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Redox Potentials of Glutaredoxins and Other Thiol-Disulfide Oxidoreductases of the Thioredoxin Superfamily Determined by Direct Protein-Protein Redox Equilibria*

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Glutaredoxins belong to the thioredoxin superfamily of structurally similar thiol-disulfide oxidoreductases catalyzing thiol-disulfide exchange reactions via reversible oxidation of two active-site cysteine residues separated by two amino acids (CX_1X_2C). Standard state redox potential ($E^{\circ\prime}$) values for glutaredoxins are presently unknown, and use of glutathione/glutathione disulfide (GSH/GSSG) redox buffers for determining $E^{\circ\prime}$ resulted in variable levels of GSH-mixed disulfides. To overcome this complication, we have used reverse-phase high performance liquid chromatography to separate and quantify the oxidized and reduced forms present in the thiol-disulfide exchange reaction at equilibrium after mixing one oxidized and one reduced protein. This allowed for direct and quantitative pair-wise comparisons of the reducing capacities of the proteins and mutant forms. Equilibrium constants from pair-wise reaction with thioredoxin or its P34H mutant, which have accurately determined $E^{\circ\prime}$ values from their redox equilibrium with NADPH catalyzed by thioredoxin reductase, allowed for transformation into standard state values. Using this new procedure, the standard state redox potentials for the *Escherichia coli* glutaredoxins 1 and 3, which contain identical active site sequences CPYC, were found to be $E^{\circ\prime} = -233$ and -198 mV, respectively. These values were confirmed independently by using the thermodynamic linkage between the stability of the disulfide bond and the stability of the protein to denaturation. Comparison of calculated $E^{\circ\prime}$ values from a number of proteins ranging from -270 mV for *E. coli* Trx to -124 mV for DsbA obtained using this method with those determined using glutathione redox buffers provides independent confirmation of the standard state redox potential of glutathione as -240 mV. Determining redox potentials through direct protein-protein equilibria is of general interest as it overcomes errors in determining redox potentials calculated from large equilibrium constants with the strongly reducing NADPH or by accumulating mixed disulfides with GSH.

Glutaredoxin (Grx1)[1] was discovered as a GSH-dependent hydrogen donor for ribonucleotide reductase in *Escherichia coli* mutants lacking the first identified electron donor, thioredoxin (1). The presence of an additional hydrogen donor system for ribonucleotide reductase was postulated since a double mutant lacking both Grx1 and Trx was viable (2). The search for this third hydrogen donor system resulted in the isolation of two additional glutaredoxins in *E. coli*, Grx2 and Grx3 (3). The recent structural characterization of Grx3 showed that in addition to the 33% amino acid sequence identity with Grx1, the two proteins have highly conserved secondary structure elements and overall fold (4). However, despite Grx1 and Grx3 being closely related 9-kDa redox proteins with identical active-site sequences (CPYC), Grx3 exhibits only a fraction of the activity of Grx1 as a reductant of ribonucleotide reductase (NrdAB and NrdEF; Refs. 3 and 5) or of insulin disulfides (4). One possible explanation for these observed differences would be a difference in redox potential between the two glutaredoxins.

Considerable experimental and theoretical effort has been focused on the role the residues between the active site cysteines (CX_1X_2C) play as a determinant for the standard state redox potential ($E^{\circ\prime}$) of the thioredoxin superfamily of proteins. The first mutagenesis studies (6, 7) demonstrated that redox potential of a protein could be made more oxidizing or reducing by simply substituting the residues X_1 and X_2 for those of a protein whose redox potential was either more oxidizing or reducing, respectively. For example, the mutant Trx_{PDI}, mimicking the active site of protein disulfide isomerase (PDI), results in a change of 35 mV in the expected direction (more oxidizing), yet this value remains far from that of PDI (7). The redox potential for the $DsbA_{Trx}$ mutant (-214 mV[2]), although 90 mV more reducing than the wild-type protein, is still far from the value of $E^{\circ\prime} = -270$ mV determined for Trx (6). A

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[1] The abbreviations used are: Grx1, *E. coli* glutaredoxin 1; Grx3, *E. coli* glutaredoxin-3; GSH, glutathione; GSSG, glutathione disulfide; DsbA, *E. coli* disulfide bond-promoting product of the gene *dsbA*; $DsbA_{Trx}$, DsbA with the active site changed into that of thioredoxin (Pro-33 replaced by Gly, and His-34 replaced by Pro); $\Delta\epsilon$, molar CD of the amide chromophore; $E^{\circ\prime}$, standard state redox potential at 25 °C; $E^{\circ\prime}_{GSH}$, standard state redox potential of glutathione; $\Delta E^{\circ\prime}_{AB}$, difference in standard state redox potentials between molecules A and B; $E^{\circ\prime}_U$, standard state redox potential of the unfolded form of the protein; f_u, fraction of unfolded protein; GdnHCl, guanidine hydrochloride; ΔG^{SS}_N and ΔG^{SS}_U, the Gibbs energy of disulfide bond formation in the folded and unfolded state, respectively. $\Delta G^{\circ}_{H_2O}$ and m_G, intercept and slope of the linear extrapolation of unfolding Gibbs energy changes *versus* denaturant concentration; K_{ox}, equilibrium constant with GSH; K', apparent concentration equilibrium constant; m_N and m_U, slopes of the pre- and post-transition region base line of the denaturation, respectively; PDI, protein disulfide isomerase; Trx, *E. coli* thioredoxin; Trx_{PDI}, *E. coli* thioredoxin with the active site changed into that of thioredoxin (Pro-34 replaced by His); HPLC, high performance liquid chromatography.

[2] M. Huber-Wunderlich and R. Glockshuber, personal communication.

similar mutation of one of the Trx-like domains of PDI decreased $E^{\circ\prime}$ from −145 to −215 mV (8). A subsequent study confirmed these findings using a more extensive set of mutants (9), demonstrating that at least for DsbA, a relationship between the redox potential and the pK_a value of the N-terminal active-site cysteine residue exists (9), as predicted by the Brønsted relationship using small thiol-containing molecules (10). Whereas the thioredoxin superfamily of oxidoreductases have redox potentials spanning a wide range, peptides containing the active-site sequences of selected oxidoreductases have surprisingly similar redox potentials (11). This observation underscores the fact that redox potentials of native proteins are determined by interactions in the folded state resulting in a rather poor predictive ability of the active site sequence even when present in a common three-dimensional framework (6, 7, 9). This is the case with Grx1 and Grx3, which have identical active-site sequences yet only 33% sequence identity in their overall similar fold (4). Knowledge of the redox potentials of these two glutaredoxins is important in light of their catalytic properties, which on one hand show similar activity in reducing GSH-mixed disulfides (3), a property that does not involve the intramolecular disulfide form of the protein (12), and on the other hand display large differences in the capacity to reduce protein disulfides, *e.g.* ribonucleotide reductase or insulin (4).

The standard state redox potential $E^{\circ\prime}$ for a protein can be calculated from the equilibrium constant of the redox reaction involving a reference with known redox potential using the Nernst equation. The commonly used references are defined glutathione/glutathione disulfide (GSH/GSSG) buffers or NADPH/$NADP^+$ coupled via an appropriate reductase (13). Standard state redox potentials within the thioredoxin family of thiol-disulfide oxidoreductases range from $E^{\circ\prime}$ = −124 (14, 15) to −270 mV (7), corresponding to an overall equilibrium constant of nearly 10^5. Since the precision in the determination of an equilibrium constant is highest the closer the equilibrium constant is to unity, one reference is insufficient to obtain accurate data within this span of redox potentials. In addition, the lack of a consensus for the value of the standard state redox potential of a commonly used redox reference standard contributes to apparently conflicting data in the literature. For example, cited values for the standard state redox potential of glutathione ($E^{\circ\prime}_{GSH}$) range from −205 to −260 mV (10, 13, 16, 17). Largely as a consequence of this ambiguity, in addition to technical difficulties in quenching a thiol-disulfide interchange reaction (18), the reported redox potential for some proteins, such as protein disulfide isomerase (PDI), ranges from $E^{\circ\prime}$ = −110 (19) to −190 mV (20), a discrepancy of no less than a factor of 500 in equilibrium constant.

We have developed a method for the determination of redox potentials by direct protein-protein equilibration that has a number of advantages compared with commonly used techniques for redox determinations. Direct equilibration techniques have been used successfully with low molecular weight thiols (10), peptides, and proteins (8, 11, 13–15) in equilibration with low molecular weight thiols (*e.g.* GSSG or β-hydroxyethylene disulfide). Here we demonstrate that direct protein-protein equilibration can also be used successfully to determine redox potential differences between proteins. When applied to *E. coli* Grx1 and Grx3, standard state redox potentials $E^{\circ\prime}$ = −198 and −233 mV were obtained, which were confirmed using a thermodynamic linkage relationship between the stability of the protein and the stability of the disulfide bond (*i.e.* the redox potential). Through multiple independent pair-wise linkages of protein-protein redox equilibria, we have established an accurate relative redox scale that is linked to the well defined standard state of NADPH of $E^{\circ\prime}$ = −315 mV (21), which presents a unifying picture of the thioredoxin superfamily of thiol-disulfide oxidoreductases.

EXPERIMENTAL PROCEDURES

Protein Preparations—A mutant of *E. coli* Grx3 with Cys-65 replaced by tyrosine, available from this laboratory,[3] was selected for this work. The absence of the third, nonactive-site cysteine in position 65 eliminates the tendency of this protein to form intermolecular disulfide-linked multimers (dimers, trimers, and tetramers) as well as the formation of a mixed disulfide with GSH with accompanying decreased catalytic activity.[3] This mutant has essentially identical enzymatic activity as the wild-type enzyme[3] and will be referred to throughout this work simply as Grx3. Overexpressed Grx3 displays some heterogeneity due to variable degrees of cleavage of the N-terminal Met residue (4), which is a potential problem when quantitating equilibrium constants using HPLC, as the reduced state of the minor component (Met form) was found to co-elute with the oxidized state of the major (non-Met) form. To circumvent this problem, we have used a preparation of Grx3 that was found to be essentially 100% in the non-Met form. *E. coli* Grx1 (22), Trx (23), and Trx_{PDI} (7) were homogenous preparations from this laboratory. The DsbA mutant with the active-site tetrapeptide sequence changed into that of thioredoxin, $DsbA_{Trx}$,[2] was generously provided by Dr. Martina Wunderlich and Professor Rudi Glockshuber, ETH-Hönggerberg, Zürich Switzerland.

Determination of Equilibrium Constants—Protein-protein equilibrium reactions (100 μl) typically contained 50 μM each of two redox-active proteins in a degassed and N_2-purged solution of 100 mM potassium phosphate, pH 7.0, 1 mM EDTA. The reduced form of a protein was prepared immediately before use by incubation of protein (1 mM) for 1 h at room temperature in the presence of 10 mM dithiothreitol, followed by Sephadex G-25 gel filtration chromatography (NAP-5, Pharmacia Biotech Inc.). Redox reactions between two proteins were initiated by adding one of the two proteins in the reduced state and the other in the oxidized state, whereafter the sample was left to equilibrate at room temperature. After 4 and 12 h, aliquots (20 μl) of each sample were quenched by the addition of 10 μl of 1 M phosphoric acid to a final pH of 2.5 followed by immediate HPLC analysis. The oxidized and reduced forms of the proteins present in the samples were separated by reverse-phase HPLC (Pharmacia SMART micropurification system) on a C_8 column (2.1 mm × 10 cm) using a gradient from 22–50% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid in 120 min at a flow rate of 0.1 ml/min at room temperature. Column effluent was monitored at 214 and 280 nm.

The amounts of oxidized and reduced forms of each protein in the quenched equilibrium mixture was obtained from peak areas after integration of the chromatograms. Essentially identical results were obtained from analysis of chromatograms recorded at 214 and 280 nm, and the former was used in subsequent analysis. The mechanism of thiol-disulfide exchange is believed to be a two-step reaction proceeding through a mixed disulfide intermediate (Eq. 1). The breakdown of the protein-protein-mixed disulfide appears to be very rapid, as it is not normally populated under standard conditions.

The apparent concentration equilibrium constant, K'_{12}, for the thiol disulfide exchange reaction between protein A and protein B involves only the oxidized forms (A^S_S, B^S_S) and reduced forms (A^{SH}_{SH}, B^{SH}_{SH}) of the two proteins involved (Eq. 2).

$$A^{SH}_{SH} + B^S_S \rightleftharpoons [A^S_{SH}\!-\!\!-\!\!-\!^S_{HS}B] \rightleftharpoons A^S_S + B^{SH}_{SH} \qquad \text{(Eq. 1)}$$

$$K'_1 \cdot K'_2 = K'_{12} = \frac{[A^S_S][B^{SH}_{SH}]}{[A^{SH}_{SH}][B^S_S]} \qquad \text{(Eq. 2)}$$

$$E^{\circ\prime}_A - E^{\circ\prime}_B = \Delta E^{\circ\prime}_{AB} = \frac{R\,T}{n\,F} \ln K'_{12} \qquad \text{(Eq. 3)}$$

The difference in redox potentials between the two proteins, $\Delta E^{\circ\prime}_{AB}$, can then be obtained using the Nernst equation (Eq. 3), where n is the number of electrons transferred in the reaction (here n = 2), F is Faraday's constant (23,040.612 cal·mol^{-1}·V^{-1}), and R is the gas constant (1.987 cal·K^{-1}·mol^{-1}).

Redox Potential of the Unfolded State—Redox equilibria were established by incubating Grx1, Grx3, and Trx_{PDI} at a concentration of 50 μM each protein in a degassed and nitrogen-purged redox buffer containing 10 mM GSH and 1 mM GSSG in 100 mM potassium phosphate, pH 7.0,

[3] A. Sandström, K. D. Berndt, F. Åslund, A. Holmgren, and G. Otting, unpublished results.

1 mM EDTA, and 6 M GdnHCl. As a control, a parallel sample containing the folded forms of the proteins was prepared in an identical buffer without GdnHCl. The reactions were equilibrated for 12 h at room temperature followed by acid quenching (as described above) and immediate HPLC separation. The concentration of GSH in the redox buffer was measured by determination of the total thiol content spectrophotometrically (ϵ_{412} = 13,600 $M^{-1}\cdot cm^{-1}$) using 5,5′-dithiobis(nitrobenzoic acid) both at the beginning and at the end of the equilibrium reaction. The concentrations of the oxidized and the reduced forms of each protein were obtained from the corresponding peak areas (214 nm) in the HPLC chromatograms. From the thiol-disulfide exchange reaction of the unfolded protein with glutathione (Eq. 4), the overall equilibrium constant K'_{12} can be calculated (Eq. 5). Given the 1 M standard state redox potential of GSH, $E^{\circ'}_{GSH}$ (−240 mV, see below), the standard state redox potential of the denatured state, $E^{\circ'}_{U}$, is obtained according to Equation 6.

$$U^{SH}_{SH} + GSSG \underset{}{\overset{K'_1}{\rightleftharpoons}} U^{S}_{SH}\text{—}{}^{S}G \overset{K'_2}{\rightleftharpoons} U^{S}_{S} \qquad \text{(Eq. 4)}$$

$$K'_1 \cdot K'_2 = K'_{12} = \frac{[U^{S}_{S}][GSH]^2}{[U^{SH}_{SH}][GSSG]} \qquad \text{(Eq. 5)}$$

$$E^{\circ'}_{U} = E^{\circ'}_{G^{SH}} - \frac{R\,T}{n\,F}\ln K'_{12} \qquad \text{(Eq. 6)}$$

GdnHCl-induced Unfolding of Grx1 and Grx3—GdnHCl-induced unfolding of oxidized and reduced Grx1 (5.6 μM) and Grx3 (4.4 μM) was performed in 100 mM potassium phosphate, pH 7.0, 1 mM EDTA, in the presence or absence of 2.0 mM dithiothreitol. Unfolding was monitored by ellipticity at 222 nm (Aviv 62DS spectropolarimeter; Aviv Associates Inc., Lakewood, NJ) as additions of GdnHCl were made automatically from an identical solution containing, in addition, 5.7 M GdnHCl, as determined by refractometry (24) using a computer-controlled microtitrator (Hamilton 540B, Reno, NV). After each addition, 5 min was allowed for equilibration, during which time the cuvette was stirred with the spectrophotometer shutters closed. Parallel experiments using manual titration and protein concentrations of 17 and 43 μM for Grx1 and Grx3, respectively, were analyzed after an overnight equilibration. The manual experiments were designed to contain a minimum of 10 points in each of the pre-transition, transition, and post-transition regions of the unfolding curves. The raw CD data (θ, in units of millidegrees were first transformed to units of molar CD of the amide chromophore $\Delta\epsilon$ ($M^{-1}\cdot cm^{-1}$) using the relationship,

$$\Delta\epsilon = \frac{\theta}{32980 \cdot l \cdot c} \qquad \text{(Eq. 7)}$$

where l is the sample path length (cm) and c is the residue molar concentration of protein. The data ($\Delta\epsilon$ *versus* [GdnHCl]) were fit using nonlinear regression (Igor Pro software package; WaveMetrics, Lake Oswego, OR) to a general equation (Eq. 8) describing the experimentally observable $\Delta\epsilon$ as a function of denaturant concentration, [D], which includes the linear extrapolation method (25).

$$\Delta\epsilon = \frac{(\Delta\epsilon_N + m_N[D]) + (\Delta\epsilon_U + m_U[D]) \cdot e^{\left[\frac{(-(\Delta G^{\circ}_{H_2O} + m_G[D]))}{RT}\right]}}{1 + e^{\left[\frac{(-(\Delta G^{\circ}_{H_2O} + m_G[D]))}{RT}\right]}} \qquad \text{(Eq. 8)}$$

Here $\Delta\epsilon_N$ and $\Delta\epsilon_U$ are the molar CD values of the native and unfolded protein, respectively, extrapolated to zero M denaturant, and m_N and m_U are the slopes of the pre- and post-transition region base lines. The parameters m_G and $\Delta G^{\circ}_{H_2}O$ are the values of the slope and intercept, respectively, of the linear extrapolation of unfolding Gibbs energy changes *versus* denaturant concentration. For purposes of presentation only, $\Delta\epsilon$ was converted to fraction of unfolded protein (f_u) with the relationship $f_u = [(\Delta\epsilon_N + m_N) - \Delta\epsilon]/[(\Delta\epsilon_N + m_N) - (\Delta\epsilon_U + m_U)]$ using the values of $\Delta\epsilon_N$, $\Delta\epsilon_U$, m_N, and m_U obtained from Eq. 8.

RESULTS

Establishing a Network of Redox Potential Differences—The popular method of determining a redox potential using defined ratios of GSH/GSSG as redox references (Eq. 4–6) is complicated in the case of glutaredoxins due to the presence of significant amounts of the GSH-mixed disulfide intermediates (Fig. 1*A*), which may be unique to the glutaredoxins that have been shown to contain a binding site for glutathione (4, 12). Under certain circumstances, β-mercaptoethanol-mixed disulfides with Trx have been trapped successfully and were used for the determination of the equilibrium constants K'_1 and K'_2 (26). We found that the amounts of the GSH-mixed-disulfide form of the glutaredoxins recovered by HPLC varied substantially depending on the quenching procedure. In view of the apparent problem of perturbing the equilibrium of this mixture upon quenching in the presence of the large excess of GSH/GSSG, we searched for more direct methods of determining redox potentials.

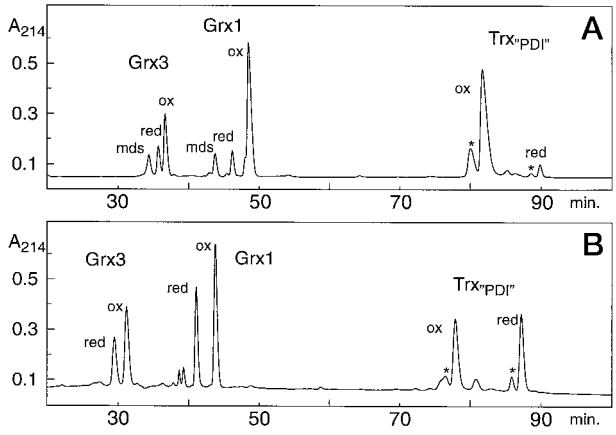

FIG. 1. **Redox equilibria of *E. coli* Grx1, Grx3, and Trx_{PDI} with GSH/GSSG in the native or unfolded states.** *A*, HPLC chromatogram of species present after incubation of *E. coli* Grx1, Grx3, and Trx_{PDI} in a defined redox buffer containing 10 mM GSH and 1 mM GSSG corresponding to a redox potential of −210 mV. *B*, as *A* but in the presence of 6 M of GdnHCl. Peaks are labeled as *ox* (oxidized), *red* (reduced), and *mds* (glutathione-mixed disulfide). An *asterisk* indicates the position of an N-terminal methionine-containing isoform of the protein (see "Experimental Procedures" for details).

In principle, differences in redox potential between two proteins, $\Delta E^{\circ'}_{AB}$, could be obtained by determining the concentrations of their oxidized and reduced forms (Eq. 1–3) at equilibrium. The success of such a method would rely on the nonspecificity of the thiol-disulfide exchange and the ability to quench and subsequently quantify equilibrium populations. The latter two are conveniently achieved by acidification followed by reverse-phase HPLC separation and quantification of the individual species. To ensure that a true state of redox equilibrium between the two proteins in question was obtained in incubations for 4 h, we verified with each protein pair that essentially identical equilibrium constants were obtained irrespective of the redox state of the initial mixture (*i.e.* A^{S}_{S} and B^{SH}_{SH} or B^{S}_{S} and A^{SH}_{SH}). Several reactions were subjected to a second analysis after 12 h of incubation at room temperature to ensure that the reactions were at equilibrium at 4 h. The elution times of the fully oxidized and fully reduced (with excess dithiothreitol) proteins were obtained in separate runs by injecting identical amounts of each protein. This procedure also revealed essentially identical molar extinction coefficients for the reduced forms compared with the oxidized forms of each protein, as might be expected at 214 and 280 nm.

Using this method, we determined a value for of K'_{12} = 15.2 between Grx1 and Grx3 at pH 7.0 (Fig. 2*A*). Thus, according to Eq. 3, Grx3 is 35 mV more oxidizing than Grx1, a fact that is rationalized at least qualitatively by noticing that in Fig. 2*A*, the equilibrium concentration of $Grx3_{red}$ is greater than that of Grx_{ox}, whereas the opposite is true of Grx1. The validity of the redox potential difference measured in this way can be assessed by independently measuring the pair-wise differences of Grx1 and Grx3 to a third, common redox-active partner. As an example, Figs. 2, *B* and *C* show chromatograms of the quenched

pair-wise equilibria of Grx3 and Grx1, respectively, with Trx_{PDI}. This mutant was chosen over wild-type Trx, as it has a more favorable redox potential for comparison with the more oxidizing glutaredoxins. From these chromatograms, we determined $K_{12} = 17.78$ for the Grx3/Trx_{PDI} pair and $K_{12} = 1.17$ for the Grx1/Trx_{PDI} pair, corresponding to redox potential differences of $\Delta E'^{\circ}_{AB} = 37$ and 2 mV, respectively. The difference between these values (35 mV) confirms the result obtained from direct equilibration between Grx1 and Grx3. Following this strategy, we extended our comparisons to establish a network of differences based on pair-wise equilibria between selected thiol-disulfide oxidoreductases, which resulted in an unambiguous relative scaling of their reducing capacities summarized in Fig. 3.

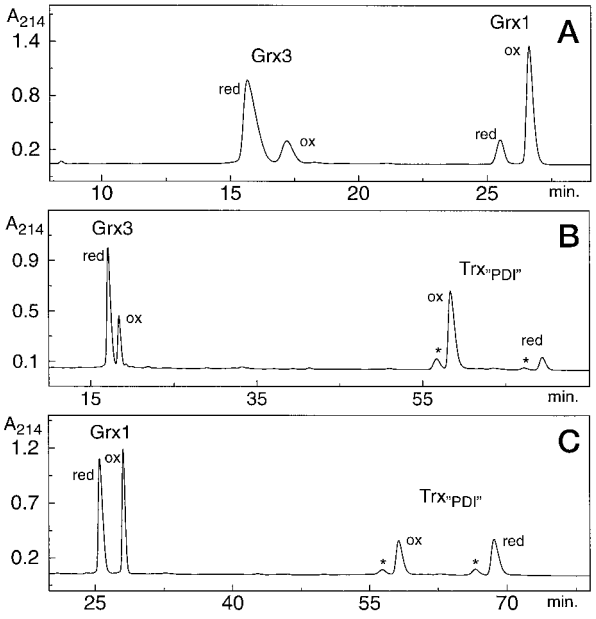

FIG. 2. **Reverse-phase HPLC chromatogram of the species present in different redox equilibria (Eq. 1).** The proteins were incubated for 12 h at room temperature at a concentration of 50 μM each in 100 mM potassium phosphate, 1 mM EDTA, pH 7.0, followed by acid quenching to pH 2.5 before HPLC analysis. *A*, *E. coli* Grx1 and Grx3; *B*, *E. coli* Grx3 and Trx_{PDI}; *C*, *E. coli* Grx1 and Trx_{PDI}. An *asterisk* indicates the elution position of a second species of Trx_{PDI} (see text for details).

Given the possibility of obtaining the equilibrium constant of thiol-disulfide interchange between two proteins using this method, one can obtain accurate standard state redox potentials for a set of proteins through pair-wise relation to other proteins, as long as one member of the set has an independently determined standard state redox potential. The standard state redox potential of NADPH has been established to be −315 mV (21) by several techniques, and unlike glutathione, is widely accepted in the literature. The standard state redox potentials of *E. coli* Trx and Trx_{PDI}, based on a thioredoxin reductase-coupled assay system with NADPH, was previously determined to be −270 and −235 mV, respectively (7).

In the network of linkages presented in Fig. 3, we have also included the relationships between GSH and Trx (27, 28), Trx_{PDI} (Fig. 1*A*), and $DsbA_{Trx}$,[2] which allows for an independent evaluation of the standard state redox potential of GSH. In the case of $DsbA_{Trx}$, we found that the equilibrium between Trx_{PDI} and $DsbA_{Trx}$ results in a redox potential of −217 mV, which agrees with the −214 mV obtained for $DsbA_{Trx}$ in GSH/GSSG buffers[2] (which used $E'^{\circ}_{GSH} = -240$ mV). Therefore a value of −240 mV for the standard state redox potential of glutathione is entirely consistent with the equilibrium constants between these three proteins and glutathione.

Redox Potential from Thermodynamic Linkage—To calculate the Gibbs energy of disulfide bond formation in the folded state (ΔG^{SS}_{N}) using the thermodynamic linkage relationship (Fig. 4), one also needs to determine the value of the Gibbs energy of disulfide bond formation in the unfolded state (ΔG^{SS}_{U}) and the conformational stabilities of both the oxidized ($\Delta G^{ox}_{H_2O}$) and

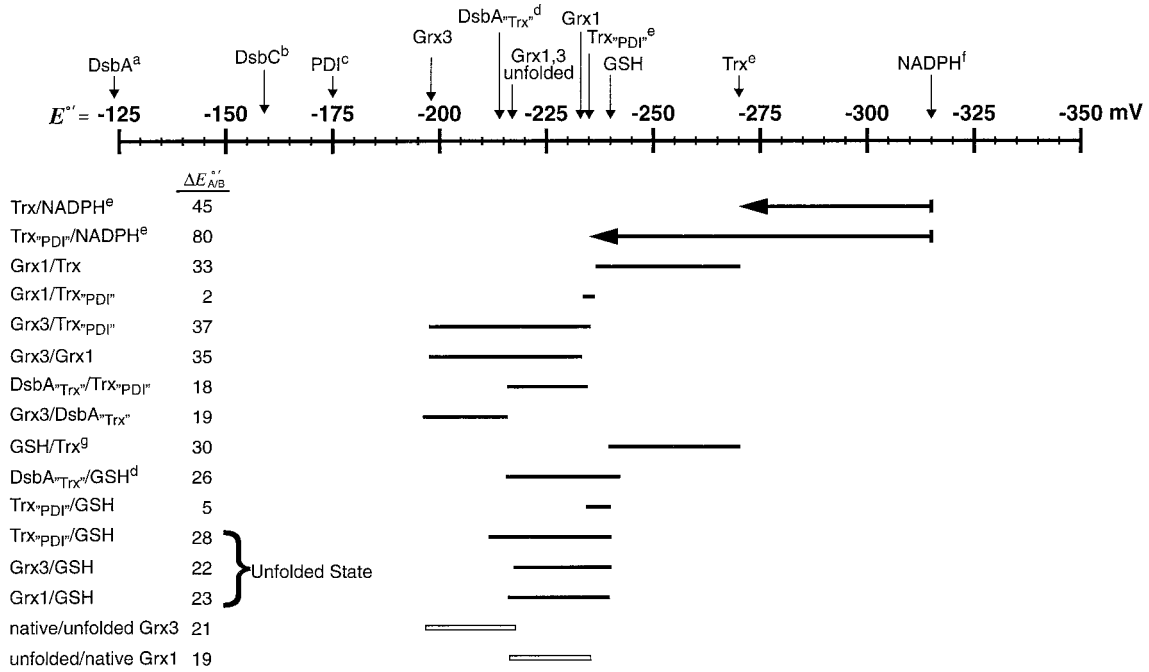

FIG. 3. **Overall scheme of standard state redox potentials of the thioredoxin family of thiol-disulfide oxidoreductases.** Differences derived from the determinations of equilibrium constants between pairs of redox active proteins in this study are displayed as *black bars*. The differences in redox potential between the various proteins was calculated using the Nernst equation (Eq. 3). *Open bars* denote differences in redox potential between the folded state and the unfolded state, calculated from thermodynamic data (see text for details). *Arrows* denote redox potential differences determined by NADPH/$NADP^+$ in the presence of thioredoxin reductase (7). [a], Ref. 42 and footnote 2; [b], Ref. 31; [c], Ref. 20; [d], see footnote 2; [e], Ref. 7; [f], Ref. 21; [g], Ref. 28.

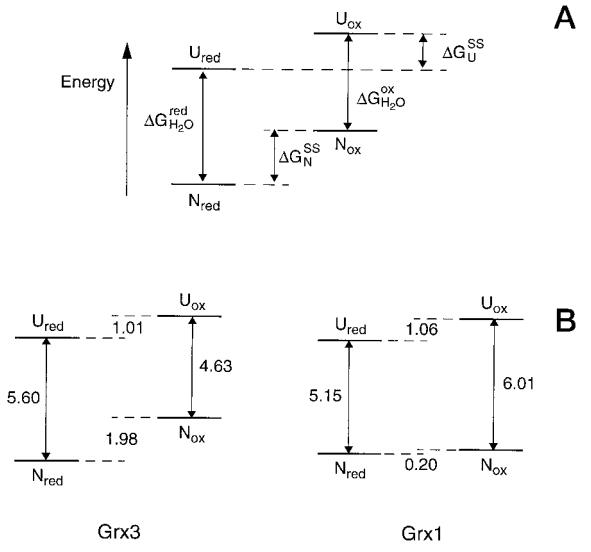

FIG. 4. **Plot of the relative Gibbs energies for native and unfolded states of the oxidized (N_{ox}, U_{ox}), and reduced (N_{red}, U_{red}) forms of a protein as given by the thermodynamic linkage relationship.** The values of the conformational stabilities, $\Delta G_{H_2O}^{ox}$ and $\Delta G_{H_2O}^{red}$, are determined from GdnHCl unfolding of oxidized and reduced protein, respectively (Fig. 5). ΔG_U^{SS} is determined from equilibration with GSH/GSSG-defined redox buffers in the presence of 6 M GdnHCl as in Fig. 3*A*, and ΔG_N^{SS} is determined using the linkage relationship. Alignment of the relative Gibbs energies of Grx3 and Grx1 is arbitrary.

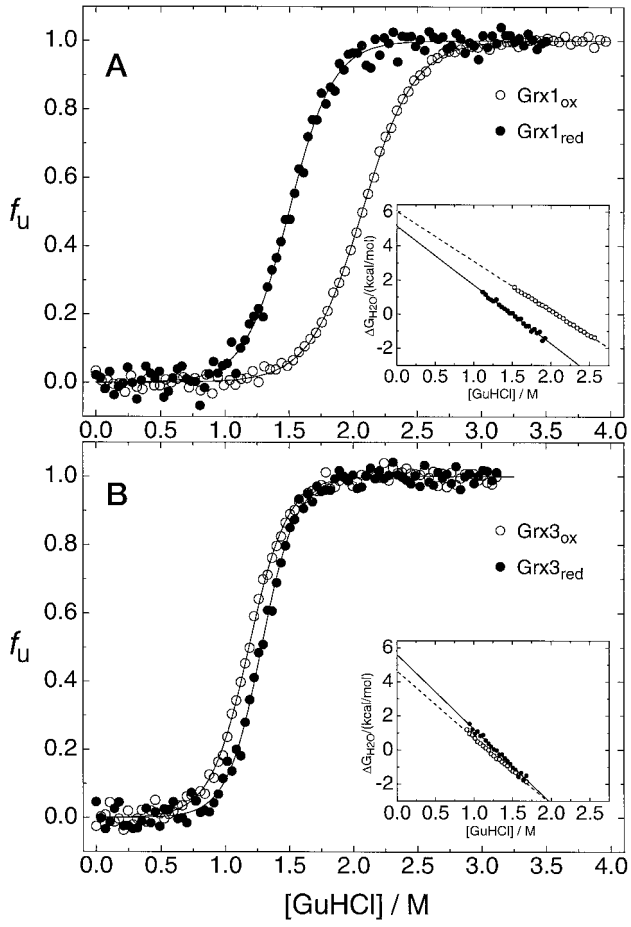

FIG. 5. **GdnHCl unfolding of Grx1 (*A*) and Grx3 (*B*) monitored by CD spectroscopy at 222 nm of the reduced (●) and oxidized (○) forms of the proteins.** The parameters were obtained from the molar ellipticities by nonlinear regression using Eq. 8. For graphical comparison, the raw CD signal was converted into fraction unfolded (f_u) using the fitted values. The *insets* show the respective proteins in the transition regions. The fitted curves in *A* (*solid lines*) and fitted lines in *B* (*solid* and *dashed lines* for oxidized and reduced forms of the proteins, respectively, were calculated using the values obtained from the nonlinear regression analysis performed before conversion to f_u.

reduced ($\Delta G_{H_2O}^{red}$) forms. One way to estimate ΔG_U^{SS} is to assume that the only contribution comes from the difference in entropies of the unfolded state due to disulfide bond formation. This has been estimated to be between 1.8 and 2.6 kcal/mol at 298 K (29, 30). It was demonstrated using a model dithiol peptide that the equilibrium constant with GSH/GSSG (Eq. 5) does not change in the presence of increasing concentrations of urea (from 0 to 7 M) (27). This allows for the determination of the stability of the disulfide bond in the unfolded state experimentally by equilibration of a given protein in defined GSH/GSSG redox buffers in the presence of a denaturant, *e.g.* 6 M GdnHCl. Under such denaturing conditions, the GSH-mixed disulfide forms of Grx1 and Grx3 were insignificant (Fig. 1*B*). Using $E_{GSH}^{\circ\prime} = -240$ mV, we found unfolded Grx1 and Grx3 to have nearly identical redox potentials ($E^{\circ\prime} = -217$ and -218 mV, respectively), whereas unfolded Trx_{PDI} yielded $E^{\circ\prime} = -212$ mV (Fig. 1*B*). These numbers are remarkably similar to the redox potentials reported for the denatured state of DsbA, DsbC, and for the individual Trx-like domains of PDI (9, 14, 31). This indicates that the range of redox potentials for the unfolded state is as restricted as found using model peptides resembling the active-site region of members of the thioredoxin superfamily (11).

The conformational stabilities, $G_{H_2O}^{ox}$ and $G_{H_2O}^{red}$, of Grx1 and Grx3 in oxidized and reduced forms were ascertained by denaturation experiments using GdnHCl and monitored by CD spectroscopy at 222 nm (Fig. 5, Table I). Both the oxidized and reduced forms of each protein were shown to undergo a reversible two-state denaturation as shown by the recovery of the expected molar ellipticity (222 nm) after dilution of samples dissolved in 6 M GdnHCl into 100 mM potassium phosphate, pH 7.0, 1 mM EDTA. The reduced form of Grx3 proved to be more resistant to GdnHCl denaturation than the oxidized form (0.97 kcal/mol), whereas the opposite was found for Grx1, where the oxidized form was more stable (0.86 kcal/mol) than the reduced form (Fig. 5, Table I). This difference (1.76 kcal/mol) corresponds to $\Delta E^{\circ\prime} = 38$ mV (Eq. 3). Using the thermodynamic linkage, the redox potentials of the folded states of Grx1 and Grx3 were determined to be $E^{\circ\prime} = -236$ and -197 mV, respectively, which is in excellent agreement with the values of $E^{\circ\prime} = -233$ and -198 mV obtained by the direct equilibration method.

TABLE I
Gibbs energy changes for unfolding of Grx1 and Grx3

Results of nonlinear least squares fitting of data to Equation 8 with standard error are shown.

Protein	$[D]_m$ [a]	m_G (M GdnHCl)$^{-1}$	$\Delta G_{H_2O}^{\circ\prime}$	$\Delta\Delta G_{H_2O}^{\circ\prime}$ [b]
	M GdnHCl	*cal mol*$^{-1}$	*cal mol*$^{-1}$	*cal mol*$^{-1}$
$Grx1_{red}$	1.50 ± 0.01	−3441 ± 130	−5153 ± 186	−858
$Grx1_{ox}$	2.07 ± 0.01	−2906 ± 44	−6011 ± 88	
$Grx3_{red}$	1.28 ± 0.01	−4364 ± 194	−5597 ± 259	+969
$Grx3_{ox}$	1.18 ± 0.01	−3908 ± 132	−4628 ± 169	

[a] Midpoint of the GdnHCl denaturation, $[D]_m = \Delta G_{H_2O}^{\circ\prime}/m_G$.
[b] $\Delta\Delta G_{H_2O}^{\circ\prime} = \Delta G_{H_2O}^{ox} - \Delta G_{H_2O}^{red}$.

It should be noted that our data clearly demonstrate the greater stability of the oxidized form of Grx1 compared with the reduced form (Fig. 5, Table I). This is in contrast to the data for Grx1 reported by Sandberg *et al.* (32) where the opposite conclusion was reached using significantly fewer titration points. Additionally, we have found that in buffers of low ionic strength such as those used in their study, Grx1 readily dimerizes, whereas under the conditions used here the protein re-

mained monomeric (data not shown). Grx1 dimerization has been subsequently confirmed in another study (33). In addition to the data presented here, we have verified our observations using urea (data not shown) and various titration procedures (manual and automatic) and are therefore confident about the accuracy of the data presented here.

DISCUSSION

The redox potentials (summarized in Fig. 3) among members of the thioredoxin superfamily of thiol-disulfide oxidoreductases range from $E^{\circ\prime} = -124$ mV for *E. coli* DsbA (9, 10) to −270 mV for *E. coli* Trx (6). Since members of the thioredoxin family share a common three-dimensional fold, the source of these differences in reducing/oxidizing capacity is of considerable interest. The importance of the two residues between the active-site cysteines has been demonstrated for T4 glutatredoxin (6), *E. coli* Trx (7), and DsbA (9). In the case of DsbA, a linear correlation between redox potential and the pK_a value of the nucleophilic thiol of the active site has been demonstrated (7). Apparently, a major function of the active site motif (CX_1X_2C) is to modulate the pK_a value of the nucleophilic thiol and thereby the stability of the reduced form of the protein relative to the oxidized form. Thus, in the case of DsbA, the very low pK_a value of 3.5 (36) is an important factor for its highly oxidizing properties. The role of the N-terminal active-site thiol as a leaving group in mixed disulfides of human glutaredoxin (thioltransferase) has been recently reported (37), and in this case, the rate of dethiolation of GSH-mixed disulfide substrates was higher than predicted by the pK_a = 3.5 of the active-site thiol.

The method of pair-wise equilibration used here for obtaining $\Delta E^{\circ\prime}$ is based on accurate determinations of the equilibrium constant, K'_{12} (Eq. 2), for the reversible thiol-disulfide exchange reaction between various pairs of redox active proteins. Standard state redox potentials are then obtained through equilibration with known standards, *e.g.* either Trx_{PDI} or Trx, whose redox potential has been determined independently (7) via coupling to NADPH ($E^{\circ\prime} = -315$ mV). Using direct protein-protein equilibration, we were able to determine an $E^{\circ\prime}$ value of −233 for Grx1 and an $E^{\circ\prime}$ of −198 mV for Grx3. Using the thermodynamic linkage relationship (Fig. 4), we obtained values for the redox potentials independently. This analysis requires an accurate determination of the redox potential of the unfolded state of the protein, $E^{\circ\prime}_U$, Fig. 1*B*. It is interesting that whereas the Gibbs energy of active-site disulfide bond formation in the unfolded forms of Grx1 and Grx3, G^{SS}_U, is essentially equivalent (G^{SS}_U = 1.1 and 1.0 kcal/mol) (the value for Trx_{PDI} is slightly higher (G^{SS}_U = 1.3 kcal/mol)), each is smaller than the computed values G^{SS}_U = 1.9 and 2.6 kcal/mol (29, 30), assuming only an entropic contribution to the Gibbs energy of disulfide bond ring closure at 298 K. Thus for these proteins, either the estimated entropic contribution is inaccurate or there is a substantial enthalpic contribution. The experimental value of G^{SS}_U = 2.3 kcal/mol obtained for Trx (28) is in closer agreement with these estimates. In this perspective, it is noteworthy that a Trx-like model peptide was found to be less reducing, $E^{\circ\prime}_U$ = −190 mV, than those resembling Grx1, PDI, or thioredoxin reductase (11).

To this end one is compelled to interpret the slopes (m_G) of the plots of $E^{\circ}_{H_2O}$ *versus* [GdnHCl] (Fig. 5, Table I). The magnitude of m_G has been shown to correlate with changes in solvent-accessible surface exposed during the folding/unfolding transition (38). In both Grx1 and Grx3, the magnitude of m_G for the reduced form is greater than for the oxidized form. Assuming an approximately constant solvent-accessible surface for the folded protein regardless of redox state, this suggests that the unfolded, oxidized form of the protein retains some residual structure. Denaturant m_G values obtained from other oxidoreductases with the active-site sequence CX_1X_2C and mutants thereof also follow this trend with very few exceptions (9, 39, 40). Perhaps the inability of the oxidized form to fully unfold may be a factor in determining the redox potential of these proteins.

The method of determining redox potentials employed in this paper is straightforward and allows quantification of all species present in a thiol-disulfide interchange reaction in a single HPLC run. Compared with the GSH/GSSG redox buffer method, this procedure offers several advantages. The relationship between the equilibrium constant and the square of the GSH concentration (Eq. 2) will serve to magnify any error present in the quantification of GSH. Difficulties in determining the equilibrium constants for DsbA (8) and PDI (15) have been shown to be the result of incomplete quenching of the thiol-disulfide exchange reaction (14). This problem must necessarily be more pronounced in the presence of millimolar concentrations of GSH/GSSG than in this new direct protein-protein method, which may be performed at micromolar concentration of only oxidized and reduced proteins. For members of the thioredoxin superfamily, it is perhaps not surprising that equilibrium was readily achieved, considering that these proteins have an exposed active-site nucleophilic thiol (34, 35). When extending this new method to include other thiol-disulfide oxidoreductases, it may be necessary to employ longer equilibrium times. The redox potential difference between two proteins that have not been assessed directly can also be obtained if equilibrium constants between each protein and a third common protein have been determined. This may help extend the technique to include pairs of proteins that have prohibitively slow kinetics.

A significant error in $E^{\circ\prime}$ may accumulate as one moves from protein to protein, further away from the independently determined standard state references such as NADPH. With the formation of a more extended network of redox potential differences and inclusion of thermodynamic data, such errors should be minimized. We have found that using equimolar amounts of the equilibrating proteins, redox potential differences of up to 40 mV can be determined readily, provided all species can be quantified. To allow for the determination of larger differences in redox potential, non-stoichiometric ratios of the proteins can be used. It should also be possible to obtain a reliable estimate of the redox potential difference between two proteins even if the HPLC separation fails to resolve the oxidized and reduced forms of one of the two proteins, provided that accurate determination of the initial protein concentrations and extinction coefficients is employed.

With the addition of the well determined redox potentials for Grx1 and Grx3 as well as a DsbA active-site mutant presented in this work, an initial framework of reference proteins with known redox potentials has been established that should prove as useful references for the determination of the redox potentials of other proteins. A further consequence of the proposed map of differences in redox potentials (Fig. 3) is support for $E^{\circ\prime}_{GSH} = -240$ mV for the 1 M standard state of GSH. This value of −240 mV is also supported by the equilibrium constant (also referred to as K_{ox}) of 10 M (corresponding to $\Delta E^{\circ\prime} = 30$ mV) that has been determined experimentally for the equilibration between Trx (−270 mV) and GSH (28). This value is in contrast to the often cited K_{ox} of 2 M (calculated using $E^{\circ\prime}_{GSH} = -260$ mV; Ref. 13), which is without experimental support in the literature. One consequence of the difference in redox potential is that from a thermodynamic point of view, Grx1 is a 15-times better disulfide reductant than Grx3. This may help to explain why Grx3 has only 5–8% activity of Grx1 as a hydrogen donor

for ribonucleotide reductase (3, 4). The redox potential difference between Grx1 and Grx3 may also explain the better ability of Grx1 to reduce all protein disulfide substrates tested. However, Grx1 is a better reductant of ribonucleotide reductase (3, 41) than Trx (lower K_m value) despite the lower redox potential of the latter. Other factors like structural details in the protein-protein interaction surfaces will also contribute to the kinetics of the reaction in matching the initial noncovalent complex postulated in thiol-disulfide exchange reactions (34). Obviously, biochemical characterizations of these proteins by determining both equilibrium constants and kinetic rates will remain a necessity, and this new method should prove useful in such studies.

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Redox Potentials of Glutaredoxins and Other Thiol-Disulfide Oxidoreductases of the Thioredoxin Superfamily Determined by Direct Protein-Protein Redox Equilibria

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