulfide isomerase (PDI) share the thioredoxin fold and a catalytic disulfide bond with the sequence Cys-Xaa-Xaa-Cys (Xaa corresponds to any amino acid). Despite their structural similarities, the enzymes have very different redox properties, which is reflected by a 100,000-fold difference in the equilibrium constant (K_{eq}) with glutathione between the most oxidizing member, DsbA, and the most reducing member, thioredoxin. Here we present a systematic study on a series of variants of thioredoxin from *Escherichia coli,* in which the Xaa-Xaa dipeptide was exchanged by that of glutaredoxin, PDI, and DsbA. Like the corresponding natural enzymes, all thioredoxin variants proved to be stronger oxidants than the wild-type, with the order wild-type < PDI-type < DsbA-type < glutaredoxin-type. The most oxidizing, glutaredoxin-like variant has a 420-fold decreased value of K_{eq}, corresponding to an increase in redox potential by 75 mV. While oxidized wild-type thioredoxin is more stable than the reduced form ($\Delta\Delta G_{ox/red}$ = 16.9 kJ/mol), both redox forms have almost the same stability in the variants. The pH-dependence of the reactivity with the alkylating agent iodoacetamide proved to be the best method to determine the pK_a value of thioredoxin's nucleophilic active-site thiol (Cys32). A pK_a of 7.1 was measured for Cys32 in the reduced wild-type. All variants showed a lowered pK_a of Cys32, with the lowest value of 5.9 for the glutaredoxin-like variant. A correlation of redox potential and the Cys32 pK_a value could be established on a quantitative level. However, the predicted correlation between the measured $\Delta\Delta G_{ox/red}$ values and Cys32 pK_a values was only qualitative.

Keywords: Cys-Xaa-Xaa-Cys motif; DsbA; glutaredoxin; pK_a values; protein disulfide isomerase; protein stability; redox potentials; thioredoxin

Thiol/disulfide oxidoreductases are ubiquitous in eukaryotes and prokaryotes, and catalyze important redox reactions in the cell, including ribonucleotide reduction in the cytosol and disulfide bond formation during folding of secretory proteins in oxidizing cell compartments. All members of this enzyme family for which a three-dimensional structure has been determined have a common fold, the so-called thioredoxin motif, which consists of a central five-stranded β-sheet flanked by 3–4 α-helices (Martin, 1995). The active site of the enzymes is formed by a catalytic disulfide bond that undergoes reversible disulfide exchange reactions with the substrates. The active-site disulfide is located at the N-terminus of an α-helix and has the consensus sequence Cys-Xaa-Xaa-Cys (Xaa corresponds to any amino acid). In the reduced enzymes, the N-terminal cysteine is generally solvent-exposed and acts as a nucleophile, whereas the more C-terminal cysteine is buried (Kallis & Holmgren, 1980; Freedman et al., 1994; Zapun et al. 1994; Wunderlich et al., 1995). Despite these structural similarities, the individual members of the enzyme family differ strongly in their primary structures and physico-chemical properties (Holmgren, 1995; Chivers et al., 1997). While the cytoplasmic members thioredoxin and glutaredoxin 1 and 3 are reductants with redox potentials (E'_o)[1] of −270 mV (Lin & Kim, 1989; Krause et al., 1991), −198 and −233 mV (Åslund, 1996), respectively, the enzymes that catalyze disulfide bond formation in proteins are oxidants, with redox potentials of −147 to −175 mV[1]) for eukaryotic protein disulfide isomerase (PDI) (Lundström & Holmgren, 1993; Darby & Creighton, 1995), −130 mV for DsbC (Zapun et al.,

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Abbreviations: A_x, absorbance at x nm; CD, circular dichroism; DTT, (±)1,4-dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GdmCl, guanidinium chloride; Grx, glutaredoxin; GSH, reduced glutathione; GSSG, oxidized glutathione; HPLC, high pressure liquid chromatography; IAM, iodoacetamide; IPTG, isopropyl-β-D-thiogalactoside; NMR, nuclear magnetic resonance; PDI, protein disulfide isomerase; TFA, trifluoroacetic acid; TR thioredoxin reductase; Tris, Tris(hydroxymethyl)aminomethane.

[1] The values of E'_o given in this paper were calculated using Equation (3) in Materials and methods, using a redox potential of −240 mV for the glutathione redox couple (Rost & Rapoport, 1964).

1995) and −122 mV for DsbA from *E. coli* (Wunderlich & Glockshuber, 1993; Zapun et al., 1993). The equilibrium constants of the enzymes with glutathione thus vary by a factor of 100,000 between the most oxidizing member, DsbA, and the most reducing member, thioredoxin (Lin & Kim, 1989; Wunderlich & Glockshuber, 1993a).

Recent biophysical studies have revealed some important factors that determine the redox properties of the individual enzymes. It was shown that the pK_a of the enzymes' nucleophilic active-site thiol is significantly lower than 9.5, the pK_a of a normal cysteine thiol. The different pK_a values can explain, at least qualitatively, the different redox properties of the enzymes and the destabilizing disulfide bond in oxidized DsbA (Nelson & Creighton, 1994; Grauschopf et al., 1995). However, the overall situation is obviously more complex, as deviations from theory have been measured for a number of DsbA variants (Jacobi et al., 1997).

It is believed that it is mainly the dipole of the active-site helix which stabilizes the negative charge of the N-terminal catalytic thiolate (Hol, 1985; Nelson & Creighton, 1994; Kortemme & Creighton, 1995). This view is supported by the lack of positively charged residues in the neighborhood of the nucleophilic cysteine in the structures of reduced thioredoxin (Jeng et al., 1994; Weichsel et al., 1996) and reduced glutaredoxin (Qin et al., 1994). Therefore, the amino-acid composition of the active-site helix, and in particular the Xaa-Xaa dipeptide sequence between the active-site cysteines, is likely to be a critical determinant of the redox properties of thiol-disulfide oxidoreductases. The Xaa-Xaa dipeptides are indeed characteristic for the individual members of the enzyme family, and there is increasing experimental evidence for the importance of the dipeptide. The first experiment in this direction was performed by Holmgren and coworkers who replaced the dipeptide Gly-Pro in *E. coli* thioredoxin against that of PDI (Gly-His) and obtained a variant with redox properties shifted toward those of PDI, corresponding to an increase in redox potential by 35 mV (Krause et al., 1991). Analogous results were reported for the catalytic *a*-domain of PDI, in which the exchange of the dipeptide Gly-His against that of thioredoxin led to a decrease in redox potential by 66 mV, and the TlpA protein of *Bradyrhizobium japonicum*, whose redox potential increased from −259 mV to −193 mV after the PDI-like dipeptide mutation Val-Pro → Val-His (Kortemme et al., 1996; Rossmann et al., 1997). Variants of DsbA, thioredoxin, and PDI obtained after random mutagenesis of the dipeptide and functional screening/selection have further confirmed the general importance of the Xaa-Xaa dipeptide (Grauschopf et al., 1995; Chivers et al., 1996; Holst et al., 1997)

In this study, we present a systematic and comparative study on variants of thioredoxin in which the enzyme's natural Xaa-Xaa dipeptide (Gly-Pro) was replaced by the corresponding sequences of glutaredoxin (Pro-Tyr), DsbA (Pro-His), and PDI (Gly-His). In addition, we have introduced the dipeptide of *E. coli* thioredoxin reductase (Ala-Thr), which does not possess the thioredoxin fold, but also a Cys-Xaa-Xaa-Cys sequence by which it undergoes $NADP^+$/NADPH-mediated disulfide exchange reactions with thioredoxin (for review see Holmgren, 1995).

We have measured the redox potentials, pK_a values of the nucleophilic cysteine (Cys32) thiol and the thermodynamic stabilities of the purified thioredoxin variants. With the exception of the glutaredoxin-like variant, the order of their redox potentials is the same as that one observed for the corresponding natural proteins. We analyze the interrelation between E_o', the pK_a of Cys32 and $\Delta\Delta G_{ox/red}$ of the variants and discuss possible reasons for discrepancies between their measured and theoretically expected properties.

Results

Expression, purification, and spectroscopic properties of the thioredoxin variants

Wild-type thioredoxin and its active-site variants were expressed in the cytoplasm of *E. coli* BL21(DE3) under control of the T7 promoter. The proteins were purified from the soluble fraction of the cell extracts in the absence of reducing agents by ion exchange chromatography and gel filtration. The yields of the purified, homogeneous proteins varied between 50 and 100 mg/L of bacterial culture. Ellman's assay revealed that all proteins were fully oxidized after purification.

The far- and near-UV CD spectra of the oxidized and reduced variants were practically indistinguishable from those of wild-type thioredoxin (data not shown), indicating that none of the amino acid exchanges had affected the three-dimensional structure of the variants. The same was observed for the fluorescence emission spectra of the variants, which showed a threefold to fourfold increase in tryptophan fluorescence upon reduction of the active-site disulfide at pH 7.0 (data not shown). This is in good agreement with the 3.4-fold fluorescence increase observed for the wild-type (Holmgren, 1972).

Equilibrium constants with glutathione and redox potentials

The equilibrium constants (K_{eq}) of the thioredoxin variants with glutathione were measured at pH 7.0 and 25 °C using the redox state-dependent fluorescence of the proteins, assuming no significant equilibrium concentrations of thioredoxin/glutathione mixed disulfides (Wunderlich & Glockshuber, 1993). The equilibrium measurements and the deduced redox potentials of the variants are shown in Figure 1 and Table 1. All variants exhibit lower values of K_{eq}, and are thus more oxidizing than wild-type thioredoxin. The

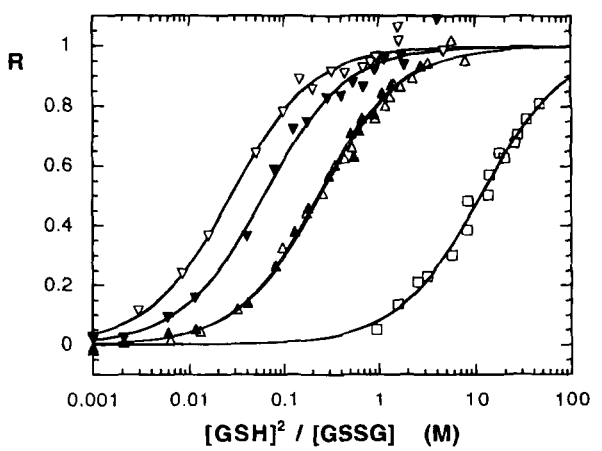

Fig. 1. Determination of the equilibrium constants of thioredoxin and its active-site variants with glutathione at pH 7.0 and 25 °C. The fraction of reduced thioredoxin at equilibrium (R) was determined by the redox-state dependent fluorescence of the proteins at 345 nm (excitation at 280 nm). The original fluorescence data were evaluated according to Equation 2 and normalized (Equation 11 in Wunderlich et al., 1993). The solid line corresponds to a fit of the normalized data according to the overall thioredoxin/glutathione equilibrium (Equation 6). (▽) Grx-type variant (Pro-Tyr); (▼) DsbA-type (Pro-His); (▲) PDI-type (Gly-His); (△) TR-type (Ala-Thr); (□) wild-type thioredoxin (Gly-Pro).

redox potential of the thioredoxin variants increased in the following order: wild-type < PDI-type < DsbA-type < glutaredoxin-type. Consequently, all variants are similar to the corresponding natural enzymes with respect to their redox properties (thioredoxin < glutaredoxin < PDI < DsbA), except for the glutaredoxin-like variant, which proved to be the most oxidizing protein with an 420-fold lower equilibrium constant with glutathione compared to wild-type thioredoxin (corresponding to an increase in redox potential by 75 mV).

Thermodynamic stabilities of the oxidized and reduced forms of the variants

The thermodynamic stabilities (ΔG_{stab}) of the oxidized and reduced variants were measured at pH 7.0 and 25 °C with equilibrium unfolding/refolding experiments induced by guanidinium chloride (GdmCl), assuming a two-state model of folding (Pace, 1986) which is well established for wild-type thioredoxin (Lin & Kim, 1989). The transitions were followed by the far-UV CD signal at 220 nm and were fully reversible for all variants (Fig. 2).

Oxidized wild-type thioredoxin was 16.9 kJ/mol more stable than the reduced protein at pH 7.0, which is in good agreement with the previously measured value of 13.0 kJ/mol at pH 8.0 (Lin & Kim, 1989). All variants showed a strongly decreased stability difference between the oxidized and reduced form ($\Delta\Delta G_{ox/red}$). In the case of the PDI-, DsbA-, and glutaredoxin-like variant, both redox forms have almost identical stabilities. The oxidized forms of all variants were found to be less stable than the oxidized wild-type, and all reduced variants were more stable than reduced wild-type thioredoxin (Table 2). The correlation between the values of $\Delta\Delta G_{ox/red}$ and the equilibrium constants with glutathione was only qualitative (cf. Fig. 6A).

Determination of pK_a values of the nucleophilic, active-site cysteine (Cys32)

We analyzed the influence of the amino acid replacements in the Xaa-Xaa sequence on the pK_a of the nucleophilic active-site cysteine (Cys32). As all variants are more oxidizing than wild-type thioredoxin, we expected a decrease in the pK_a of the Cys32 thiol for all variants. In view of previously reported and partly contradictory results for the pK_a values of Cys32 (Kallis & Holmgren, 1980; Li et al., 1993; Takahashi & Creighton, 1996; Dyson et al., 1997), we tried to establish a reliable and generally applicable method to measure the pK_a of the nucleophilic cysteine in thiol/disulfide oxidoreductases. We first investigated the pH-dependence of the thiolate-specific absorbance difference between oxidized and reduced wild-type thioredoxin at 240 nm (ΔA_{240}) and observed exactly the same two-step transition profile reported recently by Dyson et al. (1997). The first transition, which had been assigned to the Cys32 thiol, had a pK_a of 7.4 (data not shown). In the case of the active-site variants of thioredoxin, it proved to be difficult to evaluate the transitions, indicating that additional factors influence the pH-dependent absorbance properties of the variants at 240 nm (data not shown). However, we obtained a single transition for the unfolded, reduced wild-type and all unfolded variants in the presence of 5 M urea, demonstrating identical pK_a values of both active-site cysteines of 10.1 in the unfolded proteins.

Due to the difficulties associated with the absorbance measurements, we tested the pH-dependent reactivity of the Cys32 thiol with iodoacetamide (IAM) as a tool to determine its pK_a (Kallis & Holmgren, 1980). Reduced thioredoxin was mixed with excess IAM in the range of pH 4–9. After different incubation times, the reaction was quenched with acid and the reaction products were separated by reversed phase HPLC. Figure 3A shows the time course of the reaction at pH 7.5 and demonstrates that IAM exclusively reacts with the thiol group of Cys32, as only a single reaction product was obtained whose mass corresponded to mono-alkylated thioredoxin. When the reaction was carried out under denaturing conditions (4 M GdmCl), the doubly alkylated form of thioredoxin was generated (Fig. 3B). Both mono-alkylated species (alkylation at Cys32 or Cys35) were observed as intermediates of the reaction and were populated to the same extent, indicating that both cysteines have the same reactivity and pK_a values in unfolded thioredoxin. The identity of doubly alkylated thioredoxin was confirmed by mass spectrometry.

Figure 4 shows the pH-dependence of the apparent second-order rate constant (k_{IAM}) of the alkylation of Cys32 in reduced thioredoxin wild-type and all variants by IAM. The reduced wild-type exhibits a biphasic transition (Fig. 4A). The first transition from zero to approximately 155 M^{-1} s^{-1} has a pK_a value of 7.1. The second transition above pH 8 is characterized by a further increase of the rate constant (up to 1000 M^{-1} s^{-1} at pH 10; data not shown). The second transition is not caused by additional alkylation of Cys35 at higher pH values, as thioredoxin modified at Cys32 proved to be the only reaction product formed between pH 4 and pH 10. The second transition obviously results from the titration of another amino acid side chain which increases the reactivity of Cys32 with IAM, whereas the first transition corresponds to the pK_a of Cys32 (see below).

Table 1. *Equilibrium constants with glutathione and redox potentials of thioredoxin wild-type and its active-site variants at pH 7.0 and 25 °C and pK_a values of the Cys32 thiols*

Xaa-Xaa dipeptide	Gly-Pro (wild-type)	Ala-Thr (TR-type)	Gly-His (PDI-type)	Pro-His (DsbA-type)	Pro-Tyr (Grx-type)
K_{eq} (M)	11.3 ± 0.41	0.243 ± 0.008	0.228 ± 0.008	0.0610 ± 0.0042	0.0273 ± 0.0017
E_o' (mV)	−270	−222	−221	−204	−195
pK_a of Cys32	7.13 ± 0.03[a]	6.21 ± 0.05	6.34 ± 0.05	6.12 ± 0.07	5.86 ± 0.05

[a]Deduced from the first acid/base equilibrium observed for the pH-dependent reactivity of Cys32 with iodoacetamide (cf. Fig. 4A).

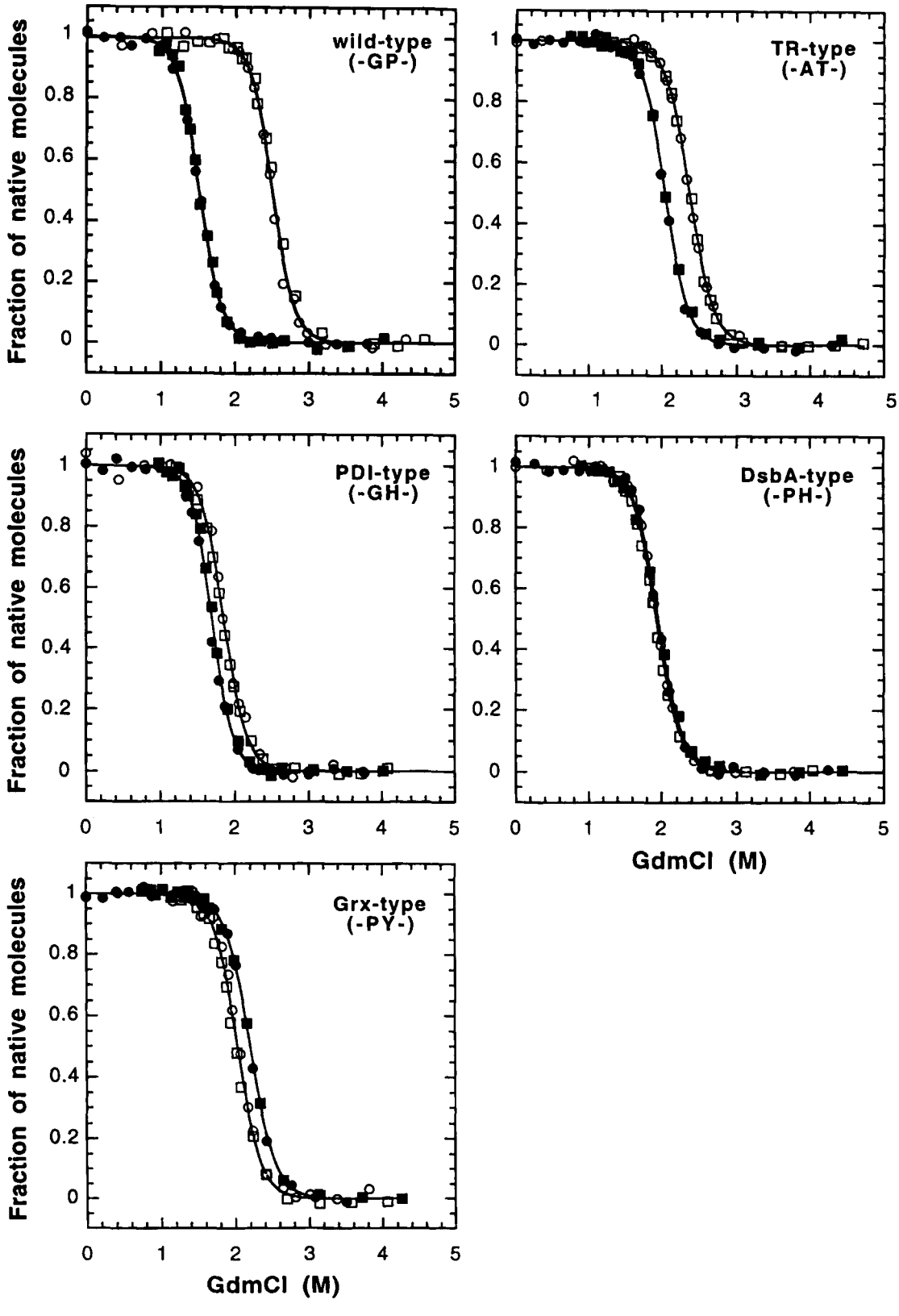

Fig. 2. GdmCl-induced unfolding/refolding equilibria of thioredoxin wild type and its active-site variants at pH 7.0 and 25 °C. Unfolding was followed by the far-UV CD signal at 220 nm. Open symbols represent oxidized proteins and closed symbols represent reduced proteins. Circles correspond to the unfolding transitions and squares to the refolding transitions. The solid lines correspond to a fit according to the two-state model of folding. Amino acids are abbreviated in the single letter code.

Table 2. *Redox state-dependent thermodynamic stabilities of thioredoxin wild-type and its active-site variants at 25 °C and pH 7.0*

Thioredoxin variant	Redox state	Midpoint of transition (M GdmCl)	Cooperativity of transition ($kJ \cdot mol^{-1} \cdot M^{-1}$)	ΔG_{stab} (kJ/mol)	$\Delta\Delta G_{ox/red}$[a] (kJ/mol)
Wild-type	Oxidized	2.50	16.6 ± 0.7	−41.4 ± 1.8	−16.9 ± 2.4
Gly-Pro	Reduced	1.50	16.2 ± 0.4	−24.5 ± 0.6	
TR-type	Oxidized	2.38	15.3 ± 0.2	−36.4 ± 0.5	−3.7 ± 1.4
Ala-Thr	Reduced	2.06	16.0 ± 0.4	−32.7 ± 0.9	
PDI-type	Oxidized	1.85	16.1 ± 0.6	−29.7 ± 1.1	0.5 ± 2.3
Gly-His	Reduced	1.69	17.9 ± 0.7	−30.2 ± 1.2	
DsbA-type	Oxidized	1.94	15.6 ± 0.3	−30.1 ± 0.7	−0.1 ± 1.6
Pro-His	Reduced	1.96	15.3 ± 0.4	−30.0 ± 0.9	
Grx-type	Oxidized	2.04	15.6 ± 0.6	−31.7 ± 1.3	0.6 ± 2.0
Pro-Tyr	Reduced	2.20	14.7 ± 0.3	−32.3 ± 0.7	

[a] A possible entropic contribution of the disulfide bond to the free energy of the unfolded state was not considered.

Unfolded thioredoxin in 4 M GdmCl only showed a single transition with a pK_a of 10.5 (Fig. 4A), which is consistent with identical pK_a values of Cys32 and Cys35 in the unfolded protein and in reasonable agreement with the absorbance measurements mentioned above, which yielded identical pK_a values of 10.1 at lower ionic strength in 5 M urea.

In contrast to native, reduced wild-type thioredoxin, the native variants exclusively showed one-step transitions and lacked the second transition in the investigated pH range of 3–9 (Fig. 4). The pK_a values of Cys32 were lowered in all variants compared to the wild-type. As expected from its highest redox potential, the most oxidizing, glutaredoxin-like variant showed the lowest Cys32 pK_a of 5.9 (Fig. 4B, Table 1). At acidic pH, the values of k_{IAM} dropped to zero in all cases. Far-UV CD measurements proved that reduced thioredoxin is native at acidic pH values (Fig. 4A, inset). Therefore, the reactivity of thioredoxin with IAM at acidic pH goes to zero due to the protonation of Cys32 and not due to unfolding of the protein. The maximal reactivity of the Cys32 thiolate anion was significantly reduced in all variants, with k_{IAM} values of 30–90 $M^{-1}\ s^{-1}$ compared to 155 $M^{-1}\ s^{-1}$ for the first transition in the wild-type (Fig. 4B). In particular, the maximal reactivities of the Cys32 thiolates differed up to threefold even if the pK_a values of

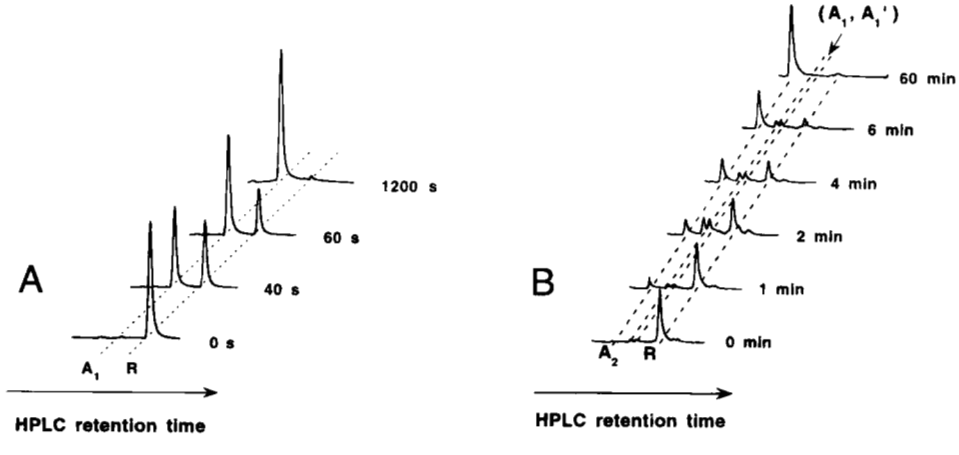

Fig. 3. HPLC analysis of the reaction of native **(A)** and unfolded **(B)** reduced wild-type thioredoxin with iodoacetamide at 25 °C. The reactions were performed under pseudo-first-order conditions with excess iodoacetamide. The reactions were quenched after different times with formic acid/GdmCl and separated on an analytical C18 reversed phase HPLC column. **A:** Reaction of native thioredoxin (4 μM) with iodoacetamide (150 μM) at pH 7.5. The dashed lines indicate identical retention times of reduced thioredoxin (R) and thioredoxin alkylated at Cys32 (A1). **B:** Reaction of unfolded thioredoxin (3 μM) with iodoacetamide (3.1 mM) at pH 9.0 in 4 M GdmCl. Both mono-alkylated species of thioredoxin, A_1 and A_1' (corresponding to alkylation at Cys32 and Cys35, respectively), are equally populated intermediates during formation of doubly alkylated thioredoxin (A_2). The decrease in reduced thioredoxin was used to determine the apparent second-order rate constants of the reaction at different pH values (c.f. Fig. 4A), assuming identical pK_a values of Cys32 and Cys35 in unfolded thioredoxin.

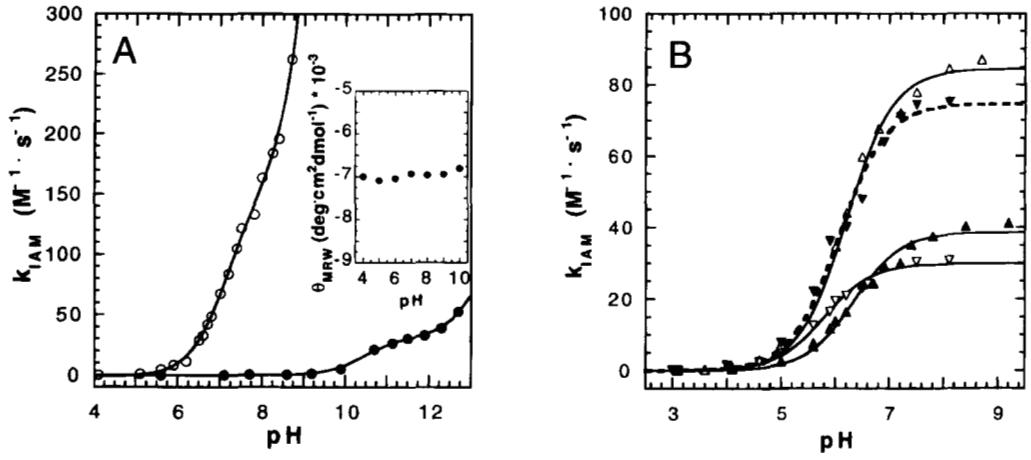

Fig. 4. Determination of the pK_a value of the Cys32 thiol of wild-type thioredoxin and its active-site variants. The apparent second-order rate constants (k_{IAM}) for the reaction of Cys-32 with iodoacetamide were determined with analytical HPLC as described under Figure 3. **A:** Alkylation of native (○) and GdmCl-denatured (●) wild-type thioredoxin. Inset: pH-independent far-UV CD signal of reduced wild-type thioredoxin at 220 nm, proving that the protein is native between pH 4 and pH 10 in the absence of denaturants. **B:** pH-dependent reactivities with iodoacetamide of the Grx-type (PY) variant (▽), the DsbA-type (PH) (▼), the PDI-type (GH) (▲), and the TR-type (AT) variant (△) of thioredoxin.

Cys32 were very similar as in the TR-, DsbA-, and PDI-like variants (6.2, 6.3, and 6.1, respectively).

To test whether the first transition of the reactivity of Cys32 from zero to 155 M^{-1} s^{-1} in wild-type thioredoxin really corresponds to the pK_a of Cys32 and whether the reaction with iodoacetamide can generally be used to measure the pK_a of the active-site thiol of disulfide oxidoreductases, the alkylation method was also applied to the most oxidizing thiol/disulfide oxidoreductase, DsbA. Like wild-type thioredoxin, DsbA shows two pH-dependent transitions that determine the reactivity of the nucleophilic active-site cysteine (Cys30), but both transitions are more separated than in thioredoxin. The first transition with a pK_a of approximately 3.5 corresponds to the pK_a of Cys30, and was measured by the change in absorbance at 240 nm (Nelson & Creighton, 1994), whereas the second transition, which was measured by alkylation with IAM, has a pK_a of 6.7 and is linked with an increase in the reactivity of Cys30 with IAM from about 20 to 80 M^{-1} s^{-1} (Nelson & Creighton, 1994). We measured the pK_a of Cys30 of DsbA both by alkylation with IAM and by the thiolate-specific absorbance at 240 nm under identical experimental conditions and observed identical titration profiles with pK_a values of 3.29 and 3.34, respectively (Fig. 5). Therefore, the pH-dependent reactivity with IAM is generally applicable to determine the pK_a of active-site cysteines.

Using the theory of Szajewski and Whitesides (1980), which allows the prediction of the rate constants of disulfide interchange reactions when all pK_a values of the involved thiols are known (see Materials and methods, Equations 6–8), we calculated the expected changes of the equilibrium constants of the variants caused by their lowered Cys32 pK_a values. In this simulation, constant and variable pK_a values were used for the buried thiol of Cys35. A reasonable fit with the experimental data was obtained when the pK_a of Cys35 was assumed to be identical to that of Cys32 (Fig. 6B). The experimental data could however not be described adequately when a constant pK_a of 9.5 was assumed for Cys35, the value determined for Cys35 wild-type thioredoxin (Dyson et al., 1997).

Discussion

The influence of the Xaa-Xaa dipeptide on the redox properties of thioredoxin

The aim of this study was to determine the general role of the Xaa-Xaa dipeptide sequence between the active-site cysteines of thiol/disulfide oxidoreductases for the physical properties of this

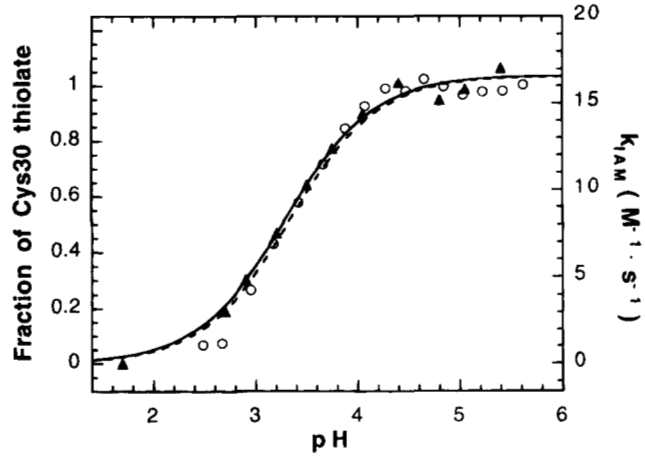

Fig. 5. Comparison of the absorbance at 240 nm (A_{240}) and the reactivity with iodoacetamide as a measure to determine the pK_a of the nucleophilic thiol (Cys30) of reduced *E. coli* DsbA at 25 °C. Oxidized DsbA was used as a reference to calculate the fraction of the Cys30 thiolate from the $A_{240\,nm}$ measurements. The apparent second-order rate constants (k_{IAM}) of the reaction with iodoacetamide were determined under pseudo-first-order conditions (excess of iodoacetamide) and followed by reverse-phase HPLC after quenching the reactions after different times with acid (see legend of Fig. 3 and Materials and methods for details). The normalized absorbance data (○) and the values of k_{IAM} (▲) were fitted according to an acid/base equilibrium (dashed and solid line, respectively).

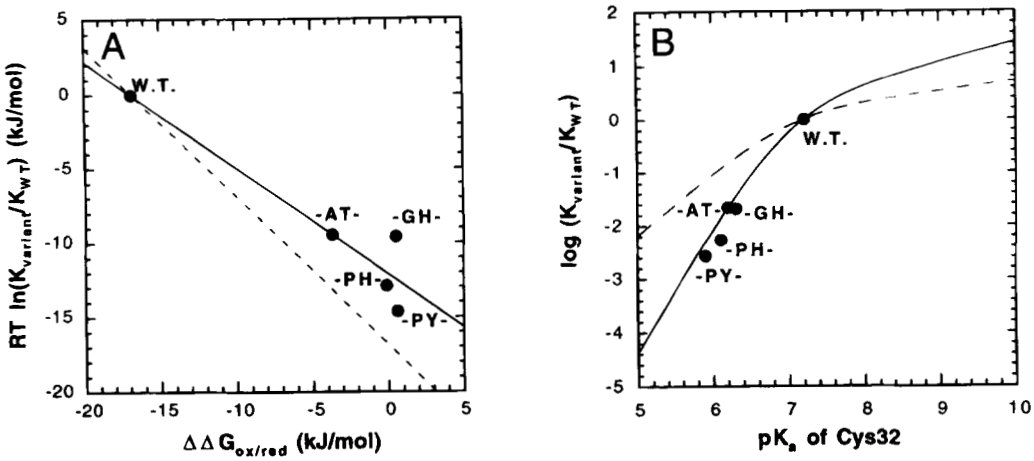

Fig. 6. Comparison of the measured equilibrium constants of the thioredoxin variants with glutathione ($K_{Variant}$) with those predicted from the difference in stability between their oxidized and reduced forms ($\Delta\Delta G_{ox/red}$) and their Cys32 pK_a values. **A:** Plot of the experimentally determined values of $RT \cdot \ln(K_{variant}/K_{WT})$ versus $\Delta\Delta G_{ox/red}$. The solid line corresponds to a linear fit of the data (slope: −0.717; correlation coefficient: 0.946), and the dashed line represents the theoretically expected correlation (slope: −1). **B:** Plot of $\log(K_{variant}/K_{WT})$ versus the measured pK_a values of Cys32. Using the theory of Szajewski and Whitesides (1980), the following parameters were tested to simulate the dependence of the equilibrium constants with glutathione on the pK_a values of Cys32: (i) (dashed line), a constant pK_a of 7.5 was assumed for the buried thiol of Cys35 (the same result is obtained for a constant Cys35 pK_a value of 9.0). (ii) the pK_a of Cys35 was assumed to be variable and identical to the pK_a of Cys32 (solid line). Closed circles represent the experimentally determined values. A pK_a of 8.7 was used for the thiol of GSH in all simulations.

enzyme family. Using the most reducing member, *E. coli* thioredoxin, as a model system, we have systematically exchanged the Xaa-Xaa dipeptide against those of the more oxidizing enzymes glutaredoxin, PDI and DsbA. The measured redox potential of the PDI-like variant (−221 mV) is in good agreement with the values of −220 and −235 mV previously published by Holmgren and coworkers (Krause et al., 1991). Overall, all thioredoxin variants were stronger oxidants than wild-type thioredoxin, with the order wild-type < PDI-type < DsbA-type < glutaredoxin-type. The variants thus behaved similar to the corresponding natural proteins, with the exception of the glutaredoxin-like variant, which proved to be the most oxidizing protein. A parallel study on variants of *E. coli* DsbA harboring the Xaa-Xaa sequences of the more reducing enzymes yielded exactly the expected order of redox potentials for the variants (thioredoxin-type < glutaredoxin-type < PDI-type < wild-type) (Huber-Wunderlich & Glockshuber, 1998). Thus, it appears that the redox properties of a thiol/disulfide oxidoreductase can be modified in a predictable manner by introducing the Xaa-Xaa sequence of another enzyme with different redox properties.

The Xaa-Xaa replacements in thioredoxin alone were however not sufficient to generate variants with exactly the same redox properties as the corresponding natural enzymes. As an example, the redox potential of the DsbA-like thioredoxin variant was increased by 66 mV, compared to a difference of 145 mV between wild-type DsbA and wild-type thioredoxin. Conversely, the redox potential of the a DsbA variant with the Xaa-Xaa dipeptide of thioredoxin decreased by 92 mV compared to wild-type DsbA (Huber-Wunderlich & Glockshuber, 1998). Therefore, the nature of the Xaa-Xaa dipeptide accounts for approximately one half of the factors that determine the redox properties of a thiol-disulfide oxidoreductase. The other factors obviously arise from the individual protein context. This is underlined by the 35 mV difference in redox potential between glutaredoxin 1 and 3 from *E. coli*, which both have the dipeptide Pro-Tyr (Åslund, 1996). The same is valid for DsbA from *E. coli* and *V. cholerae*, which both have the dipeptide Pro-His but different pK_a values of the nucleophilic thiolate of 3.5 and 5.1, respectively (Nelson & Creighton, 1994; Ruddock et al., 1996)

Overall, the effects of the Xaa-Xaa exchanges were slightly weaker in thioredoxin compared to DsbA: The measured equilibrium constants (K_{eq}) with glutathione differed by a factor of 410 for thioredoxin wild-type and its most oxidizing glutaredoxin-like variant (Table 1) compared to a factor of 1,200 for DsbA wild-type and its thioredoxin-like variant (Huber-Wunderlich & Glockshuber, 1998). Random mutagenesis of the Xaa-Xaa sequence in DsbA yielded a variant with the dipeptide Pro-Pro, with an even 1,700-fold increased K_{eq} (Grauschopf et al., 1995). The pK_a shifts of the nucleophilic thiol were also smaller in the thioredoxin variants ($\Delta pK_a = -1.3$ for the glutaredoxin-like variant) than in the Xaa-Xaa variants of DsbA, where the pK_a increased by 3.3 and 2.9 units in the most reducing variants "Pro-Pro" and "Gly-Pro" (Grauschopf et al., 1995; Huber-Wunderlich & Glockshuber, 1998). The lower sensitivity of thioredoxin towards exchanges of the Xaa-Xaa dipeptide compared to DsbA has also been reported for random dipetide variants of thioredoxin (Chivers et al., 1997).

The determination of the pK_a of the nucleophilic thiol in thiol/disulfide oxidoreductases

There has been much debate on the pK_a value of Cys32 in *E. coli* thioredoxin. The tryptophan fluorescence of thioredoxin proved to be unsuitable for measuring the pK_a of Cys32, as the fluorescence is strongly influenced by the Asp26 side chain (Dyson et al., 1997). Therefore, the pK_a value of 6.7 obtained by Chivers et al. (1996) must be considered with care. While NMR and Raman measurements had yielded similar pK_a values of 7.4 and 7.1, respectively (Dyson et al., 1997; Li et al., 1993), measurements of the pH-

dependence of the thiolate-specific absorbance of reduced thioredoxin at 240 nm had been evaluated differently, yielding values of 7.1 (Dyson et al., 1997) and 9.9 (Takahashi & Creighton, 1996). The measured data differed mainly by the almost complete absence of the first transition at pH 7.1 in the measurements of Takahashi and Creighton (1996), which could be explained by partial oxidation of thioredoxin during the experiment (Dyson et al., 1997). We could reproduce the absorbance data of Dyson et al. and also detected a first transition with a pK_a of 7.1. However, we had difficulties to obtain unambiguously interpretable absorbance data for some of the thioredoxin variants, possibly due to their susceptibility to air oxidation (Dyson et al., 1997) or the sensitivity of A_{240} toward the titration of other amino acid side chains and/or small pH-dependent conformational changes in the protein.

In search for a generally applicable method to determine the pK_a of the reactive cysteine in thiol-disulfide oxidoreductases, we have examined the pH-dependent reactivity with excess iodoacetamide (IAM) as a measure of the pK_a of Cys32 in thioredoxin. IAM was chosen instead of iodoacetic acid to exclude complicating effects by a pH-dependent charge (and reactivity) of the alkylating agent. In all titration profiles the apparent second-order rate constant of the reaction (k_{IAM}) dropped to zero at acidic pH due to protonation of the Cys32 thiol (Fig. 4). Our data on reduced wild-type thioredoxin differ significantly from the alkylation experiments described by Kallis and Holmgren (1980) who obtained a pK_a of 6.7 for Cys32 and found that k_{IAM} reached a finite value of about 45 M^{-1} s^{-1} at the lowest pH value investigated (pH 5.7). As the reaction was followed by quantification of residual thioredoxin activity with a thioredoxin reductase assay, a further reaction of unmodified thioredoxin with IAM during the assay might explain the discrepancy between our data and those of Kallis and Holmgren (1980). In our experiments, the alkylation reaction was quenched with acid (pH below 2) and the reaction products were separated by HPLC at acidic pH. Acid quenching is well established to prevent further reaction of cysteines with alkylating agents (Weissman & Kim, 1993), and acid-quenched samples were stable at room temperature for 1–2 days without any change in the composition of the reaction products. We also confirmed that the second transition observed for thioredoxin at higher pH values does not correspond to the alkylation of Cys35, but to a further increase in the reactivity of the thiolate anion (cf. Takahashi & Creighton, 1996)

Measurements of the reactivity of the Cys32 thiol of thioredoxin to determine its pK_a have been questioned recently by Takahashi and Creighton (1996) who argued that the reactivities might rather reflect the ionization properties of the transition state of thioredoxin-mediated disulfide exchange reactions and not the true pK_a of Cys32 in thioredoxin. Therefore, they favored the pH-dependence of A_{240}, which is a specific property of the thiolate. To determine possible differences between the absorbance and alkylation method, we compared both methods using *E. coli* DsbA as a model catalyst, where the absorbance method is well established (Nelson & Creighton, 1994; Grauschopf et al., 1995; Jacobi et al., 1997), but where the reactivity with IAM has so far not been applied in the range of the pK_a (3.3–3.5) of the nucleophilic cysteine (Cys30). Figure 5 shows that indistinguishable titration profiles were obtained which yielded a pK_a of 3.3. Therefore, the reactivity with IAM offers another valid method to determine the pK_a of catalytic thiols in disulfide oxidoreductases. The second transition for the reactivity of DsbA with IAM with a pK_a of 6.7 corresponds to an increase in the reactivity of Cys30 from 20 M^{-1} s^{-1} to 80 M^{-1} s^{-1} and may be linked to the titration of Glu24 (Nelson & Creighton, 1994). The pK_a values of Cys32 and Cys35 in unfolded thioredoxin were also determined with both the alkylation and the absorption method, yielding similar values of 10.5 and 10.1, respectively. The small difference is most likely due to the different ionic strength used in both experiments.

Overall, the following conclusions can be drawn: (1) the pK_a value of Cys32 in thioredoxin is between 7.1 and 7.4. The slightly different values obtained from absorbance, Raman and NMR measurements might result from small differences in ionic strength and may also be within the range of experimental error. (2) The alkylation with IAM is a valid method to measure the pK_a values of reactive cysteines in thiol-disulfide oxidoreductases. (3) The first transition with the lowest pK_a, starting at a reactivity with IAM of zero, corresponds to the pK_a of the nucleophilic thiol. Further transitions at higher pH values correspond to the titration of other side chains that increase (or decrease) the reactivity of the thiolate anion.

Correlation between redox potentials, the pK_a of the nucleophilic cysteine and $\Delta\Delta G_{ox/red}$

There appears to be at least a qualitative correlation between the pK_a of the nucleophilic cysteine and the redox potentials of thiol/disulfide oxidoreductases. While the nucleophilic thiol (Cys30) in DsbA, the most oxidizing member, has an extremely low pK_a of about 3.5 (Nelson & Creighton, 1994), values between 6.3 and 7.4 are observed for Cys32 in human and *E. coli* thioredoxin (Forman-Kay et al., 1992; Li et al., 1993; Dyson et al., 1997). A correlation between decreased redox potentials and increased pK_a values of Cys30 was shown for a series of DsbA variants (Grauschopf et al., 1995; Huber-Wunderlich & Glockshuber, 1998). This correlation is expected from the theory of Szajewski and Whitesides, which predicts the rate constants of disulfide exchange reactions from the pK_a values of all involved thiols (Szajewski & Whitesides, 1980; Nelson & Creighton, 1994; Grauschopf et al., 1995). The experimental data on the thioredoxin variants could only be described with this theory when a variable pK_a of the buried Cys35 was assumed (Table 1), while the simplifying assumption of a constant Cys35 pK_a of 9.5 did not describe the data properly (Fig. 6B). The deviations from the theory may indeed be caused by an additional shift of the pK_a of Cys35 in the dipeptide variants of thioredoxin, as a variable pK_a of Cys35 has already been demonstrated for other thioredoxin variants (Dyson et al., 1997).

Another reason for the deviation of the theory from the experiment may be the different reactivities of the Cys32 thiolates in wild-type thioredoxin and the thioredoxin variants (Fig. 4A,B). Despite almost identical pK_a values, differences by a factor of three were measured for the reactivity of Cys32 in the TR-, PDI-, and DsbA-like thioredoxin variants, whereas the theory of predicts identical reactivities if the Cys32 thiols had identical pK_a values (cf. k^{obs} in Equation 7). Therefore, the pK_a value of Cys32 is an important, but not the only factor that determines the reactivity of thioredoxin. In general, the expected correlation between the pK_a of the nucleophilic thiol and redox potential appears to be much better for DsbA variants compared to thioredoxin variants. This was also reported for random Xaa-Xaa dipeptide variants of thioredoxin generated by random mutagenesis (Chivers et al., 1997).

The stability difference ($\Delta\Delta G_{ox/red}$) between the oxidized and reduced forms of thiol/disulfide oxidoreductases at pH 7.0 should principally be correlated with their redox potentials and the pK_a of

nucleophilic thiol (Nelson & Creighton, 1994). We found a reasonable, qualitative agreement between the measured values of $\Delta\Delta G_{ox/red}$ and the redox properties of the thioredoxin variants (Fig. 6A). Again, the detailed analysis of the data shows that the correlation between $\Delta\Delta G_{ox/red}$, pK_a, and redox potential is complex: The lowered pK_a of Cys30 in DsbA (3.5) can explain that its oxidized form is less stable than the reduced form if one assumes that the oxidation of DsbA is nothing but the elimination of the negative charge at Cys30 (Nelson & Creighton, 1994). However, the same consideration fails for wild-type thioredoxin, whose oxidized form is more stable (Lin & Kim, 1989). In addition, this simplifying view would predict that only the stabilities of the reduced forms of the thioredoxin variants would be affected by the dipeptide replacements (Nelson & Creighton, 1994). However, the stabilities of the oxidized variants were affected to the same extent by the dipeptide exchanges as in the reduced forms. The situation is even more surprising and difficult for DsbA, where the dipeptide mutations mainly increased the stabilities of the oxidized variants, while the reduced forms had stabilities similar to the reduced wild type (Grauschopf et al., 1995; Huber-Wunderlich & Glockshuber, 1998). Interestingly, there are no differences between the structures of oxidized and reduced thioredoxin that could convincingly explain the higher stability of the oxidized protein (Jeng et al., 1994). The X-ray structures of oxidized wild-type DsbA and its oxidized glutaredoxin-like variant His32Tyr also revealed no obvious reason for the dramatically increased stability (22 kJ/mol) of the oxidized variant (Guddat et al., 1997a, 1997b; Huber-Wunderlich & Glockshuber, 1998). Overall, the interrelation between $\Delta\Delta G_{ox/red}$, pK_a, and E'_o in thiol-disulfide oxidoreductases is complex. The best correlations are generally obtained for the dependence of the redox potential on the pK_a of the nucleophilic cysteine, although significant deviations have also been observed for this correlation (Jacobi et al., 1997).

The stabilization of the nucleophilic thiolate

Several explanations for the lowered pK_a of the nucleophilic cysteine in thiol/disulfide oxidoreductases have been discussed and include electrostatic interaction of the Cys32 thiolate with lysine36 or with the dipole of the helix itself (Kallis & Holmgren, 1980; Hol, 1985; Kortemme & Creighton, 1995). Another possible stabilization of the thiolate would be a charged hydrogen bond with either the thiol or the amide proton of the buried active-site cysteine (Katti et al., 1990; Forman-Kay et al., 1991; Jeng et al., 1995; Katti et al., 1995). A stabilization of the thiolate by a salt bridge with a basic side chain was not observed in the structures of reduced *E. coli* and human thioredoxin (Qin et al., 1994; Jeng et al., 1995; Weichsel et al., 1996) and reduced glutaredoxin (Bushweller et al., 1992; Xia et al., 1992). The only case where a salt bridge might stabilize the nucleophilic thiolate is pig liver thioltransferase, where Arg26 is likely to make a ionic interaction with Cys22 within the active-site helix (Cys22-Pro23-Phe24-Cys25-Arg26) (Katti et al., 1995). Thus, the most likely explanation for the lowered pK_a values remains the favorable interaction of the N-terminal nucleophilic thiolate with the partial negative charge of the helix dipole (Hol, 1985; Kortemme & Creighton, 1995). Consequently, the amino acid sequence of the active-site helix should be critical for the pK_a of the nucleophilic thiol. In this context, the two amino acids of the Xaa-Xaa dipeptide appear to be especially important: first, a proline residue follows the nucleophilic cysteine (Cys-Pro-Xaa-Cys) in the enzymes with an extremely low pK_a (3.5) of the nucleophilic thiol, *E. coli* DsbA and yeast glutaredoxin (Gan et al., 1990; Nelson & Creighton, 1994), whereas a proline at the second position of the dipeptide (Cys-Xaa-Pro-Cys) is characteristic for the reductant thioredoxin. The role of the prolines is supported by studies on helical model peptides, where an N-terminal Cys-Pro sequence had the most favorable interaction with the α-helix, with a pK_a of the cysteine lowered by 1.6 units (Kortemme & Creighton, 1995). The critical role of the N-terminal proline is confirmed by our studies, as the DsbA- and glutaredoxin-like thioredoxin variants, that contain the N-terminal proline had the lowest Cys32 pK_a values (Table 1). The corresponding mutations in the Xaa-Xaa sequence of DsbA yielded the analogous result, with even stronger effects on the pK_a of the nucleophilic thiol (Cys30) (Huber-Wunderlich & Glockshuber, 1998). Second, as shown for the *a*-domain of PDI, the histidine present at the second position of the active-site dipeptide (Cys-Xaa-His-Cys) in the oxidizing enzymes DsbA and PDI appears to interact electrostatically with the N-terminal cysteine thiolate. It lowers the pK_a by about one pH unit in the *a*-domain of PDI (Kortemme et al., 1996), which agrees with the decrease of the pK_a from 7.1 to 6.2 caused by the Gly-Pro → Gly-His mutation in thioredoxin (Table 1) and with the crystallographic analysis of a glutaredoxin-like dipeptide variant of DsbA (Pro-His → Pro-Tyr) (Guddat et al., 1997b).

Conclusions

The present study on rationally designed Xaa-Xaa variants of thioredoxin, in conjunction with the characterization of rationally designed variants of PDI, TlpA, and DsbA (Kortemme et al., 1996; Rossmann et al., 1997; Huber-Wunderlich & Glockshuber, 1998) and previously reported Xaa-Xaa variants of DsbA and thioredoxin obtained by random mutagenesis (Grauschopf et al., 1995; Chivers et al., 1996) underline the critical function of the Xaa-Xaa dipeptide for the redox properties of thiol-disulfide oxidoreductases. In particular, this study supports the idea first suggested by Krause et al. (1991) that these enzymes can be converted to oxidants or reductants by rational means, simply by exchange of the Xaa-Xaa dipeptide against that of a related enzyme. We believe that this concept will prove to be useful for applications of thiol-disulfide oxidoreductases in biotechnology and the study of their function *in vivo*.

Materials and methods

Materials

5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB), 1,4,-dithio-DL-threitol (DTT), reduced glutathione (GSH) and oxidized glutathione (GSSG) were from Sigma (Deisenhofen, Germany). Isopropyl-β-D-thiogalactoside (IPTG) was purchased from Biomol (Hamburg, Germany) and guanidinium chloride (GdmCl) was from ICN (Orangeburg, New York). Yeast glutathione reductase and NADPH were from Boehringer (Mannheim, Germany), DE52 cellulose was from Whatman (Maidstone, United Kingdom), the Superdex 200 HiLoad 26/60 column was obtained from Pharmacia (Uppsala, Sweden), and the Vydac™ 218TP54 HPLC column was from Vydac (Hesperia, U.S.A.). Oligodeoxynucleotides were purchased from MWG Biotech (Ebersberg, Germany). All other chemicals were from Merck (Darmstadt, Germany) and of analytical grade.

Methods

Construction of the expression plasmid and site-directed mutagenesis

Molecular cloning techniques were based on Sambrook et al. (1989). The thioredoxin wild-type gene was amplified from the genome of the *E. coli* K12 strain W3110 (Bachmann, 1972) by the polymerase chain reaction (PCR) using the following oligonucleotide primers: N-terminal primer: 5′-CAACACGCCAGGTCTAG ATAACGAGGGCAAAAAATGAGCGATAAAATTAT-3′; C-terminal primer: 5′-GGCACC CGAACAGGATCCAAGCTTAC GCCAGGTTAGCGTCGAGGAAC-3′. The amplified thioredoxin gene was cloned into the phagemid pRBI-PDI-T7 (Strobl et al., 1995) via the XbaI and HindIII restriction sites after the *dsbA* gene in pRBI-PDI-T7 had been removed by cutting with EcoRI and BamHI and religating the vector. In the resulting expression phagemid, termed pTrx, the thioredoxin gene is under control of the T7 promoter/lac operator sequence (Studier & Moffat, 1986). The correct sequence of the thioredoxin gene was verified by dideoxy sequencing. Site-directed mutagenesis Cys32 sis was performed according to Kunkel (Kunkel et al., 1987) with uridinylated, single-stranded DNA of pTrx and the following oligonucleotide primers (mismatches are underlined):

variant (GH): 5′-GGCGATCATTTTGCAGTGCCCGCACC ACTCTGCC-3′

variant (PH): 5′-GGCGATCATTTTGCAGTGCGGGCACC ACTCTGCCCAG-3′

variant (PY): 5′-GGCGATCATTTTGCAGTACGGGCACC ACTCTGCCCAG-3′

variant (AT): 5′-GGCGATCATTTTGCAGGTTGCGCACC ACTCTGCCC-3′. (1)

All mutations were confirmed by sequencing of the whole thioredoxin genes.

Protein purification

E. coli BL21(DE3) (Studier & Moffat, 1986) harboring pTrx or the corresponding derivative was grown at 37 °C in 10 L LB medium containing 100 μg/mL ampicillin until an optical density at 546 nm of 1.0 was reached. After addition of IPTG to a final concentration of 1 mM the cells were further grown for 5 h and harvested by centrifugation (5,000 × *g*). The cells were suspended at 4 °C in 100 mL 20 mM Tris/HCl pH 7.5 and disrupted twice in a French Pressure cell (18,000 PSI). The lysate was centrifuged (15,000 × *g*, 40 min, 4 °C) and the supernatant was dialyzed against 20 mM Tris/HCl pH 7.5 and applied to a DE52-cellulose column (40 mL). The protein was eluted with a linear gradient (900 mL) from 0 to 0.4 M NaCl in 20 mM Tris/HCl pH 7.5. Fractions containing thioredoxin were pooled, concentrated to 6 mL by ultrafiltration (YM10 membrane, Amicon) and applied to a Superdex 200 HiLoad 26/60 gel filtration column equilibrated with 50 mM sodium phosphate pH 7.0. Proteins were eluted with the same buffer, fractions containing pure thioredoxin were combined, dialyzed against distilled water and stored at −20 °C. Typically, 500–1000 mg of homogeneous protein were obtained from a 10 L culture.

Wild-type thioredoxin and all thioredoxin variants were fully oxidized after purification, as judged by Ellman's assay (Ellman, 1959). The molecular mass of each protein was confirmed by electrospray mass spectrometry (accuracy: ±3 Da). Protein concentrations were determined by the absorbance at 280 nm (Gill & von Hippel, 1989). The following extinction coefficients (ϵ_{280}) were used for the oxidized proteins: 16,100 $M^{-1}cm^{-1}$ for the Grx-type variant and 14,500 $M^{-1}cm^{-1}$ for wild-type thioredoxin and all other variants.

Circular dichroism and fluorescence spectra

Far- and near-UV CD spectra were recorded on a JASCO J-710 spectropolarimeter at protein concentrations of 40 μM in 1 mM sodium phosphate pH 7.0, 10 mM sodium sulfate. One-centimeter cuvettes were used for the near-UV region (240–350 nm) and 0.2 mm cuvettes for the far-UV region (180–260 nm). Fluorescence spectra were measured between 300 and 400 nm (excitation at 280 nm) on a Hitachi F-4500 fluorescence spectrometer at protein concentrations of 1–2 μM in 100 mM sodium phosphate pH 7.0. DTT (3 mM) and EDTA (100 μM) were included for all spectroscopic measurements with the reduced proteins.

Determination of the equilibrium constants with glutathione (K_{eq})

The about 3.5-fold increase in tryptophan fluorescence at 345 nm (excitation at 280 nm) which is observed at pH 7.0 upon reduction of thioredoxin (Holmgren, 1972) was used to measure their equilibrium constants with glutathione (K_{eq}) (Wunderlich & Glockshuber, 1993). The oxidized proteins (1–2 μM) were incubated under nitrogen atmosphere for 16 h at 25 °C in 100 mM sodium phosphate pH 7.0, 1 mM EDTA containing 10 μM GSSG and different concentrations of GSH (0–200 mM). Due to impurities of GSSG in the GSH stock solution, the true concentration of GSSG in each redox buffer was determined independently with the glutathione reductase assay as described previously (Loferer et al., 1995). The equilibrium constants (K_{eq}) were determined by fitting the original fluorescence data according to Equation 2:

$$F_m = F_{ox} + ([GSH]^2/[GSSG]) \times (F_{red} - F_{ox})/(K_{eq} + [GSH]^2/[GSSG]) \quad (2)$$

where F_m is the measured fluorescence and F_{ox} and F_{red} are the fluorescence intensities of the oxidized and reduced protein, respectively (cf. Equation 6). No systematic deviations from Equation 2 were observed for the equilibrium fluorescence data of any of the variants, demonstrating that thioredoxin/glutathione mixed disulfides were not significantly populated. A value of −240 mV was used for the standard redox potential of glutathione (Rost & Rapoport, 1964) to calculate the redox potentials (E_o') of the thioredoxin variants (Equation 3):

$$E_o' = -240\ \text{mV} - (RT/2F) \cdot \ln K_{eq}. \quad (3)$$

GdmCl-induced unfolding equilibria

Unfolding/refolding equilibria of oxidized and reduced thioredoxins were measured by the far-UV CD signal at 220 nm in 0.2 mm

cuvettes. Protein solutions (30–50 μM) were equilibrated at 25 °C for 2 days in 100 mM sodium phosphate pH 7.0, 100 μM EDTA containing different concentrations of GdmCl. In the case of the reduced proteins, DTT was included (final concentration of 3 mM). The ellipticities at 220 nm were recorded for 3 min and averaged. The original data were evaluated according to the two-state model of folding (Pace, 1986; Lin & Kim, 1989) with a six-parameter fit (Santoro & Bolen, 1988).

pK_a values of Cys32 of thioredoxin and Cys30 in DsbA

The ionization of Cys32 in thioredoxin and Cys30 in DsbA was measured by the pH-dependent reactivity of the active-site cysteines with iodoacetamide (Kallis & Holmgren, 1980; Yang & Wells, 1991). The reactions were performed at 25 °C in buffer A (200 mM KCl, 10 mM *di*-sodium hydrogen phosphate, 10 mM boric acid, 10 mM succinic acid, adjusted with HCl or NaOH to pH 2–13). Reduced thioredoxin and DsbA were obtained after reduction by DTT and gel filtration on a PD10 column (Pharmacia) as described previously (Wunderlich et al., 1993). Pseudo-first-order conditions were applied in all reactions (excess of iodoacetamide). The concentrations of reduced proteins were 2–4 μM, and the iodoacetamide concentrations were varied between 75 μM and 3 mM for the reactions with thioredoxin and between 0.4 and 1.1 mM for the reactions with DsbA. The reactions were stopped after different times by addition of 0.5 volumes of 30% (v/v) formic acid (final pH $\leq$ 2), and 0.5 mL aliquots were applied to a Vydac™ 218TP54 C18 reverse-phase HPLC column (4.6 × 250 mm). After application to the column the reaction products were separated within 30 min at 55 °C and a flow rate of 0.5 mL/min with a linear gradient from 41 to 53% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid (TFA) in the case of thioredoxin and a gradient from 45 to 48% (v/v) acetonitrile in 0.1% (v/v) TFA in the case of DsbA. Eluted proteins were detected by absorbance at 280 nm and quantified by integration of the peak areas using the software DS 450-MT1-EMS (Kontron). The apparent second-order rate constants (k_{IAM}) were calculated from the initial velocities of the reactions (0–25% product formation). The pK_a values of the active-site cysteines (pK^{SH}) were obtained from fitting the data according to Equation 4:

$$k_{IAM} = k_{S^-}/(1 + 10^{pK^{SH} - pH}) \quad (4)$$

where k_{S^-} corresponds to the second-order rate constant for the reaction between the active-site thiolate anion and iodoacetamide. If another side chain (X) titrating above pK^{SH} influenced the reactivity of the nucleophilic cysteine, Equation (5) was applied:

$$k_{IAM} = \frac{k_{S^-} \times 10^{pK^{SH} - pH} + k_{S^-X^-} \times 10^{2pH - pK^{SH} - pK^X}}{1 + 10^{pK^{SH} - pH} + 10^{2pH - pK^{SH} - pK^X}} \quad (5)$$

where $k_{S^-X^-}$ is the rate constant for the reaction of the thiolate with iodoacetamide when X is deprotonated and pK^X is the pK_a of the side chain X.

The pK_a of Cys30 of DsbA was alternatively measured by the pH-dependent absorbance at 240 nm as described previously, using oxidized DsbA as a reference (Nelson & Creighton, 1994; Jacobi et al., 1997). Reduced DsbA (30 μM) was titrated in the range of pH 8–2 by stepwise addition of HCl in buffer A containing 140 μM β-mercaptoethanol. The same conditions were applied to measure the pH dependence of A_{240} in thioredoxin.

Simulation of the dependence of Keq on the pK_a values of Cys32 and Cys35 of thioredoxin

Equation 6 shows the microscopic rate constants involved in the overall equilibrium between thioredoxin and glutathione, assuming that the buried active-site cysteine (Cys35) is not capable to form a mixed disulfide with glutathione.

$$Trx_{SH}^{SH} + GSSG \underset{k_2}{\overset{k_1}{\rightleftharpoons}} Trx_{SH}^{SSG} + GSH \underset{k_4}{\overset{k_3}{\rightleftharpoons}} Trx_S^S + 2GSH. \quad (6)$$

The observed rate constants of disulfide exchange reactions (k^{obs}) at any pH can principally be predicted from the pK_a values of all involved thiols by Equation 7

$$\log k^{obs} = (6.3 + 0.59\,pK_a^{nuc} - 0.40\,pK_a^c - 0.59\,pK_a^{lg}) - \log(1 + 10^{pK_a^{nuc} - pH}) \quad (7)$$

where pK_a^{nuc}, pK_a^c, and pK_a^{lg} are the pK_a values of the nucleophilic, central and leaving group sulfur, respectively (cf. Equations 38 and 39 in Szajewski & Whitesides, 1980). Using the measured pK_a values of Cys32 of the variants, changes in the equilibrium constants with glutathione at pH 7 relative to the wild-type were predicted by applying Equations 7 and 8.

$$K_{eq} = (k_1^{obs} \times k_3^{obs})/(k_2^{obs} \times k_4^{obs}). \quad (8)$$

It was assumed that the effective concentration of Cys35 which determines k_3 is the same in all variants. A pK_a of 8.7 was used for the thiol of GSH in all simulations, whereas the pK_a values for the thiol of Cys35 were set to a constant value of 9.0 or were set to be identical to the pK_a of Cys32 (cf. legend of Fig. 6B).

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