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Redox Dependent Dynamics of a Dual Thioredoxin-Fold Protein: Evolution of Specialized Folds

Andrea Hall[‡], Derek Parsonage[¶], David Horita[¶], P. Andrew Karplus[‡], Leslie B. Poole[¶], and Elisar Barbar^{‡,*}

[‡]Department of Biochemistry and Biophysics, Oregon State University, Corvallis, OR 97331

[¶]Department of Biochemistry, Wake Forest University School of Medicine, Winston-Salem, NC 27157

Abstract

An enzyme system protecting bacteria from oxidative stress includes the flavoprotein AhpF and the peroxiredoxin AhpC. The N-terminal domain of AhpF (NTD), with two fused thioredoxin (Trx) folds, belongs to the hyperthermophilic protein disulfide oxidoreductase family. The NTD is distinct in that it contains a redox active *a* fold with a CxxC sequence and a redox inactive *b* fold that has lost the CxxC motif. Here we characterize the stability, the ¹⁵N backbone relaxation, and the hydrogen deuterium exchange properties of reduced (NTD-(SH)₂) and oxidized (NTD-S₂) NTD from *Salmonella typhimurium*. While both NTD-(SH)₂ and NTD-S₂ show similar equilibrium unfolding transitions and order parameters, R_{ex} relaxation terms are quite distinct with considerably more intermediate timescale motions in NTD-S₂. Hydrogen exchange protection factors show that the slow exchanging core corresponds to residues in the *b* fold in both NTD-(SH)₂ and NTD-S₂. Interestingly, folded state dynamic fluctuations in the catalytic *a* fold are significantly increased for residues in NTD-S₂ compared to NTD-(SH)₂. Taken together, these data demonstrate that oxidation of the active site disulfide does not significantly increase stability but results in a dramatic increase in conformational heterogeneity in residues primarily in the redox active *a* fold. Differences in dynamics between the two folds of the NTD suggest that each evolved a specialized function which, in the *a* fold, couples redox state to internal motions which may enhance catalysis and specificity, and in the *b* fold, provides a redox insensitive stable core.

Keywords

backbone dynamics; N-terminal domain; AhpF; thioredoxin; disulfide; peroxiredoxin; alkyl hydroperoxide reductase; protein disulfide oxidoreductase; NMR; slow exchanging core

The N-terminal domain of the flavoprotein AhpF (NTD) is composed of two fused and intimately interacting thioredoxin (Trx) folds. The NTD is part of a system that provides protection from oxidative stress in bacteria (1-3) by catalyzing the reduction of hydrogen peroxide and organic hydroperoxides to their corresponding alcohol and water. Two enzymes are required: AhpC, a bacterial peroxiredoxin (Prx) (4), and AhpF, a flavoenzyme disulfide

Corresponding author: Elisar Barbar, Department of Biochemistry and Biophysics, Oregon State University, Corvallis, OR 97331. Tel: 541-737-4143, Fax: 541-737-0481 barbar@science.oregonstate.edu.

SUPPORTING INFORMATION AVAILABLE H/D exchange rates and protection factors, size exclusion chromatograms, ¹H-¹⁵N HSQC spectra, ¹⁵N backbone relaxation data and model free analysis results are available as additional information. This material is available free of charge via the internet at <http://pubs.acs.org>.

reductase. In addition to the NTD, AhpF contains a thioredoxin reductase (TrxR)-like domain (3,5,6) (Figure 1).

The NTD acts as an appended substrate for the TrxR-like portion of AhpF (6-8) and is a highly specific and effective reductant of AhpC (3). A single redox-active disulfide (-Cys₁₂₉-His-Asn-Cys₁₃₂-), characteristic of Trx-like proteins, is located in the C-terminal Trx fold and provides redox activity while the N-terminal Trx fold has no such motif and is thus inactive in redox reactions (Figure 2). Using nomenclature for Trx-like domains in other proteins (9,10), we refer to the redox active C-terminal fold as the **a** fold and the redox inactive N-terminal fold as the **b** fold. In addition to the CxxC motif, the active site of the **a** fold makes use of a conserved glutamic acid from the **b** fold that reaches across the interface of the two folds. Sequence and structural comparisons indicate that the NTD is a subgroup of the protein disulfide oxidoreductase (PDO) family of proteins important for redox chemistry and isomerization in Archaea (11). A PDO-like protein present in hyperthermophilic organisms is thought to represent an intermediate in the evolution of the NTD (5,12) (Figure 2C).

A construct of the NTD (residues 1 through 202 of AhpF) retains the function (7) and structure (13) of the NTD in the full AhpF (residues 1 through 521) and provides an excellent model for high resolution structure/function characterization of a redox active protein in solution. To determine if and how the two Trx folds of the NTD function as a single unit and to gain insight into changes that occur with oxidation in this class of Trx-related proteins, we have analyzed the dynamics and stability of the reduced (NTD-(SH)₂) and oxidized (NTD-S₂) NTD from *Salmonella typhimurium*. Our results indicate that the two Trx folds behave as a single cooperative folding unit, but have distinct dynamic properties not detected in crystal structures. Each fold has a specialized function such that the **b** fold provides protein stability in both redox states while the **a** fold is more dynamic and shows redox state-specific changes in dynamics; upon oxidation, residues in the **a** fold show increased internal flexibility and conformational heterogeneity. From these analyses, we propose that protein dynamics are adaptable features in the evolutionary divergence of the broader Trx-like family.

MATERIALS AND METHODS

Protein Preparation

All buffers and stock solutions were freshly prepared. Unlabeled NTD was expressed and purified as previously described (7). Uniformly ¹⁵N-labeled NTD was expressed from pAF [1-202] (7) in *Escherichia coli* strain BL21*DE3 using N-5052 medium (14) containing 2.5 g/L ¹⁵NH₄Cl. ¹⁵N-¹³C-labeled NTD was purified from cells grown in M9 minimal medium containing 1 g ¹⁵NH₄Cl and 2 g ¹³C-U-glucose per liter. After purification on a Superose 12 column, all concentrated samples were further purified on a Superdex 75 column (GE Healthcare) with a running buffer of 50 mM sodium phosphate, 200 mM sodium sulfate and 1 mM sodium azide (pH 7.3). The purified protein was exchanged into 50 mM potassium phosphate buffer (pH 6.5), with 50 mM potassium chloride. For NTD-(SH)₂, 10-fold excess DTT was added during buffer exchange. Protein concentration was determined from absorbance at 280 nm using an extinction coefficient of 15100 M⁻¹ cm⁻¹ (6).

Size Exclusion Chromatography

NTD-S₂ and NTD-(SH)₂ were run on a Superdex 75 HR 10/30 size-exclusion column (flow rate 0.5 mL/min) as described above with a 5 - 20 µL injection of a 0.8 mM protein solution. For NTD-(SH)₂, 5 mM βME was added to the running buffer. Proteins were detected by absorbance at 280 nm, refractive index and multiangle light scattering (miniDawn, Wyatt).

Unfolding Measured by Intrinsic Fluorescence and Circular Dichroism

Urea stock solutions for use in denaturation unfolding studies were prepared as described elsewhere (15) using 50 mM potassium phosphate buffer (pH 6.5) with 50 mM potassium chloride. Intrinsic fluorescence emission spectra of the single tryptophan in the NTD were acquired on a Jobin Yvon/Spex spectrofluorometer. The excitation wavelength was set to 295 nm and fluorescence emission spectra were scanned from 310 to 380 nm. A sample cell of 0.5 cm and slit widths of 2 and 4 nm or 2 and 2 nm were used for data collection. To limit the impact of photobleaching, only one fluorescence measurement was recorded on each sample. Two sets of samples were prepared with urea concentrations ranging from 0 to 7.8 M in 50 mM potassium phosphate buffer (pH 6.5), with 50 mM potassium chloride: the first contained 2 μ M protein (200 μ M DTT added for NTD-(SH)₂) and the second contained 20 μ M protein (2 mM DTT added for NTD-(SH)₂). Blanks were prepared for each data point and the fluorescence of the blank was subtracted from the fluorescence of the protein sample.

Circular dichroism (CD) experiments were conducted on a Jasco J-720 spectropolarimeter. Experiments were recorded in a 0.1 cm sample cell and spectra were scanned from 200 to 250 nm. After fluorescence data collection, the same 20 μ M protein samples (2 mM DTT added for NTD-(SH)₂) were used for CD experiments. Additional samples prepared with 20 μ M protein and 200 μ M DTT for NTD-(SH)₂ were also measured. For all unfolding experiments, samples were equilibrated for 12 h at 4 °C before data collection at 25 °C.

The resulting transition curves were analyzed by nonlinear least squares curve fitting using a two-state model for unfolding following methods developed by Bolen and Santoro (16,17) and described in Hall *et al.* (18). Individual and global fits to the data were calculated. Data were normalized to set the population of folded protein at 0 M urea equal to 1.

Attempts to perform thermal unfolding experiments were not successful due to decreased protein solubility at elevated temperature.

NMR Spectroscopy

NMR spectra were collected on a 600 MHz Bruker DRX spectrometer at 298 K. Samples were prepared with a protein concentration of 1 mM in 50 mM potassium phosphate buffer (pH 6.5) with 50 mM potassium chloride, 10% (v/v) D₂O, and 1 mM DSS, maleic acid, sodium azide and protease inhibitor (Roche). For NTD-(SH)₂ samples, 10 mM DTT was added.

¹H-¹⁵N HSQC experiments were recorded using Echo/Antiecho-TPPI gradient selection. Backbone assignments of NTD-S₂ were obtained using standard 3D triple resonance CBCACONH and HNCA experiments collected on ¹⁵N-¹³C-labeled NTD. Backbone resonance assignments for NTD-(SH)₂ have previously been determined (19). Backbone chemical shift assignments for NTD-S₂ have been deposited in the BioMagResBank with accession number 16265.

Backbone Relaxation Measured by NMR

Backbone amide relaxation parameters (¹⁵N R₁, R₂ and steady-state ¹H-¹⁵N heteronuclear NOE) were measured using pulse sequences described by Farrow *et al.* (20). The R₁ experiments were recorded with relaxation delay times of 50 (2x), 100 (2x), 250, 500, 1000, 2000 and 3000 ms, and the R₂ relaxation data were acquired using relaxation delays of 20, 35, 48 (2x), 65, 80, 100 ms. Duplicate measurements (marked with “2x”) were used to verify experimental error. Steady-state heteronuclear NOE experiments were recorded in the presence and absence of amide proton saturation. Spectra with proton saturation utilized a 3 s period of saturation and an additional delay of 1.5 s.

NMR Data Analysis

NMR spectra were processed using NMRPipe (21) and analyzed with Burrow Owl (19). The change in ^1H - ^{15}N HSQC chemical shifts (Δ N-H) between NTD-S₂ and NTD-(SH)₂ was determined using the equation $\Delta\text{N-H} = [(\Delta^1\text{H})^2 + (\Delta^{15}\text{N})^2]^{1/2}$ after multiplying the ^1H chemical shift by 6.2 (fractional difference in ^{15}N : ^1H spectral widths) to eliminate ^{15}N chemical shift bias (22).

For all dynamics experiments, peak intensities were measured as peak height at the highest point and the error associated with peak height measurement was taken to be the baseline noise. R_1 and R_2 values were determined by fitting the measured peak heights versus time plots to the relationship $I = I_0 e^{-\text{Rate} \cdot t}$, where t is the relaxation delay, I is the intensity of the peak at time t and I_0 is the initial peak intensity. Curve fitting was performed using the program Curvefit (<http://cpmcnet.columbia.edu/dept/gsas/biochem/labs/palmer/software/curvefit.html>) and visualized using Grace (<http://plasma-gate.weizmann.ac.il/Grace/>).

Steady state NOE values were obtained from the ratio of peak intensities in the presence and absence of amide proton saturation. The error associated with the NOE value was calculated from the equation $\sigma/\text{NOE} = [(\sigma I_A/I_A)^2 + (\sigma I_B/I_B)^2]^{1/2}$, where I and σI denote the intensity of the peak and its baseline noise, and the subscripts A and B denote spectra recorded in the presence and absence of proton saturation, respectively (23).

Lipari-Szabo Analysis of Relaxation Data

Backbone amide relaxation parameters were analyzed with the extended Lipari-Szabo formalism (24-27) using the program TENSOR2 (28) to assess global tumbling and internal motions. The ^{15}N CSA was set to -170 ppm and an N-H bond length of 1.02 Å was used. For each data set, a global tumbling correlation time (τ_c) was calculated from the residues assumed to have a negligible exchange contribution to ^{15}N relaxation determined using the method described by Tjandra *et al.* (29) and verified using the more stringent method described by Pawley *et al.* (30).

Internal motions were determined using Monte Carlo sampling methods and F-tests validation incorporated in TENSOR2 (28). Five standard models were used to describe internal mobility with motion complexity increasing with model number (24,27). A model for the internal motions was rejected if the experimental χ^2 value was higher than the simulated χ^2 value at the 90% confidence limit. Residues that were not adequately fit by any of the five models for motion were omitted from further analysis. For anisotropic analyses, pdb codes 1ZYP and 1ZYN were used for NTD-(SH)₂ and NTD-S₂, respectively (13).

Hydrogen-Deuterium Exchange

Samples were prepared for NMR as described above but with a buffer pH of 6.2, flash-frozen and lyophilized. Hydrogen-deuterium (H/D) exchange was initiated by dissolving the lyophilized sample in 100% $^2\text{H}_2\text{O}$. The pH of the samples was verified to be 6.2 both before and after the experiment. The deadtime of the experiment, defined as time between first exposure to $^2\text{H}_2\text{O}$ and the middle of the first HSQC experiment, was 27 min for NTD-S₂ and 33 min for NTD-(SH)₂. ^1H - ^{15}N HSQC spectra were collected continuously for the first 18 hours. Data were collected on the NTD-S₂ sample for only an additional four days due to protein precipitation. Data collection continued on the NTD-(SH)₂ sample once every two days for 26 days to monitor the disappearance of peaks. No precipitation was observed for the NTD-(SH)₂ sample during this interval. The H/D exchange rates were determined using the equation $I = I_0 e^{-\text{Rate} \cdot t} + \Omega$ where t is the exchange time, I is the intensity of the peak at time t , I_0 is the initial peak intensity and Ω is the extrapolated offset value from zero intensity caused by residual hydrogen present in the sample. Curve fitting was done as previously described for

R₁ and R₂ relaxation rate determination. Exchange rates were estimated for residues where peak intensity loss could not be fit to an exponential decay. A minimum exchange rate for peaks not detected at the first data point was estimated assuming $I/I_0 = 0.1$, and a maximum exchange rate for peaks present at the last time point of the reduced data set (and unable to be exponentially fit) was estimated assuming $I/I_0 = 0.9$. Protection factors were determined using the program Sphere (31,32). The estimated stability of the protein based on H/D exchange rates was calculated using

$$\Delta G^\circ_{\text{HX}} = -RT \ln(k_{\text{ex}}/k_{\text{int}}) \quad (1)$$

where $\Delta G^\circ_{\text{HX}}$ is the free energy of unfolding and k_{ex} and k_{int} are the observed (estimated) and intrinsic exchange rates for the slowest exchanging residues (33).

Molecular graphics were created using pymol (34) and figures were prepared using Gimp.

RESULTS

Size Exclusion Chromatography

Size exclusion chromatography confirms that at the concentrations used for NMR, both NTD-(SH)₂ and NTD-S₂ are monomeric with similar molecular shapes (Supplemental Figure 1). Molecular weight determined from multiangle light scattering is $22,620 \pm 450$ Da, consistent with the 22,292 Da calculated mass for a monomeric protein.

NTD-(SH)₂ and NTD-S₂ Chemical Shifts Comparison

Backbone amide resonance assignments for NTD-(SH)₂ were completed for 191 of the 192 non-proline residues (19). Here, the equivalent assignments for NTD-S₂ were completed for 188 residues; resonances were missing for residues 28, 85 and 134 and were weaker for residues 128 and 129. Comparison of the ¹⁵N and ¹H chemical shifts between NTD-(SH)₂ and NTD-S₂ shows that the residues with the largest changes cluster around the active site; chemical shift differences greater than 0.1 ppm occur for residues 86-89, 124-129, 136, 137, 171-173, 184, 185 (Supplemental Figure 2). Residues with signal loss in NTD-S₂ are also localized near the active site suggesting their intensity loss reflects changes in active site dynamics due to exchange broadening.

Unfolding Studies

Protein stabilities were determined by monitoring changes in both fluorescence emission and CD signal with increasing urea concentration. The NTD's single tryptophan residue, Trp96, is partly buried at the interface of the two Trx folds (Figure 2A); atoms Nε1, Cζ2 and Cη2 are ~50% accessible (35). Consistent with the partial solvent exposure of Trp96, its fluorescence maximum occurs at 347 nm in the folded state (compared to ~327 nm for a fully buried tryptophan) and shifts to 357 nm in the unfolded protein. Initial fluorescence intensity is the same for NTD-(SH)₂ and NTD-S₂, consistent with earlier results (36).

Urea-induced denaturation at pH 6.5 yields similar profiles for NTD-(SH)₂ and NTD-S₂ with an apparent single-step transition irrespective of the spectral probe used to follow unfolding (Figure 3). Each of the four curves was individually fit to a two-state unfolding model. The extrapolated $\Delta G^\circ_{\text{H}_2\text{O}}$ value for NTD-(SH)₂ unfolding is 6.5 kcal/mol as measured by either CD or fluorescence. Similarly, the extrapolated $\Delta G^\circ_{\text{H}_2\text{O}}$ value for NTD-S₂ unfolding is 6.6 kcal/mol as measured by either technique. Despite the equivalence of $\Delta G^\circ_{\text{H}_2\text{O}}$ values determined from the unfolding transition monitored by the two techniques, the curves are clearly not superimposable, and the calculated urea concentration for the midpoint of unfolding

is 0.3 M higher when measured by CD (Table 1). This difference necessitates a closer examination of the two-state unfolding assumption.

In the standard two-state model of protein unfolding, a global (i.e. simultaneous) fit of both fluorescence and CD data should give parameters that fit well to both of the individual curves (37). Simultaneous fits of the transition curves of NTD-(SH)₂ and NTD-S₂ give midpoints of unfolding of 3.9 and 4.1 M urea. The respective extrapolated $\Delta G^{\circ}_{\text{H}_2\text{O}}$ values for unfolding are 6.5 and 6.6 kcal/mol. The globally determined parameters fit the data well (Figure 3, Table 1). As no apparent intermediate was detected in the unfolding data, a three-state unfolding model was not pursued.

Unfolding of the NTD-(SH)₂ in the presence of 10 and 100-fold DTT (0.2 and 2 mM DTT) gave similar results when monitored by CD (data not shown).

¹⁵N Backbone Relaxation Dynamics

Backbone relaxation dynamics were measured with R_1 , R_2 and steady-state heteronuclear NOEs for 149 and 146 residues in NTD-(SH)₂ and NTD-S₂, respectively. Excluded residues correspond to overlapping peaks or to those that are too weak for accurate measurements. The relaxation rates along the backbone of NTD-(SH)₂ and NTD-S₂ show similar trends including a disordering of the C-terminus (Supplemental Figure 3). Overall, NTD-(SH)₂ and NTD-S₂ have comparable R_1 and NOE values, but NTD-S₂ has notably higher R_2 values (Figure 4, Table 2).

Model free analysis of backbone relaxation data was carried out using the axially symmetric anisotropic diffusion model. Global tumbling correlation times of 13.08 ± 0.01 ns and 13.63 ± 0.01 ns were calculated for NTD-(SH)₂ and NTD-S₂ using the filter described by Tjandra *et al.* (29). For internal motions (Supplemental Figure 4), the majority of residues were fit by models 1 and 3 in NTD-(SH)₂ and model 4 in NTD-S₂. Despite the differences in model type, the S^2 terms are similar across the backbone for both redox states (Figure 4, Table 2) with an average of 0.89 ± 0.01 for NTD-(SH)₂ and 0.94 ± 0.02 for NTD-S₂ which indicate that overall, both redox states are well ordered on the fast timescale. In contrast, a clear difference is seen in the R_{ex} values, with NTD-S₂ having both more residues modeled with R_{ex} terms (79 versus 48) and a higher average value (3.7 ± 0.5 versus 2.2 ± 0.4) (Figure 4, Table 2). As R_{ex} terms measure intermediate chemical exchange, these data suggest more intermediate timescale conformational heterogeneity in NTD-S₂. To verify that the R_{ex} terms described by the model free analysis, especially those for the NTD-S₂, were not due to incorrect global tumbling correlation times, the two-part filter described by Pawley *et al.* (30) was used to determine a more accurate description of the tumbling of the protein. With this method, global correlation times of 13.47 ± 0.01 ns and 14.35 ± 0.03 ns were determined for NTD-(SH)₂ and NTD-S₂. The internal motions determined from the model free analysis using these more stringently determined τ_c values slightly altered the qualitative results for both NTD-(SH)₂ and NTD-S₂ but led to no quantitative changes (data not shown).

H/D Exchange Monitored by NMR

The EX2 exchange limit (38) was determined to be the more likely mechanism for hydrogen exchange in the NTD; plots of k_{ex} versus k_{int} for secondary structure elements in the **a** and **b** folds for both NTD-S₂ and NTD-(SH)₂ give straight lines with slopes ranging from 0.8 to 1.1 (data not shown). This is close to the expected slope of 1 for an EX2 exchange mechanism (39,40). Protection factors were calculated at pH 6.2 and 25 °C for fast, intermediate and the slowest exchanging residues for both NTD-(SH)₂ and NTD-S₂ (Figure 5, Supplemental Table 1). Fast exchanging residues are defined as those fully exchanged at the first time point (~30

min) for which a minimum exchange rate of $\sim 1 \times 10^{-3} \text{ s}^{-1}$ is estimated. The fast exchanging residues are located primarily on the surface of the protein.

For intermediate exchanging residues, rate constants determined by fits of a single exponential to decreasing peak intensity with time range from 1×10^{-3} to $3 \times 10^{-7} \text{ s}^{-1}$. Comparison of protection factors for intermediate exchanging amides reveals that many residues distributed throughout the sequence are ~ 10 -fold more slowly exchanging (more protected) in NTD-(SH)₂. These residues cluster in $\beta 1$, $\alpha 1$, $\beta 2$, $\beta 3$ and $\beta 4$ of the *a* fold and in $\beta 4$ of the *b* fold. Only four amides are > 5 -fold more protected in NTD-S₂ (42, 44, 110 and 190); these are located on the protein surface distant from the active.

The slowest exchanging residues, those with less than 10% intensity loss over the course of the experiment (~ 27 days), were given an estimated upper limit exchange rate constant of $6 \times 10^{-8} \text{ s}^{-1}$. Although the data sets used for NTD-(SH)₂ and NTD-S₂ were collected for different lengths of time, both identify the same group of slowest exchanging residues; all are associated with secondary structure elements $\beta 1$, $\alpha 1$, $\beta 2$, $\beta 3$ and $\alpha 3$ of the *b* fold. Based on the maximal estimated protection factors for NTD-(SH)₂, its apparent stability by hydrogen exchange criteria ($\Delta G^\circ_{\text{HX}}$) is ~ 10 kcal/mol.

DISCUSSION

Oxidation Does Not Significantly Change Global Stability of the NTD

The combined use of CD and fluorescence quenching monitors both secondary and tertiary structural changes that occur with increasing denaturant. While the fluorescence and CD unfolding profiles are not completely overlapping, the finding that global (simultaneous) fits to the urea unfolding data for NTD-(SH)₂ and NTD-S₂ give parameters that agree with those obtained from fits to individual curves (Table 2) and fit both curves well (Figure 3) supports a cooperative two-state unfolding model (37). Very similar $\Delta G^\circ_{\text{H}_2\text{O}}$ values of 6.5 and 6.6 kcal/mol are obtained for NTD-(SH)₂ and NTD-S₂, respectively. The apparent two-state unfolding behavior is consistent with the absence of detectable stable intermediates and suggests that the two folds of the NTD do not significantly separate before the protein unfolds.

The apparent stability obtained by H/D exchange analysis is higher (~ 10 kcal/mol) than that determined by fluorescence and CD unfolding studies, probably due to residual structure in the unfolded state (41,42), however both results indicate that the NTD is quite stable. The extrapolated $\Delta(\Delta G^\circ_{\text{H}_2\text{O}})$ value between NTD-(SH)₂ and NTD-S₂ of 0.1 kcal/mol derived from urea denaturation (Table 1) is our best measure of the increased stability of NTD-S₂. This difference is considerably lower than the 1.2 kcal/mol net stabilization expected solely from disulfide bond oxidation decreasing the conformational freedom of the unfolded state by closing a loop of four residues in length (43). The difference between expected and observed values implies that upon disulfide oxidation, other factors collectively decrease $\Delta(\Delta G^\circ_{\text{H}_2\text{O}})$.

Oxidation Increases Conformational Heterogeneity of the NTD

To probe the effect of disulfide bond oxidation and reduction on the structure and dynamics of residues around the active site and the protein as a whole, we compare chemical shifts, peak broadening, relaxation parameters and hydrogen exchange rates between the two redox forms. ¹H-¹⁵N HSQC spectra of NTD-(SH)₂ and NTD-S₂ show chemical shift changes primarily localized to residues near the active site, and along the active site ends of helices $\alpha 1$ of the *a* fold and $\alpha 3$ of the *b* fold (Supplemental Figure 2). These data suggest that while the overall structure is similar in the two redox forms, there are local structural changes in the active site that may be propagated down the helices containing active site residues. In addition to peak shifts, apparent chemical exchange broadening for active site residues Ser128 and

Cys129 and nearby residues in the NTD-S₂ spectrum indicates conformational heterogeneity on the ms- s timescale that is absent in NTD-(SH)₂. This qualitative measurement of the differences between NTD-(SH)₂ and NTD-S₂ supports the more quantitative model free analyses of internal motions which show that intermediate timescale motions differ significantly in the two redox forms; NTD-S₂ has substantially more residues with motions reflected in a higher R_{ex} term (Figures 4 and 6).

Dynamics differences between NTD-(SH)₂ and NTD-S₂ are strongly evident from H/D exchange results which indicate that amides in NTD-S₂ are faster exchanging (Figure 5, orange and red colors in Figure 7A). The consistent pattern is a ~10-fold (1 log unit) decrease in protection factor for five elements of secondary structure in the catalytic **a** fold and one in the **b** fold (β4 at the interface of the two folds). It is noteworthy that NTD-S₂ shows decreased protection despite being slightly more stable. This implies that the higher exchange rates for intermediate exchanging residues in NTD-S₂ do not arise from exchange processes involving global unfolding, but rather from increased internal fluctuations *within the folded state ensemble* of NTD-S₂ (Figure 8).

What are the possible origins of the increased motions in NTD-S₂? Although chemical exchange broadening, larger R_{ex} values and faster H/D exchange rates indicate increased internal fluctuations in NTD-S₂, the motions responsible for the increases are not necessarily the same. This is apparent as increased values of R_{ex} are observed throughout the **a** and **b** folds of NTD-S₂ and report motions on the ms- μs timescale. However, increased H/D exchange rates are observed primarily for residues in the **a** fold and some residues near Glu86 in the **b** fold. These motions reported by folded state H/D exchange of amide N-H groups, that in the crystal structure are buried and intramolecularly H-bonded, cannot at present be attributed to any specific timescale (42). The fact that buried amides exchange by a folded state mechanism means that the folded protein moves in ways that permits access of buried N-H groups to water. The pattern of increased exchange rates in the **a** fold of NTD-S₂ (Figure 7A) suggests movement of helix α1 relative to the β-sheet it is packed against. Collective motion of the entire helix α1 as in local unfolding or unraveling is unlikely as an inside/outside pattern of slow and fast exchange rates is observed. The data are consistent with changes in numerous, local and small-scale motions in α1 and in strands β1, β2, β3 and β4 of the **a** fold (red and yellow in Figure 7A). In any case, there is little or no change in average secondary structure as indicated by similar CD spectra for NTD-S₂ and NTD-(SH)₂ (data not shown) and published crystal structures (13).

The Two Trx Folds Have Distinct Dynamics Not Detected in Crystal Structures

Protein crystal structures provide both a three dimensional image and an estimate of time-averaged atomic mobility reported as the B-factor (temperature factor). For the NTD, crystal structures show no significant conformational or mobility differences between NTD-(SH)₂ and NTD-S₂ as is expected based on Trx and Trx-like domains in other proteins (44). Also, as measured by B-factors, the **a** and **b** folds have remarkably similar apparent mobility along the chain (Figure 9). In both folds, helix α is highly mobile and in the rest of the fold, core regions with B-factors near 20 - 25 Å² alternate with loop regions having higher B-factors.

In contrast, H/D exchange data convincingly show that the dynamic/thermodynamic behavior of the **a** and **b** folds are quite distinct. Typically the final group of amides to exchange in a protein identify the packed secondary structure that constitutes the core of the protein as these regions are accessible to exchange only when the protein undergoes global unfolding (45). For the NTD, these are all in the **b** fold (blue in Figure 7A). In contrast, corresponding secondary structure elements in the **a** fold are 10 to 100-fold faster exchanging in NTD-(SH)₂ and 100-1000 fold faster exchanging in NTD-S₂ (Figure 5). Relative to the **b** fold, this substantially more rapid exchange in the **a** fold, even in the most protected residues, implies a much greater

folded state mobility in the **a** fold. The dramatic differences between the folds and striking redox sensitive internal fluctuations (discussed above) are likely to be related to the biological role of the NTD in the redox cascade (Figure 1). This is supported by the observations that (1) upon oxidation, the largest changes in both R_{ex} and H/D exchange are clustered around the active site and in the catalytic **a** fold, and (2) that in the **b** fold, strand $\beta 4$ (which packs under the active site), and residues in the vicinity of Glu86, exchange faster in NTD-S₂, along with others in the **a** fold.

The clear divergence of a Trx fold that has very low mobility independent of redox state (the **b** fold) from a Trx fold that displays redox-dependent dynamics (the **a** fold) suggests that the functions of the two folds have specialized. The **b** fold not only contributes key residues to the active site (Glu86) but also provides the core stability of the protein. This might allow the **a** fold, which contains most of the active site structure, to acquire dynamic properties which enhance catalysis.

Similarities and Differences Between the NTD and EcTrx

The overall structure of *Escherichia coli* Trx (EcTrx) determined by NMR (46) or crystallography (47,48) is very similar for the two redox forms with only small conformational changes observed for active site residues. This is consistent with our data for the NTD. Interestingly, there is a significantly larger difference in stability between oxidized and reduced EcTrx (2.4 kcal/mol) (49) compared to the NTD (0.1 kcal/mol).

¹⁵N backbone relaxation analyses of EcTrx (50) show that the overall fast motions (S^2 values) are very similar in both redox states, with three loops (before $\beta 1$, $\alpha 2$ to $\beta 3$, and $\beta 4$ to $\alpha 3$) being particularly dynamic on this timescale and the latter two important to catalysis. In the NTD, there are similar low S^2 values for the $\alpha 2$ to $\beta 3$ loop (and to a lesser extent for the $\beta 4$ to $\alpha 3$ loop) in the **a** fold, but no hint of such dynamics are present in the **b** fold. The conservation of these fast motions in loops that pack on the NTD active site disulfide and not in the equivalent loops of the **b** fold is consistent with their importance in catalysis.

The slow exchanging core in EcTrx (51,52) contains residues mostly from $\beta 1$, $\beta 2$ and $\beta 3$ (Figure 7B). In the NTD, the slowest exchanging residues which include the slow exchanging core, are only present in the **b** fold and are located in strands $\beta 1$, $\beta 2$ and $\beta 3$ as well as helices $\alpha 1$ and $\alpha 3$ (Figures 5 and 7A). The **a** fold shows similar relative features (β -strands being local maxima of protection) but the absolute protection levels are much lower, and all amides in the **a** fold exchange from the folded state with no contribution from global stability.

The most dramatic differences between EcTrx and the NTD are redox associated changes in dynamics. In EcTrx, oxidation does not cause a change in fast motions but decreases conformational exchange for a few active site residues (between $\alpha 2$ and $\beta 3$) (50), and increases protection from H/D exchange specifically for those residues at the active site (51). In stark contrast, oxidation of the NTD is associated with widespread increased conformational exchange across the backbone (Figures 4 and 6), and decreased protection from H/D exchange across the **a** fold (Figures 5 and 7A). A schematic summary of the changes in protein thermodynamics with oxidation is given in Figure 8.

Evolutionary Divergence of the Two Trx Folds

The opposite trends with oxidation observed for EcTrx and the NTD fit the idea of evolutionary divergence and specialization of the two Trx folds. Folding thermodynamics and H/D exchange show that the NTD is not comprised of two independent Trx folds, each retaining the original functional and dynamic properties of Trx. Instead, we see evidence for an evolved merging of two Trx folds into a single cooperative folding unit along with specialization of function of

each Trx fold to produce unique redox functions of a new protein. The **b** fold (all but $\beta 4$ and the residues around Glu86 in $\alpha 3$) provides a largely redox insensitive stable core, and the **a** fold (together with $\beta 4$ from the **b** fold) provides the biological redox activity with dynamic behavior coupled to redox state.

The proposed evolutionary origins for the NTD purports a gene duplication and fusion (53) that took place in hyperthermophilic archaea (12) (Figure 2C). In this context, the covalent linking of the two Trx-like folds could provide a selective advantage as oligomerization is one strategy for stabilization of hyperthermophilic proteins (54-56). We propose that initially, each Trx fold had its own stable core and redox activity. Then, with one “superfluous” active site, what would become the **b** fold diverged to gain stability and lose activity. Subsequent divergence led to further differentiation of the functional roles of the two folds with strand $\beta 4$ of the **b** fold becoming a functional part of the **a** fold. In the evolutionary intermediate PDO, the functions of the two folds already differ although both folds contain a CxxC motif: only the C-terminal CxxC motif (corresponding to the **a** fold of the NTD) is required for oxidoreductase activity and the N-terminal CxxC motif contributes to isomerase activity (11). Following this scenario further, after horizontal gene transfer to bacteria, the two folds were no longer needed for increased stability in a hyperthermic environment, but the requirement for catalysis of Glu86 from the **b** fold necessitated its continued conservation in the NTD. Thus divergence continued with the **b** fold losing the CxxC motif and all catalytic function. Interestingly, this result of a partial physical separation of stability and catalysis may provide a distinct advantage of permitting the protein to evolutionarily explore dynamic properties that enhance catalysis beyond what would be achieved in a single fold.

Additionally, as hyperthermophilic proteins are less dynamic at the lower temperatures supporting growth of mesophilic organisms (57), the separation of stability and dynamics may be a remnant of an hyperthermophilic adaptation - while the **b** fold still reflects the lower internal mobility at mesophilic temperatures, the **a** fold has evolved to be more dynamic at these lesser temperatures. It remains to be seen how the dynamic properties of the **a** fold impact NTD function, but one intriguing possibility is that the high level of order of NTD-(SH)₂ is related to its high specificity for reducing AhpC while the flexibility associated with reduced *Ec*Trx is important for its broad specificity as a protein reductant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

AhpR, alkyl hydroperoxide reductase
 AhpF, alkyl hydroperoxide reductase F component (flavoprotein reductase)
 AhpC, alkyl hydroperoxide reductase C component (peroxidase)
StAhpF, *Salmonella typhimurium* AhpF
StAhpC, *Salmonella typhimurium* AhpC
 NTD, the N-terminal domain (residues 1-202) of *StAhpF*

NTD-S₂, oxidized NTD
 NTD-(SH)₂, reduced NTD
 TrxR, thioredoxin reductase
 Trx, thioredoxin
EcTrx, *Escherichia coli* Trx
 Prx, peroxiredoxin
 HSQC, heteronuclear single quantum correlation
 NOE, nuclear Overhauser effect
 H/D exchange, hydrogen-deuterium exchange
 DTT, 1,4-dithiothreitol
 CD, circular dichroism
 DSS, 2, 2-dimethyl-2-silapentane-5-sulfonate sodium salt
 βME, beta-mercaptoethanol

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Figure 1.

Two pathways for the reduction of peroxides by flavoproteins. A) The bacterial AhpF/AhpC system (also known as the alkyl hydroperoxide reductase system) uses the two enzymes AhpF and AhpC to reduce hydrogen peroxide and organic hydroperoxides using NADH. A ribbon diagram of AhpF (pdb code 1HYU), depicting only one subunit of the dimer, is shown. B) The TrxR/Trx/Prx system is analogous to the AhpF/AhpC system. Ribbon diagrams of TrxR (pdb code 1TDE) and Trx (pdb code 2TRX) are shown. A comparison of the proteins in the two systems reveals that AhpF consists of a fused TrxR-like domain and a Trx-like domain. To highlight this homology, the same coloring scheme is used in both A) and B) with red and blue shades for the FAD binding (FAD) and the NADH binding redox-active disulfide-containing (NADH/SS) domains of the TrxR(-like) protein, and green shades for the Trx(-like) protein/domain. The N-terminal domain in AhpF (NTD) is made up of two interacting Trx folds and acts like an appended substrate for the TrxR-like domain of AhpF (linker region is colored cyan). The bound FAD molecule and redox active disulfides in both A) and B) are shown as ball and stick with disulfide sulfur atoms colored yellow.

Figure 2.

Comparison of the NTD and *Ec*Trx. A) Ribbon diagram of the NTD (pdb code 1ZYN). The two Trx folds are colored in grey (*b* fold) and green (*a* fold). The redox active cysteines (Cys129 and Cys132 with sulfur atoms colored yellow), conserved glutamic acid (Glu86 with oxygen atoms colored red) and fluorescence reporter (Trp96 with side chain colored purple) are shown as ball and stick. Secondary structure elements are labeled; conserved secondary structure elements of the Trx fold are labeled with subscripts. Helix α_2 of the Trx fold is not present in the *b* fold of the NTD. The only secondary structure element in the NTD that is not in the conserved Trx fold is an α -helix, labeled α , that is present at the beginning of both the *a* and *b* folds. The letters, N and C mark the N and C-termini of the NTD. B) Ribbon diagram of the NTD. C) Schematic diagram showing the evolutionary path from Trx to NTD. Trx (D-CxxC) undergoes gene duplication and fusion to form a dimer (D-CxxC-D-CxxC), which then diverges into two forms (D-CxxC-E and E-D-CxxC). Loss of aspartates leads to PDO (CxxC-E and E-CxxC), which then diverges into NTD (E-CxxC).

EcTrx (pdb code 2TRX) in the same orientation as the **a** fold of the NTD in A). The redox active cysteines and conserved aspartic acid are shown as in A) and only the conserved structural elements of the Trx fold are labeled. A structure-based sequence alignment of *EcTrx* on the **a** and **b** folds of the NTD gives a 1.3 Å (for 73 Cα atoms) and 1.5 Å (for 51 Cα atoms) RMSD with 15% and 16 % sequence identity, respectively, while alignment of the two NTD folds gives an RMSD of 1.3 Å (for 46 Cα atoms) with 14% sequence identity. C) The proposed evolution of the NTD begins with an ancestral Trx-like protein that has an active site aspartic acid and redox active disulfide (D and CxxC in top green bar). Gene duplication and fusion (53) creates a hypothetical ancestral protein that has two folds and two active sites (each containing a buried aspartic acid). Sequence divergence introduces a buried glutamic acid in the **b** fold (shown as an E). With the addition of this charged group to the active site, it has been speculated that the oxidoreductase activity of the protein could be rescued after the loss of the buried aspartate in the **a** fold (5). The active site CxxC motif in the **b** fold seen in the evolutionary intermediate, protein disulfide oxidoreductase (PDO), is lost with time. Fold coloring is as in A).

Figure 3. Global, cooperative denaturation of the NTD by urea. The reversible unfolding of NTD-S₂ (triangles) and NTD-(SH)₂ (circles) in urea was followed by fluorescence (grey shapes) and CD (black shapes). Solid curves are global fits of the unfolding data to a two-state unfolding model.

Figure 4. Select data from ^{15}N backbone relaxation experiments and model free analyses. Shown are R_2 , S^2 and R_{ex} data for NTD- S_2 (left panels) and NTD- $(\text{SH})_2$ (right panels) plotted per residue. Secondary structure elements with labels shown above the plots are colored by fold as in Figure 2A. Additional relaxation data are shown as Supplemental Information.

Figure 5.

H/D exchange protection factors for the NTD-S₂ are lower than for NTD-(SH)₂. Overlay of protection factors for NTD-S₂ (black bars) and NTD-(SH)₂ (grey bars). Residues for which there is no bar or only one bar (all grey or all black) are those amides that were not measured due to overlap or unassigned peaks. Blue bars are shown for the estimated protection factors of the slowest exchanging residues (21-26, 35, 37, 41, 53, 65-68, 92, 93, 95, and 96). For residues where a protection factor was measured in both redox states, a grey and black bar are overlaid and the color on top indicates which form is more protected (more slowly exchanging). Residues 77-80, 83, 90, 121-125, 136, 137, 141-143, 148, 152-156, 174-176, 179-181 and 191 are 5 to 100 fold more protected in NTD-(SH)₂ while only residues 42, 44, 110 and 190 are more protected in NTD-S₂. The residues that are significantly faster exchanging in NTD-S₂ (shorter bars) cluster in $\alpha 1$, $\beta 1$, $\beta 2$, $\beta 3$ and $\beta 4$ of the *a* fold and in $\beta 4$ and near Glu86 of the *b* fold. Secondary structure elements are shown with labels above the plot and are colored by fold as in Figure 2A. The positions of active site Cys129 and Cys132 are indicated. Protection factors are computed for exchange rate constants determined at pH 6.2 and 25 °C.

Figure 6.

Different intermediate exchange motions for NTD-S₂ and NTD-(SH)₂. Model free analysis results for intermediate timescale motions (R_{ex}, Figure 4 bottom graphs) are plotted on the crystal structure. Comparison of A) NTD-S₂ and B) NTD-(SH)₂ reveals an increase in R_{ex} motions across the entire protein for NTD-S₂ (green, yellow and red colors). The same coloring scale is used for both panels and the redox active disulfide is shown as ball and stick in both structures. Residues with R_{ex} less than 2 s⁻¹ or with no measurements are colored grey.

Figure 7.

The two Trx folds of the NTD display different protection from exchange. A) Data shown in Figure 5 are mapped on the crystal structure. The slowest exchanging residues (blue) are located in the *b* fold while residues with significant differences in protection factor with oxidation (colored yellow to red by magnitude of difference) are located around the active site and found primarily in the *a* fold. The redox active disulfide is shown as ball and stick and residues with no measurements or with a difference less than 0.5 in protection factor are colored grey. B) The slow exchanging residues (blue) of *Ec*Trx are located in the core of the Trx fold (data from Jeng and Dyson, 1995). The redox active disulfide is shown as ball and stick with sulfur atoms colored yellow. *Ec*Trx is aligned with the *b* fold of the NTD.

Figure 8.

Inferred energy landscapes for the NTD and *EcTrx*. Schematic energy wells illustrate changes in energy landscapes that accompany oxidation: the width of the wells represents qualitative conformational heterogeneity as inferred from H/D exchange behavior and the roughness at the bottom of the wells represents qualitative intermediate timescale motions as inferred from R_{ex} (see text). The reduced states of the NTD and *EcTrx* (grey wells) are shown with the same energy landscape that has the bottom of the well arbitrarily set to zero. The oxidized states (black wells) are drawn to represent the inferred quantitative changes in thermodynamics relative to the reduced state. The dotted line marks the expected 1.2 kcal/mol stability increase for the oxidized form due to the formation of the disulfide bond (43). Oxidation of the NTD slightly increases stability and causes increased internal fluctuations (wider well) of rapidly interconverting states on an intermediate timescale (rougher well). Oxidation of *EcTrx* increases stability (49) and decreases the conformational heterogeneity (51,52) (narrower well) without large changes in intermediate timescale motions (50) (similar roughness).

Figure 9.

The *a* and *b* folds show similar mobility in crystal structures. The B-factors along the chain from NTD-S₂ (pdb code 1ZYN) for the *a* fold (green) and *b* fold (grey) are mapped onto the *b* fold by a structure-based sequence alignment. Secondary structure elements are indicated - helix α_2 is present only in the *a* fold and is colored green. Other NTD crystal forms (pdb codes 1ZYP and 1HYU) behave similarly suggesting that crystal packing is not a major influence. Minor local differences in B-factor between the two folds occur in regions where structural elements have different solvent accessibilities due to the fold-fold packing.

Table 1

Thermodynamic parameters for urea-induced unfolding.

	$\Delta G^{\circ}_{\text{H}_2\text{O}}$ (kcal/mol)	m (kcal/mol/M)	Unfolding Midpoint (M)
<i>NTD-S₂</i>			
CD (220 nm) ^a	6.6	1.6	4.2
Fluorescence ^b	6.6	1.7	3.9
Global ^c	6.6	1.6	4.1
<i>NTD-(SH)₂</i>			
CD (220 nm) ^a	6.5	1.6	4.1
Fluorescence ^b	6.5	1.7	3.8
Global ^c	6.5	1.7	3.9

^aParameters determined by fitting unfolding data obtained from CD at 220 nm.^bParameters determined by fitting unfolding data obtained from fluorescence.^cParameters determined by simultaneously fitting unfolding data obtained from CD and fluorescence.

Table 2Overall ^{15}N backbone dynamics summary.

	NTD-S ₂	NTD-(SH) ₂
R ₁ (s ⁻¹)	1.09 ± 0.02	1.04 ± 0.01
R ₂ (s ⁻¹)	20.8 ± 0.4	17.3 ± 0.3
NOE	0.76 ± 0.06	0.79 ± 0.03
τ _c (ns)	13.63 ± 0.03	13.08 ± 0.01
S ²	0.94 ± 0.02	0.89 ± 0.01
R _{ex} (s ⁻¹)	3.0 ± 0.5	2.0 ± 0.4