# Effect of pH on the Oxidation−Reduction Properties of Thioredoxins†

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*Received September 17, 2003*

ABSTRACT: Oxidation−reduction midpoint potential ($E_m$) versus pH profiles were measured for wild-type thioredoxins from *Escherichia coli* and from the green alga *Chlamydomonas reinhardtii* and for a number of site-directed mutants of these two thioredoxins. These profiles all exhibit slopes of approximately −59 mV per pH unit, characteristic of the uptake of two protons per reduction of an active-site thioredoxin disulfide, at acidic, neutral, and moderately alkaline pH values. At higher pH values, these profiles exhibit slopes of either −29.5 mV per pH unit, characteristic of the uptake of one proton per disulfide reduced, or are pH-independent, indicating that neither proton uptake nor proton release is associated with reduction of the active-site disulfide. Reduction of the two wild-type thioredoxins is accompanied by the uptake of two protons even at pH values where the more acidic cysteine thiol group of the reduced proteins would be expected to be completely unprotonated. The effect of site-directed mutagenesis of two highly conserved aspartate residues that play important structural and/or catalytic roles in both thioredoxins, and which could in principle play a role in proton transfer, on the p$K_a$ values of redox-linked acid dissociations (deduced from changes in slope of the $E_m$ versus pH profiles) has also been determined for both *E. coli* thioredoxin and *C. reinhardtii* thioredoxin *h*.

Thioredoxins are ubiquitous proteins, of ca. 11 kDa molecular mass, with a multiplicity of functions (*1*). The active site of the reduced forms of thioredoxins contains two cysteine residues, separated by two intervening amino acids (usually glycine and proline). The two-electron oxidation of thioredoxins results in the formation of a disulfide bond between the two active-site cysteines. The first thioredoxin to be discovered, that from *Escherichia coli*, can serve as a hydrogen donor to ribonucleotide reductase, plays a role in sulfur assimilation and phage assembly, and serves as a general disulfide oxidoreductase (*2*). The amino acid sequence of *E. coli* thioredoxin has been known for some time (*3*). A high-resolution three-dimensional structure is known, from X-ray crystallography, for the oxidized protein (PDB 2TRX, ref *4*), and a solution structure, determined by nuclear magnetic resonance (NMR) spectroscopy, is available for the reduced protein (PDB 1XOB, ref *5*). The tertiary structures of the reduced and oxidized forms of *E. coli* thioredoxin are very similar, with the only significant differences being at the active site (*4*, *5*).

It has been known for some time that oxygenic photosynthetic eukaryotes (i.e., plants and algae) contain at least four different types of thioredoxins, two of which (thioredoxins *f* and *m*) are located in the chloroplasts, one (thioredoxin *h*) that is located in the cytoplasm (*1*, *6*, *7*), and one (thioredoxin *o*) that is located in the mitochondria (*8*). The role of thioredoxins *f* and *m* in the regulation of metabolism has been extensively documented, and many target enzymes for these thioredoxins have been identified (*1*, *6*, *9*−*11*). Recently, two additional forms of chloroplastic thioredoxins, thioredoxin *x* (*12*−*14*) and thioredoxin *y* (*15*), have been discovered, but their specific functions have not yet been fully elucidated.

Relatively little is known about the function of the cytosolic *h*-type thioredoxins (*1*, *7*), but amylase inhibitors and storage proteins can be reduced by these thioredoxins in higher plants (*1*, *7*), and it has been demonstrated that expression of thioredoxin *h* is induced by heavy metals in the green alga *Chlamydomonas reinhardtii* (*16*). In terms of structure and chemical properties, thioredoxin *h* from *C. reinhardtii* is one of the best-characterized of this class of thioredoxins. The solution structure of the oxidized form of *C. reinhardtii* thioredoxin *h* has been determined by NMR

† Supported by a grant from the Robert A. Welch Foundation (D-0710 to D.B.K.).

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10.1021/bi0302088 CCC: $25.00 
Published on Web 11/27/2003

spectroscopy (*17*), and X-ray crystal structures are available for wild-type *C. reinhardtii* thioredoxin *h* and two site-directed variants of the protein (*18*, *19*). The structure of wild-type *C. reinhardtii* thioredoxin *h* shows many similarities to that of *E. coli* thioredoxin (*1*, *17*). In fact, the structures of other thioredoxins also resemble those of *E. coli* thioredoxin and *C. reinhardtii* thioredoxin *h*, and the common features of a central five-stranded, $\beta$-pleated sheet surrounded by several short α-helices is commonly referred to as the thioredoxin fold (*20*). *E. coli* thioredoxin and *C. reinhardtii* thioredoxin *h* also show considerable sequence homologies—a number of aspartate residues are absolutely conserved, and the two proteins have identical **WCGPCK** active-site sequences (*1*). The two proteins also have similar oxidation−reduction midpoint potentials ($E_m$) at pH 7.0, with values near −270 mV having been reported for *E. coli* thioredoxin (*21*−*23*) and a value of −290 mV having been reported for *C. reinhardtii* thioredoxin *h* (*24*).

The catalytic ability of thioredoxins as a disulfide oxidoreductase at pH values near neutrality exceeds that of dithiothreitol (DTT),[1] one of the most active nonprotein dithiol/disulfide couples, by ca. 10 000-fold (*25*). The enhanced reactivity of thioredoxins, as compared to that of molecules such as DTT, no doubt arises from the one or more unique aspects of the microenvironment of the active site of thioredoxin. In fact, the active-site disulfides of *E. coli* thioredoxin and *C. reinhardtii* thioredoxin *h* are in relatively hydrophobic environments (*1*, *4*, *5*, *17*−*20*). There are, however, important differences in the local environments of the two active-site cysteines, the only two cysteine residues present in either protein (*1*). In both proteins, the more N-terminal of the two cysteines (Cys32 in *E. coli* thioredoxin and Cys36 in C. *reinhardtii* thioredoxin *h*) is significantly more exposed to the solvent than the second cysteine (Cys35 in *E. coli* thioredoxin and Cys39 in C. *reinhardtii* thioredoxin *h*). One would expect that these differences would result in differences in the acid−base dissociation properties of the thiol groups of the two active-site cysteines. This appears to be the case for both *E. coli* thioredoxin and for *C. reinhardtii* thioredoxin *h*. The acid/base titration behavior of both thioredoxins is in fact complex, which has resulted in different interpretations of the data. Thus, while there is reasonably good agreement that the p$K_a$ of Cys32 (the more solvent-exposed cysteine) of *E. coli* thioredoxin is near 7 (reported values range from 6.3 to 7.5, refs *26*−*38*), estimates for the p$K_a$ of Cys35 (the more buried cysteine) of *E. coli* thioredoxin vary widely, ranging from 7.0 to greater than 11.0 (*29*−*39*). It appears likely that microscopic p$K_a$s are in fact being observed (*34*). NMR spectroscopy has been used (*24*) to estimate p$K_a$ values of 7.0 for Cys36 of *C. reinhardtii* thioredoxin *h* (the more solvent-exposed cysteine that corresponds to Cys32 in *E. coli* thioredoxin) and 9.5 for Cys39 of *C. reinhardtii* thioredoxin *h* (the more buried cysteine that corresponds to Cys35 in *E. coli* thioredoxin). As it is possible to estimate p$K_a$ values for acid−base groups involved in proton uptake or release coupled to redox reactions of thioredoxins from the pH-dependence of $E_m$ values (*33*), we have undertaken a detailed study of $E_m$ versus pH profiles for wild-type *E. coli* thioredoxin and *C. reinhardtii* thioredoxin *h*. To explore the possibility that amino acids other than the two active-site cysteines may be involved in proton uptake coupled to disulfide reduction, $E_m$ versus pH profiles have also been obtained for several site-directed mutants of both proteins.

[1] Abbreviations: AMP, 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid; Bis-Tris propane, 1,3-bis[tris(hydroxymethyl)methylaminopropane; CABS, 4-[cyclohexylamino]-1-butanesulfonic acid; mBBr, monobromobimane; MES, 2-[*N*-morphilino]ethanesulfonic acid; MOPS, 3-[*N*-morpholino]propanesulfonic acid; DTT, dithiothreitol; Tricine, *N*-tris[hydroxymethyl]methylglycine.

## MATERIALS AND METHODS

Wild-type *E. coli* thioredoxin (*34*) and its D26N (*34*), D26L (*34*), CWGC (*40*), and D61N (*41*) variants were expressed and purified as described previously. Wild-type *C. reinhardtii* thioredoxin *h* (*24*, *42*) and its W35A (*24*) and D30A (*18*) variants were expressed and purified as described previously. The D65A variant of *C. reinhardtii* thioredoxin *h* was generated by PCR site-directed mutagenesis, using the oligonucleotides D65A, UP (GAAGGTCGATGTTGCTGCCGTCGCGGCTGTTGCCG) and D65A, DOWN (CGGCAACAGCCGCGACGGCAGCAACATCGACCTTC). Mutagenesis, cloning, expression, and purification of this variant were performed as previously described for wild-type thioredoxin and the other variants (*42*, *43*).

Oxidation−reduction titrations were carried out using redox buffers containing different defined ratios of oxidized and reduced DTT, followed by labeling of cysteine thiols with the fluorophore monobromobimane (mBBr), as described previously (*24*, *44*). Fluorescence was measured using an Aminco-Bowman Series 2 Luminescence Spectrometer, and redox titration data were fitted to the Nernst equation for a two-electron redox couple as described previously (*24*, *44*). All the titrations used gave an excellent fit to the Nernst equation for a single two-electron component. A value of −327 mV for the $E_m$ of DTT at pH 7.0 was used in the calculations (*43*), and p$K_a$ values of 9.2 and 10.1 were used for reduced DTT in calculating $E_m$ values at pH values other than 7.0 (*45*). In the case of the CWGC variant of *E. coli* thioredoxin, because of its more positive $E_m$ value, glutathione rather than DTT redox buffers were used (*45*). Values of −240 mV for the $E_m$ and glutathione at pH 7.0 (*46*) and 8.7 for the p$K_a$ of the thiol group of reduced glutathione (*47*) were used for these titrations. The pH-dependencies of the $E_m$ values were fitted to the following equation (*33*):

$$E_m = E^\circ - (RT/nF)\ln\{1/([H^+]^2 + [H^+]K_{n1} + [H^+]K_{n2} + K_{n1}K_{n2})\} \quad (1)$$

where $R$ is the universal gas constant, $F$ is the Faraday constant, $n$ is the number of electrons transferred in the redox reaction ($n = 2$ for the case of all the disulfide/dithiol couples examined in the experiments reported next), $E^\circ$ represents the intrinsic tendency of the disulfide bond to undergo reduction, and $K_{n1}$ and $K_{n2}$ are the first and second p$K_a$ values for acid/base equilibria coupled to the redox reaction ($E^\circ$ was set so that eq 1 gave an $E_m$ value at pH 7.0 that matched the experimentally determined value, while $K_{n1}$ and $K_{n2}$ were determined as best-fit parameters using MicroSoft Excel).

The following pH buffers, all at a concentration of 100 mM, were used: acetic acid/sodium acetate for pH values

between 5.00 and 6.00; MES for pH values between 6.00 and 6.75; MOPS for pH values between 6.75 and 7.75; tricine for pH values between 7.75 and 8.75; bis-Tris propane for pH values between 8.75 and 9.50; AMP for pH values between 9.50 and 10.50; and CABS for pH values between pH 10.50 and 11.50.

The $E_m$ values measured at pH 7.0 were independent of redox equilibration time over the range from 1.0 to 3.0 h and were independent of the total DTT concentration of the redox buffer over a range from 1.0 to 5.0 mM. All titrations at other pH values were carried out using a total DTT concentration of 5.0 mM and a redox equilibration time of 2.0 h. In the case of the titrations of the CWGC variant of *E. coli* thioredoxin, where glutathione rather than DTT redox buffers were used, $E_m$ values were also independent of time and of redox buffer concentration. All $E_m$ values reported represent the average of at least two independent titrations. Although all of the redox titrations shown next were carried out under aerobic conditions, control titrations carried out under an argon atmosphere gave results that were identical, within the experimental uncertainties of the measurements, to those carried out under aerobic conditions. Furthermore, it was shown that reduced DTT and reduced glutathione were stable against oxidation under the conditions of the aerobic titrations for at least 4.0 h. In the case of wild-type *C. reinhardtii* thioredoxin *h*, a control redox-titration at pH 7.0 using $NADP^+$/NADPH mixtures for redox-poising and the NADPH-dependent thioredoxin reductase from *Arabidopsis thaliana* to catalyze redox equilibrium gave an $E_m$ value identical to that measured using the mBBr method (*24*).

## RESULTS

Figure 1A shows the results of a redox titration of wild-type *E. coli* thioredoxin carried out at pH 7.0. The data give a good fit to the Nernst equation for a single two-electron component with $E_m = -275$ mV. The average value, from five replicate titrations, for the $E_m$ value of wild-type *E. coli* thioredoxin at pH 7.0 was $-285 \pm 10$ mV. Although the $E_m = -285$ mV value is slightly more negative than the values ranging from $-260$ to $-275$ mV reported previously in the literature (*21*−*23*), it agrees with the average of values measured by other techniques within the experimental uncertainties of the methods used. Figure 1B shows the results of a redox titration of the CWGC variant of *E. coli* thioredoxin (*40*) carried out at pH 7.0. This variant, in which the CGPC active-site sequence of the wild-type protein has been replaced by the sequence CWGC, was chosen for study because both its $E_m$ value at pH 7.0 and the p$K_a$ of its more acidic cysteine, Cys32, have been reported to be changed significantly from those of wild-type *E. coli* thioredoxin (*40*). The results give a good fit to the Nernst equation for a single two-electron component with $E_m = -215$ mV. The average value, from three replicate titrations, for the $E_m$ value of this variant of *E. coli* thioredoxin at pH 7.0 was $-210 \pm 10$ mV, in good agreement (within the experimental uncertainties of the measurements) with the previously reported value of $-200$ mV (*40*). We have also carried out redox titrations of variants of *E. coli* thioredoxin in which each of two conserved aspartate residues (Asp26 and Asp61) were replaced by residues that can neither take up nor release protons. The tertiary structure of thioredoxin suggests the possibility that, at least in principle, either of these aspartates might play a role in delivering protons to the active-site disulfide during reduction of the protein. The $E_m$ values for these mutated *E. coli* thioredoxins at pH 7.0 are D26L, $-290 \pm 10$ mV; D26N, $-290 \pm 10$ mV (replacement of Asp26 by alanine had previously been shown to have little effect on the $E_m$ value of the active-site disulfide of *E. coli* thioredoxin—ref *48*); and D61N, $-280 \pm 10$ mV. Thus, with the exception of the CWGC variant, none of these mutations have a major effect on the $E_m$ value of *E. coli* thioredoxin at pH 7.0.

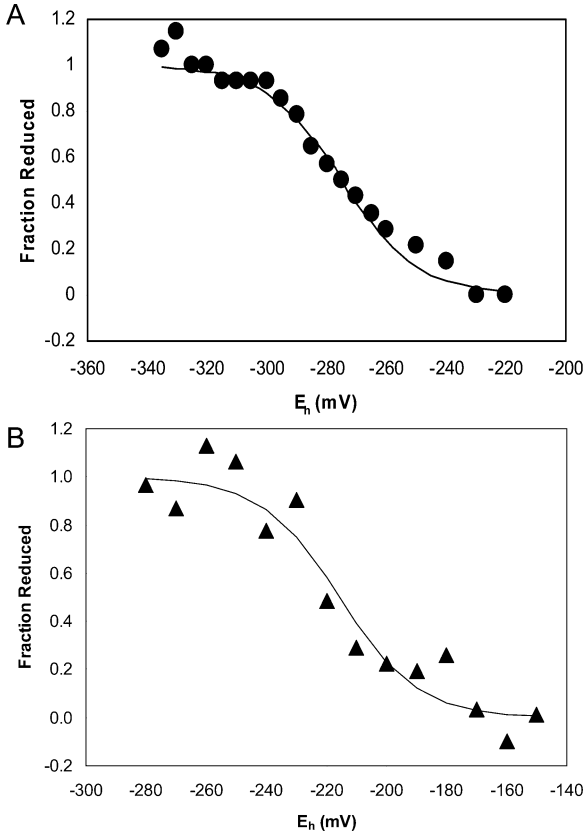


FIGURE 1: Oxidation−reduction titrations of wild-type *E. coli* thioredoxin and its CWGC variant at pH 7.0. (A) Wild-type *E. coli* thioredoxin, at a concentration of 100 $\mu$g/mL, was titrated in 100 mM MOPS buffer (pH 7.0), at a total DTT concentration of 2.0 mM, with a redox equilibration time of 2.0 h. (B) CWGC *E. coli* thioredoxin, at a concentration of 100 $\mu$g/mL, was titrated in 100 mM MOPS buffer (pH 7.0), at a total glutathione concentration of 5.0 mM.

Figure 2A shows the pH-dependence of the $E_m$ value of wild-type *E. coli* thioredoxin over the pH range from pH 6.0 to 11.0 (the protein was too unstable to titrate at pH values above 11.0). The $E_m$ versus pH profile from pH 6.0 to approximately pH 9.0 exhibits the $-59$ mV/pH unit slope expected for the uptake of 2.0 protons by thioredoxin per active-site disulfide reduced, and the $E_m$ versus pH profile at pH values near 11.0 are most consistent with the pH-independent $E_m$ value that would arise from a redox reaction associated with no net proton uptake or release. The entire data set gives a good fit to eq 1, with identical p$K_a$ values of 10.0 for two acid/base groups coupled to the redox reaction. Although thermodynamic relationships can be used to calculate both the number of protons taken up during the reduction of thioredoxin and the p$K_a$ values for the acid/base groups involved from the $E_m$ versus pH profile, these

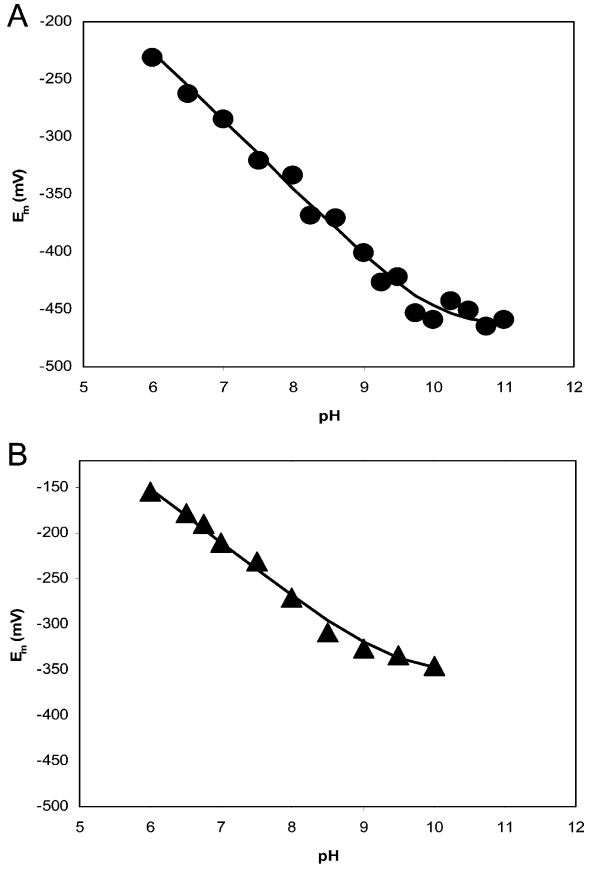


FIGURE 2: $E_m$ vs pH plots of wild-type *E. coli* thioredoxin and its CWGC variant. (A) Average values of $E_m$ (mV) for wild-type *E. coli* thioredoxin are plotted against the pH value at which $E_m$ was measured (closed circles). Eq 1 was used to calculate values of 10.0 for both p$K_{a1}$ and p$K_{a2}$. (B) Average values of $E_m$ (mV) for CWGC *E. coli* thioredoxin are plotted against the pH value at which $E_m$ was measured (closed triangles). Values of 9.4 were calculated for both p$K_{a1}$ and p$K_{a2}$.

relationships cannot identify the specific groups involved in proton uptake or release. Nevertheless, the simplest model for proton uptake accompanying thioredoxin reduction would predict the following: the uptake of two protons per disulfide reduced at pH values below the p$K_a$ values of both active-site cysteines (i.e., one proton being taken up by a sulfur on each of the two cysteine residues formed during reductive cleavage of the active-site disulfide); the uptake of one proton per disulfide reduced at pH values above the p$K_a$ of the more acidic active-site cysteine but below the p$K_a$ value of the less acidic active-site cysteine (i.e., after reduction, one cysteine exists as the protonated thiol and the other as the unprotonated thiolate anion); and no proton uptake at pH values above the p$K_a$ of the less acidic active-site cysteine (*33*). Given the consensus that the p$K_a$ value for the more acidic Cys32 is near 7.0 and that the less acidic Cys35 has a considerably more alkaline p$K_a$ value, one would thus predict a transition in the slope of the $E_m$ versus pH profile from −59 mV/pH unit to a slope of −29.5 mV/pH unit, with the transition centered near pH 7.0. In fact, as shown in Figure 2A, the $E_m$ versus pH profile for wild-type *E. coli* thioredoxin displays the −59 mV/pH unit slope characteristic of the uptake of two protons coupled to the reduction of the active-site disulfide up to pH values near 9.0, some 2.0 pH units above the p$K_a$ estimated for the thiol group of Cys32. Thus, if the values near 7.0 reported for the p$K_a$ of Cys32 are correct, some amino acid(s) other than this cysteine (or perhaps a bound water) must be involved in taking up one proton at pH values between 7.0 and 9.0 (assuming that Cys35 is responsible for the uptake of the second proton).

To test the conclusion that the reduction of *E. coli* thioredoxin can be accompanied by the uptake of two protons per active-site disulfide reduced at pH values above the p$K_a$ of Cys32, experiments similar to those summarized in Figure 2A were carried out with the CWGC variant of *E. coli* thioredoxin, a variant in which the p$K_a$ of Cys32 is approximately 1.4 units more acidic than is the case for the wild-type protein (*34*). Figure 2B shows the pH-dependence of the $E_m$ value of the CWGC variant of *E. coli* thioredoxin over the pH range from 6.0 to 10.0 (this variant was too unstable to titrate at pH values more alkaline than 10.0). The slope of the $E_m$ versus pH profile of Figure 2B is close to the −59 mV/pH unit value expected for the uptake of two protons per reduction of the active-site disulfide up to pH values slightly above 8.0, more than 2.0 units above the value of 5.9 estimated for the p$K_a$ of Cys32 in this *E. coli* thioredoxin variant (*40*). The complete data set of Figure 2B gives a good fit to eq 1, with identical p$K_a$ values of 9.4 for two acid/base groups coupled to the redox reaction.

Two aspartate residues in *E. coli* thioredoxin, Asp26 and Asp61, are well-conserved in thioredoxins; thus, it was of interest to determine whether replacement of these two residues by amino acids incapable of proton uptake or release had any significant effect on the $E_m$ versus pH profile for *E. coli* thioredoxin. A possible role for Asp26 in proton uptake coupled to the reduction of *E. coli* thioredoxin seemed of particular interest to investigate, given the reports that the p$K_a$ of the side-chain carboxyl group of this amino acid has an unusually alkaline value (*31*, *34*, *36*, *49*−*51*), that it might interact with Cys32 (*34*, *35*), and that it might play an important role as a general acid/base catalyst in the reaction cycle of thioredoxins (*35*, *52*). As the side-chain carboxyl group of Asp61 forms a structurally important hydrogen bond with the indole ring of Trp31 (*4*) and Trp31 is a functionally important part of the active-site domain of *E. coli* thioredoxin (*53*), it also seemed important to examine the possible role of Asp61 in proton uptake during reduction of *E. coli* thioredoxin.

Figure 3A shows the $E_m$ versus pH profile for the D26N variant of *E. coli* thioredoxin. The data give a good fit to eq 1 with p$K_a$ values of 8.8 and 11.0 (the instability of the protein at pH values above 11.0 that made obtaining $E_m$ values at pH values more alkaline than 11.0 make it somewhat difficult to estimate the second p$K_a$ value accurately). The $E_m$ versus pH profile for a second variant of *E. coli* thioredoxin at this position, D26L, also gives a good fit to eq 1, with p$K_a$ values of 8.9 and 9.9 (data not shown). Figure 3B shows the $E_m$ versus pH profile for the D61N variant of *E. coli* thioredoxin. Somewhat surprisingly, the profile for this *E. coli* thioredoxin variant shows no deviation from the −59 mV/pH unit slope characteristic of the uptake of two protons per disulfide reduced even at pH 11.0, the most alkaline pH value at which the protein was stable enough to titrate. One might have expected that, if either Asp26 or Asp61 is a site of proton uptake coupled to the reduction of thioredoxin at pH values more alkaline than 6.0, replacement of these residues by amino acids incapable of

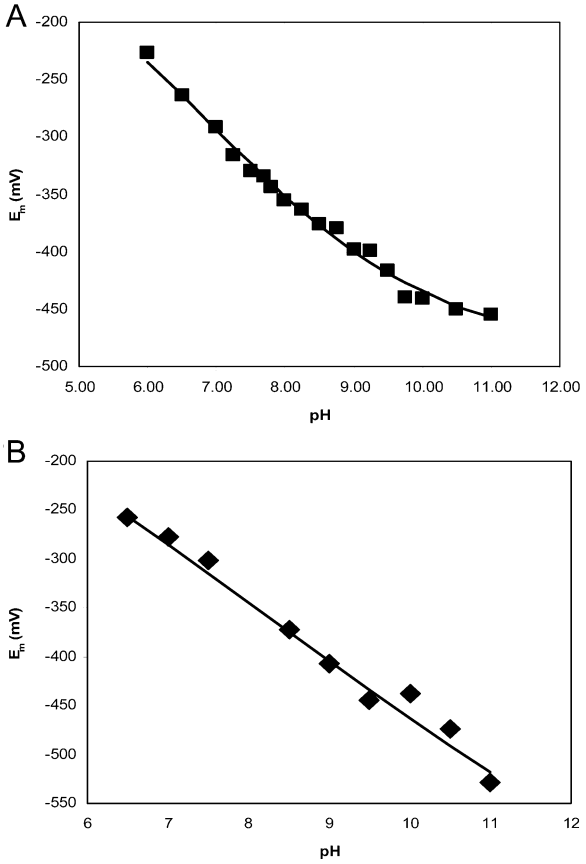


FIGURE 3: $E_m$ vs pH plots of D26N and D61N mutants of *E. coli* thioredoxin. (A) Average values of $E_m$ (mV) for D26N *E. coli* thioredoxin are plotted against the pH value at which $E_m$ was measured (closed squares). Values of 8.8 and 11.0 were calculated for p$K_{a1}$ and p$K_{a2}$, respectively. (B) Average values of $E_m$ (mV) for D61N. *E. coli* thioredoxin $E_m$ are plotted against the pH value at which $E_m$ was measured (closed diamonds). Both p$K_{a1}$ and p$K_{a2}$ were estimated to be >11.0.

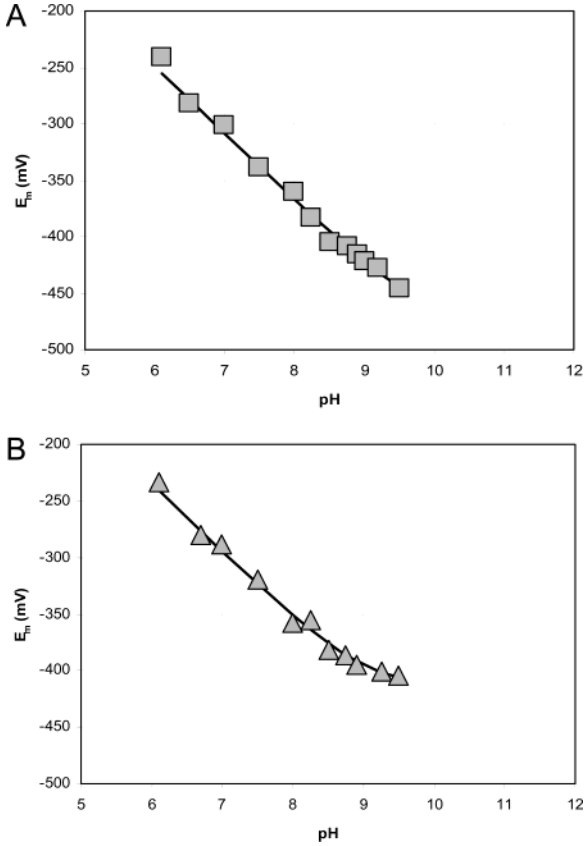


FIGURE 4: $E_m$ vs pH plots of wild-type *C. reinhardtii* thioredoxin *h* and W35A mutant of *C. reinhardtii* thioredoxin *h*. (A) Average values of $E_m$ (mV) for wild-type *C. reinhardtii* thioredoxin *h* are plotted against the pH value at which $E_m$ was measured (gray circles). Values of 9.3 and >10.0 were calculated for p$K_{a1}$ and p$K_{a2}$, respectively. (B) Average values of $E_m$ (mV) for W35A *C. reinhardtii* thioredoxin *h* are plotted against the pH value at which $E_m$ was measured (gray squares). Values of 9.0 were calculated for both p$K_{a1}$ and p$K_{a2}$.

proton uptake would result in the uptake of less than 2.0 protons over the pH range from 6.0 to approximately 9.0. The fact that this is not the case (in fact, some of these replacements extend the pH range over which 2.0 protons are taken up per disulfide reduced) suggests that neither of these conserved aspartate residues plays a significant role in proton uptake couple to the reduction of *E. coli* thioredoxin.

We have previously measured $E_m$ versus pH profiles for wild-type *C. reinhardtii* thioredoxin *h* and its W35A variant (Trp35 in *C. reinhardtii* thioredoxin *h* corresponds to Trp31 in *E. coli* thioredoxin) but carried out these measurements over a relatively narrow range of pH values (*34*). Given the unexpected ability of *E. coli* thioredoxin, described previously, to take up 2.0 protons at pH values substantially more alkaline than the likely p$K_a$ of the more acidic active-site Cys32 and the considerable structural similarity between *E. coli* thioredoxin and *C. reinhardtii* thioredoxin *h*, it seemed of interest to carry out a more extensive study of the pH-dependence of the redox properties of the wild-type algal thioredoxin and its W35A variant. Such studies are particularly attractive in light of the fact that there is high-resolution structural data for wild-type *C. reinhardtii* thioredoxin *h* and for several site-specific variants of the protein (*17*−*19*) and because p$K_a$ values have been estimated for the two active-site cysteines of the reduced protein (*24*). Although one would not expect Trp35 to serve as a proton carrier, the indole ring of Trp35 in *C. reinhardtii* thioredoxin *h* is hydrogen bonded to the side chain of Asp65 (Asp65 in the algal thioredoxin is equivalent to Asp61 in *E. coli* thioredoxin—refs *18* and *19*) and replacement of Trp35 by a nonaromatic residue affects the activities of the protein (*24*).

Figure 4A,B shows, respectively, the similar $E_m$ versus pH profiles for wild-type *C. reinhardtii* thioredoxin *h* and it W35A variant. Average $E_m$ values at pH 7.0 were −300 ± 10 mV for wild-type thioredoxin and −290 ± 10 mV for the W35A variant, in excellent agreement with the values reported previously (*24*). Both proteins proved too unstable to titrate at pH values above 9.5, and so the $E_m$ versus pH profiles are somewhat limited as compared to those obtained for wild-type *E. coli* thioredoxin and its variants. In fact, it was not possible to determine p$K_a$ values for acid/base groups coupled to the redox reaction for either protein but instead to only place lower limits, using eq 1, of 9.3 and 9.5 on the p$K_a$ values for the wild-type protein and it W35A variant, respectively. What can be said is that the slope of these profiles shows little deviation from the −59 mV/pH unit value characteristic of the uptake of 2.0 protons per disulfide reduced over the entire pH range from 6.0 to 9.0. As the p$K_a$ of Cys36 has been estimated to be 7.0 in wild-type *C.*

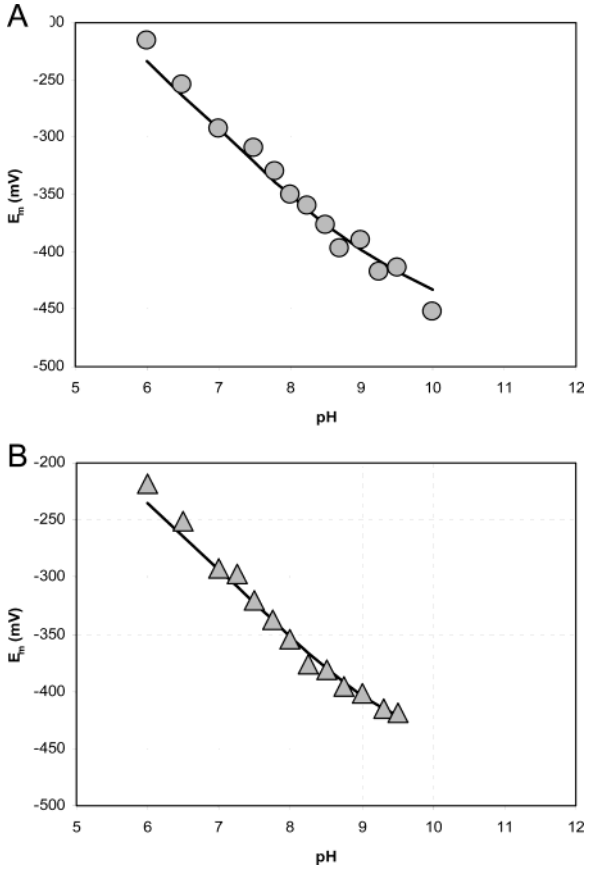


FIGURE 5: $E_m$ vs pH plots of D30A and D65A mutants of *C. reinhardtii* thioredoxin *h*. A. Average values of $E_m$ (mV) for D30A *C. reinhardtii* thioredoxin *h* are plotted against the pH value at which $E_m$ was measured (gray squares). Values of 8.7 and >10.0 were calculated for p$K_{a1}$ and p$K_{a2}$, respectively. (B) Average values of $E_m$ (mV) for D65A *C. reinhardtii* thioredoxin *h* are plotted against the pH value at which $E_m$ was measured (gray diamonds). Values of 9.4 were calculated for both p$K_{a1}$ and p$K_{a2}$.

*reinhardtii* thioredoxin *h* and 8.3 in its W35A variant (*24*), one again has a situation—as has been demonstrated above for *E. coli* thioredoxin—in which reduction of the active-site disulfide is accompanied by the uptake of 2.0 protons at pH values where Cys36 is likely to be present entirely as the thiolate anion after reduction.

We next examined a possible role for Asp30 and Asp65 in proton uptake coupled to the reduction of *C. reinhardtii* thioredoxin *h* (these two aspartate residues correspond, respectively, to Asp 26 and Asp61 in *E. coli* thioredoxin). Average $E_m$ values for these two variants at pH 7.0 were $-290 \pm 10$ mV for D30A and $-300 \pm 10$ mV for D65A, so neither substitution produces any significant change in the $E_m$ value of the protein. Figure 5A,B shows, respectively, the similar $E_m$ versus pH profiles for the D30A and D65A variants of *C. reinhardtii* thioredoxin *h*. As was the case for the wild-type protein and its W35A variant, stability considerations limited the $E_m$ data for the D65A variant of *C. reinhardtii* thioredoxin *h* to pH values ≤9.5, while the slightly greater stability of the D30A variant allowed $E_m$ determinations up to pH 10.0. The $E_m$ versus pH profile for the D30A and D65A variants of *C. reinhardtii* thioredoxin *h* are, in general, quite similar to those obtained for the wild-type protein and its W35A variant. Fitting the data sets of Figure 5 to eq 1 yields p$K_a$ values of 8.7 and >10.0 for the D30A variant of *C. reinhardtii* thioredoxin *h* and two p$K_a$ values, both >9.3, for the D65A variant.

## DISCUSSION

The disagreement between published values for the p$K_a$ of Cys32 in *E. coli* thioredoxin, obtained using a number of quite different techniques, prompted us to utilize the thermodynamic relationship between the pH-dependence of $E_m$ values for thioredoxin reduction and the p$K_a$ values of acid/base couples associated with reduction of the protein in an attempt to provide new information about this p$K_a$ and other thioredoxin p$K_a$ values. We were encouraged in this effort by the fact that the p$K_a$ value for the more acidic regulatory cysteine residue in spinach phosphoribulokinase had been successfully measured in our laboratory using this technique (*44*). Although we were unable to determine p$K_a$ values for the active-site cysteines in either *E. coli* thioredoxin or in *C. reinhardtii* thioredoxin *h* using this approach, the thermodynamic approach did prove fruitful in that it demonstrated that these two structurally similar thioredoxins both unexpectedly take up two protons on reduction at pH values significantly more alkaline than the reported p$K_a$ values for the more acidic active-site cysteine instead of the one proton that would be predicted by the simplest models.

On the basis of the complex [$^{13}$C]NMR-based acid/base titrations of reduced *E. coli* thioredoxin, it was proposed that Asp26 interacts with Cys32 at the active site of the protein in a manner that requires the invocation of microscopic p$K_a$ values of 7.5 and 9.2 for these two coupled residues (*34*). The shortest distances between an oxygen from the side-chain carboxyl group of Asp26 and the sulfur of Cys32 (i.e., 5.9 Å in reduced *E. coli* thioredoxin and 5.6 Å in oxidized *E. coli* thioredoxin (*54*)) are too large for any direct hydrogen-bonding interaction between these two amino acids. Nevertheless, support for the idea that Cys32 and Asp26 are coupled came from both site-directed mutagenesis studies and from acid/base titrations of the oxidized protein. The observation that acid/base titrations of Asp26 in oxidized, wild-type *E. coli* thioredoxin (where there is no thiol group on Cys32 to interact with Asp26) gave well-defined curves that could be fitted to the equation for an acid dissociation characterized by a single p$K_a$ value of 7.5 (*34*), in contrast to the more complex titration curves observed for the wild-type protein, is consistent with this proposal. Furthermore, if this interpretation is correct, then making the Cys32/Asp26 interaction impossible by site-directed mutagenesis of Asp26 should alter the acid/base titration behavior of Cys32 so that microscopic p$K_a$ values no longer need be invoked. In fact, acid/base titrations of Cys32 in the reduced forms of both the D26L and D26N variants of *E. coli* thioredoxin exhibit well-defined curves characteristic of a single p$K_a$ (*34*), consistent with this hypothesis. Similar considerations apply to *C. reinhardtii* thioredoxin *h*, where the recent 2.1−2.2 Å resolution X-ray crystal structures of wild-type *C. reinhardtii* thioredoxin *h* and its D30A variant (*18*) have allowed the question of a possible direct interaction between Asp30 (which corresponds to Asp26 in *E. coli* thioredoxin) and Cys36 (which corresponds to Cys32 in *E. coli* thioredoxin) in the algal protein to be examined. It was concluded that the 5.9 Å distance between the OD1 carboxylate oxygen of Asp30 and the SG sulfur of Cys36 is too great to allow any direct, proton-sharing interaction between these two amino

acids (*18*). However, molecular dynamics simulations and solvent isotope effect measurements suggest that an oriented water molecule may bridge the gap between Asp30 and Cys 36 (*18*).

In conclusion, it should be stressed that reduction of thioredoxins involves the uptake of both electrons and protons, with the number of protons taken up per two-electron reduction of an active-site disulfide depending on the ambient pH and the p$K_a$ values of amino acids involved in redox-coupled proton uptake. *E. coli* thioredoxin and *C. reinhardtii* thioredoxin *h* are ideally suited to studying this coupling because three-dimensional structures are available for both proteins, because site-specific variants of both proteins are readily available, and because both proteins are stable over a wide range of pH values. All of the currently available models for proton uptake coupled to the reduction of the structurally related *E. coli* thioredoxin and *C. reinhardtii* thioredoxin *h* would predict a transition, centered near pH = 7.5 (the p$K_a$ of Cys32), from the uptake of 2.0 protons per thioredoxin reduced to 1.0 proton taken up per thioredoxin reduced for the D26L and D26N variants of *E. coli* thioredoxin and a similar transition, centered near pH 7.0 (the p$K_a$ of Cys36) for the D30A mutant of *C. reinhardtii* thioredoxin *h*. However, as can be seen from the data of Figures 3A and 5A, this is not the case. It thus must be concluded that either bridging water molecules and/or some other amino acid(s), perhaps including Lys57 in *E. coli* thioredoxin (*36*) and Lys61, the corresponding residue in *C. reinhardtii* thioredoxin *h*, contribute to proton uptake accompanying the reduction of the two thioredoxins in this pH region. If an extensive network of amino acids exists for the transfer of protons during thioredoxin reduction, it may be necessary to analyze thioredoxin variants mutated at multiple positions to fully understand the coupling of proton uptake to disulfide reduction in these ubiquitous proteins. Nevertheless, the data presented previously provide significant evidence for the apparent complexity of the coupling process.

## ACKNOWLEDGMENT

The authors thank Amy Sorrels, Amy Daniels, Jeremy Dalton, and Lisa Walters for their assistance with the redox titrations and Profs. Jean-Marc Lancelin and Peter Schürmann for their many helpful suggestions.

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BI0302088