DOI: 10.1002/anie.200604620

Protein Electrochemistry

Direct Electrochemical Characterization of Archaeal Thioredoxins**

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The thioredoxin (Trx) superfamily of proteins contains small soluble proteins that function as $2e^-/2H^+$ electron-transfer agents by virtue of a redox-active disulfide bond. Although the members of this superfamily are known to contain disulfide bonds that span a range of midpoint potential of at least 150 mV,[1] a detailed picture of the molecular determinants of the disulfide-bond potential has yet to be attained. Herein, we demonstrate that this goal is feasible through the application of protein-film voltammetry (PFV),[2] an electrochemical technique that we use to directly observe the reversible $2e^-$ redox couple of thioredoxins.

Successful PFV yields a fast electrochemical connection between a submonolayer of protein analyte and an electrode. Previous electrochemical investigations of plant-type and *Escherichia coli* Trx disulfides did not yield reversible $2e^-$ voltammetry: instead quasireversible $1e^-$ cyclic voltammetry for the disulfide/disulfide radical potential and an second irreversible feature (corresponding to the reduction of the radical intermediate) was observed.[3,4] Martin and co-workers have developed a modified gold electrode to investigate His-tag-labeled *E. coli* Trx; they too were unable to directly observe a reversible $2e^-$ potential[5] and could only establish reversible voltammetry for the $1e^-$ disulfide/disulfide radical couple.[5,6] As the biologically significant reaction for thioredoxins involves cooperative $2e^-$ chemistry,[7,8] we have examined a series of members of the Trx superfamily to observe a reversible $2e^-/2H^+$ reaction by using PFV. Herein, we report the successful extension of PFV to the direct measurement of a reversible $2e^-$ disulfide/dithiol couple.

Members of the Trx superfamily contain a single disulfide bond within a -CXXC- motif, and the differences in redox properties of Trx proteins are attributed to variation of the -CXXC- motif.[1,9] The difference in potential of the disulfide is correlated to function: *E. coli* Trx is reducing in the cellular milieu[8] with a reported potential from solution-based experiments of −270 mV (versus hydrogen).[10] More oxidizing members of the thioredoxin superfamily, such as *E. coli* DsbA, bear a disulfide bond with a reported reduction potential of −90[11] to −122 mV.[12] In comparison, the disulfide bond proximal to the Rieske [2Fe–2S] center of the *Thermus thermophilus* Rieske protein is electrochemically stable at sustained potentials as low as −0.85 V.[13] Trx acts as a reducing agent for a wide array of critical biological pathways (e.g., ribonucleotide biosynthesis, oxidative-stress defense, and transcription-factor activation),[14] whereas DsbA oxidizes target proteins, installing disulfides for proper protein folding and maturation.[15] Structures of the oxidized, disulfide-containing loops of *E. coli* Trx and DsbA are shown in Figure 1.[16,17]

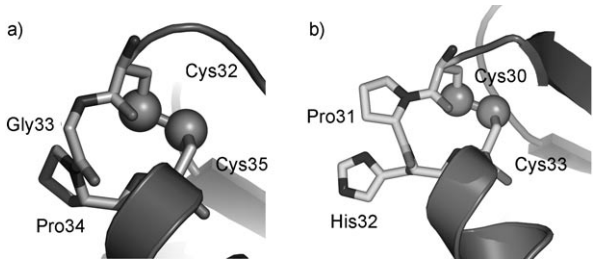

Figure 1. The -CXXC- motif of the thioredoxin superfamily is illustrated by a) *E. coli* Trx (-CGPC-) and b) DsbA (-CPHC-).

Probing the influence of sequence and structure on the reduction potential of Trx proteins has been limited by the methods used for determining $2e^-$ disulfide bond potentials. Such potentials are typically measured by coupled, solution-based processes, such as glutathione equilibria, which indirectly give a value for the disulfide potentials of interest, though such studies have yielded variable values of potential previously.[11,12,18,19]

Figure 2 shows the cyclic voltammetric response of four purported thioredoxins from the thermophilic archaeon *Archaeoglobus fulgidus*, at a pyrolytic graphite edge (PGE) electrode. The cloning, expression, and purification of these proteins as His-tagged constructs allowed us to carry out indentical PFV characterizations of the putative Trx proteins. Baseline subtraction of the non-faradaic component of the current reveals a single set of nernstian peaks in all cases. The peak height (I_p) corresponds linearly to the scan rate (v) indicating that the Trx-based signal is due to protein immobilized upon the PGE surface (data not shown).

The peaks resulting from oxidative and reductive scans appear highly symmetric, and all of the Trx proteins demonstrate a value of peak width at half height (δ) that

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[**] This work was supported by the Richard Allan Barry Fund at the Boston Foundation (SJE), the Boston University Undergraduate Research Opportunities Program (SEC), and the National Institutes of Health (GM65337 to C.L.D.; F31-GM073569 and T32-GM08334 to H.H.H.). The authors thank C. Becker and B. A. Brown II for technical assistance.

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

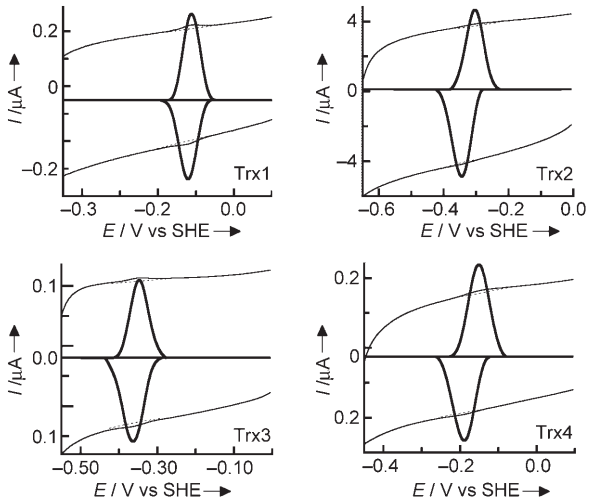

Figure 2. Voltammetric response of *A. fulgidus* Trx proteins as both raw and baseline-subtracted data at 10 °C. All data were acquired at a pyrolytic graphite edge electrode with pH 7.75 for Trx1, Trx2, and Trx3, and with pH 4.5 for Trx4. SHE = standard hydrogen electrode.

indicates a cooperative redox process; that is, the number of electrons (n) is greater than 1.[20] Figure 3a shows that at lower temperatures, measured values of δ are between those found for $n = 1\,e^-$ and $n = 2\,e^-$ process, though at 25 °C, δ approaches the anticipated value for an immobilized system undergoing a $n = 2\,e^-$ reaction (equivalent to two highly rapid $n = 1\,e^-$ steps).[20] Values of δ decrease as a function of temperature, suggesting a dispersion of Trx conformations exists at the electrode surface, yielding artificial peak broadening at low temperatures and a nonzero peak separation.

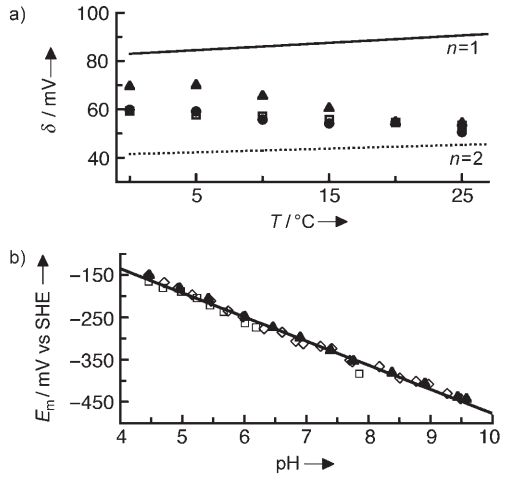

Figure 3. a) Experimentally determined values of peak width for Trx1 (□), Trx2 (▲), and Trx3 (●) (Trx4 protein films were not sufficiently robust to heating) compared with calculated values for $n = 1$ and $n = 2$ electron reactions. b) The pH-dependent behavior of Trx3 midpoint potentials for three distinct sets of experiments. The data are fit to a linear progression with a slope indicating a $1\,e^-/1\,H^+$ process. The buffer solution was composed of 10 mM β-morpholinoethanesulfonic acid (MES), MOPS, 3-[tris(hydroxymethyl)methylamino]-1-propanesulfonic acid (TAPS), and 2-cyclohexylaminoethanesulfonic acid (CHES) with 150 mM NaCl, and the pH value was determined independently for each data point.

Upon heating, conformations that result in faster electrokinetics dominate and peak narrowing is observed. All experiments gave identical data regardless of the redox poise of the Trx. The determined values of the midpoint potential (E_m) and δ are given in Table 1 for a single set of conditions, demonstrating that E_m for the $2\,e^-$ couple varies significantly between Trxs.

Table 1: Measured midpoint potentials and peak widths of Trx proteins; pH 7.0, 10 °C.

Sample	E_m [mV]	δ[a] [mV]	-CXXC-
Trx1	−32	62	CPHC
Trx2	−301	65	CPYC
Trx3	−287	56	CMPC
Trx4	−309	64	CMPC

[a] For the sake of comparison, the theoretical values of δ (at 10 °C) for $n = 1$ or $n = 2$ processes are 86 or 43 mV, respectively.[18]

The pH dependence of the Trx3 electrochemical response is shown in Figure 3b. In all cases, Trx films are stable upon the electrode surface from pH 4.5 to 9.5, though at pH values greater than 9.0, the intensity of the electrochemical signal becomes discernibly smaller (the depletion of peak height is fully reversible, indicating that protonation promotes conformations that yield reversible voltammetry). The slope of the pH dependence is −57 mV per pH unit, implying a $1\,e^-/1\,H^+$ or $2\,e^-/2\,H^+$ stoichiometry. As the observed peak widths indicate that $n = 2\,e^-$, the pH dependence indicates a $2\,e^-/2\,H^+$ process is at work. Within the pH values studied, pK_a events are not observed, that is, E_m always appears to have a linear dependence upon the pH value. The absence of a pK_a value for the buried thiol of the reduced form may be due to the instability of the Trx–electrode interaction at pH values greater than 9.5. Protein films readily desorbed at highly basic pH values and recent studies of the *E. coli* and *Chlamydomonas reinhardtii* Trx, indicate that pK_a values for the Trx thiol greater than 10.0 are common for Trx proteins.[21]

Trx1 has a strongly oxidizing potential, in analogy with DsbA from *E. coli.* This variation can be generally understood by the hypothesis of Raines and co-workers that the presence of a protonatable residue in the -CXXC- motif, which contains the Trx disulfide bond, leads to a high potential.[1] Indeed, the Trx1 disulfide loop possesses the -CPHC- sequence found in DsbA, suggesting that functionally Trx1 is not a thioredoxin at all, but an oxidizing protein. The identity of the residues in the -CXXC- motif cannot be the sole determinant of the Trx reduction potential: Trx3 and Trx4 contain the same motif (-CMPC-) and yet vary in potential by 20 mV.

In summary, we have determined that PFV is a useful tool in the study of the archaeal thioredoxins as it does not depend upon other solution equilibria and it can relate quantitative information regarding redox cooperativity. Thus, employment of PFV will enable detailed studies of the relationship between sequence, structure, and redox chemistry of thioredoxins.

Experimental Section

Protein-film voltammetry was carried out in a three-electrode configuration by using a standard calomel reference electrode and constructed PGE working electrodes. The electrochemical cell was water jacketed for temperature control and the reference electrode maintained in a separate compartment at a fixed (room) temperature. Electrodes were constructed of pyrolytic graphite (Advanced Ceramics), which was machined into cylinders, mounted onto a steel rod with silver epoxy, and then embedded in epoxy, yielding a graphite edge plane as the working surface. PGE electrodes were polished with an aqueous slurry of 1.0 μm alumina (Beuhler) and then sonicated prior to use.

Electroactive protein films were generated by rotating the working electrode at 200 rpm in a diluted protein solution (10 μM Trx in 20 mM 3-(*N*-morpholine)propanesulfonic acid (MOPS), 150 mM NaCl; pH 7.5) for 15 minutes while cycling the applied potential from 0.2 to −0.5 V. The protein-containing solution was then remove, the cell rinsed, and the working electrode then replaced in protein-free buffer solution containing 150 mM electrolyte. PFV was initially conducted at 10 °C to observe an electrochemical response at uniform conditions prior to adjusting the pH value or the temperature. Baseline subtraction was achieved by first measuring the baseline response of the electrode for a given set of buffer solution conditions prior to deposition of the protein film.

Details of *trx* gene identification, cloning, and Trx protein purification are given in the Supporting Information.

Received: November 13, 2006
Revised: March 5, 2007
Published online: April 19, 2007

Keywords: disulfide bonds · electrochemistry · electron transfer · proteins · redox chemistry

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