Proton Stoichiometry in the Reduction of the FAD and Disulfide of *Escherichia coli* Thioredoxin Reductase

**EVIDENCE FOR A BASE AT THE ACTIVE SITE***

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The oxidation-reduction midpoint potentials, $E_m$, of the FAD and active site disulfide couples of *Escherichia coli* thioredoxin reductase have been determined from pH 5.5 to 8.5. The FAD and disulfide couples have similar $E_m$ values and thus a linked equilibrium of four microscopic enzyme oxidation-reduction states exists. The binding of phenylmercuric acetate to one enzyme form could be monitored which allowed solving the four microscopic $E_m$ values. The $E_m$ values at pH 7.0 and 12°C of the four couples of thioredoxin reductase are: ($S_2$)-enzyme-FAD/FADH$_2$ = −0.243 V, (SH)$_2$-enzyme-FAD/FADH$_2$ = −0.250 V, (FAD)-enzyme-(S)$_2$ (SH)$_2$ = −0.254 V, and (FADH$_2$)-enzyme-(S)$_2$/ (SH)$_2$ = −0.271 V. Thus, at pH 7.0, the FAD and disulfide moieties have a 0.017-V negative interaction and $E_m$ values which are different by 0.011 V. The $\Delta E_m$/dpH of the FAD couples $E_m^a$ and $E_m^b$ are about 0.052 V/pH throughout the pH range studied, showing an approximately 2-proton stoichiometry of reduction of the enzyme FAD. The $\Delta E_m$/dpH of the disulfide couples $E_m^a$ and $E_m^b$ are about 0.052 V/pH from pH 5.5 to 8.5, showing an apparently nonintegral proton stoichiometry of reduction of 1.8 in this pH range. This proton stoichiometry suggests the presence of a base with an ionization behavior that is linked to the oxidation-reduction state of the disulfide. A novel method is presented for determining the pK values on oxidized and reduced enzyme which agrees with the less accurate classical method. The proton stoichiometry results are consistent with the presence of a thiol-base ion pair in which the pK of the base is elevated from 7.6 in disulfide containing enzyme to greater than 8.5 upon forming an ion pair with a thiol anion of pK 7.0 generated upon reduction of the disulfide. The fluorescence of the FAD in thioredoxin reductase decreases as the pH is lowered with a pK of 7.0, direct evidence for a base near the FAD probably distinct from the base interacting with the dithiol.

Thioredoxin reductase catalyzes the reversible transfer of electrons between NADPH and thioredoxin, a small protein containing an oxidation-reduction active disulfide (Zanetti and Williams, 1967; Moore et al., 1964). Thioredoxin reductase contains an FAD and an oxidation-reduction active site disulfide (Zanetti and Williams, 1967; Moore et al., 1964; Ronchi and Williams, 1972). It is thought that the electrons flow sequentially from NADPH to the FAD, from the FAD to the disulfide, and from the dithiol on thioredoxin reductase to the disulfide on thioredoxin. In a 4-electron reduction of thioredoxin reductase at pH 7.6, there is a gradual bleaching of the flavin absorbance throughout the titration (Zanetti and Williams, 1967). Thus, at pH 7.6, the FAD and disulfide appear to have similar oxidation-reduction potentials. The reduction of thioredoxin reductase by NADH is described by the linked equilibria of four enzyme microforms shown in Scheme 1. Use of the nonphysiological pyridine nucleotide avoids the complexes characteristic of reduction with NADPH and is faster than reduction with dithionite.

The two thiol groups generated upon reduction of the active site disulfide are expected to carry out different tasks by analogy with lipoamide dehydrogenase (Thorpe and Williams, 1976a and 1976b) and glutathione reductase (Arscott et al., 1981). Presumably, FADH$_2$ reduces the active site disulfide by transfer of electrons to the disulfide via the sulfur which is proximal to the FAD (referred to as the flavin thiol), and the electrons in the active site dithiol are passed to the disulfide of thioredoxin in the other active-site thiol, referred to as the interchange thiol (Scheme 2).

Model studies show thiol-dissulfide interchange to be initiated by attack of a thiol anion on the disulfide (Foss, 1961). Rapid reduction of the disulfide bond in thioredoxin by thioredoxin reductase proceeds at pH 7.0, well below the pK value of model thiols (Moore et al., 1964). Thus, the environment at the active site of thioredoxin reductase must lower the pK of the interchange thiol. There is substantial evidence for a low pK value of an active site cysteinyl thiol (pK = 3–5) in four enzymes: papain (Polgar, 1973; Lewis et al., 1978), glyceraldhyde-3-phosphate dehydrogenase (Polgar, 1973), lipoamide dehydrogenase (Matthews et al., 1977), and glutathione reductase (Arscott et al., 1981). A thiol-base ion pair in a relatively apolar milieu is hypothesized to explain the low pK value of the thiol in each of these enzymes. The resulting thiolate in lipoamide dehydrogenase and glutathione reductase is the donor in a charge transfer complex with the flavin (Scheme 2) (Kosower, 1966; Massey and Ghisla, 1974; Sears and Sanadi, 1961). Because the oxidation-reduction potentials of the two couples are widely separated, two-electron reduced enzyme can be considered a single species in which the sulfurs are reduced and the FAD is not reduced (in the ground state). No such charge transfer complex has been observed in thioredoxin reductase and the oxidation-reduction potentials of

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FAD and disulfide into the concentrations of the four enzyme microforms. The separate $E_m$ values of each of the four enzyme couples (Scheme 1) are calculated from the concentrations of the four enzyme microforms in equilibrium with the oxidized and reduced species of titrant of known $E_m$ using the Nernst relationship. The titrants used in the microform titrations were NADH and an analog which has a more positive $E_m$, APADH, in order to avoid the complexes formed between the enzyme and its physiological pyridine nucleotide substrate.

The detailed explanation of the measurements of the equilibrium concentrations of the oxidized and reduced macroscopic enzyme species (FAD/FADH$_2$, (SH)$_2$/((S)_2) and titrant species is given in the Miniprint. Briefly, the concentrations of enzyme-FAD and enzyme-FADH$_2$ at each titration point were calculated from absorbance measurements at 456 nm; the concentration of NADH (APADH) in equilibrium with thioredoxin reductase was obtained from absorbance increases at an isosbestic point for thioredoxin reductase reduction (= 347 nm); the concentration of NAD$^+$ (APAD$^+$) in equilibrium with the enzyme was the difference between the titrant NADH added and the NADH in equilibrium with the enzyme; the concentration of the reduced active site disulfide (enzyme-(SH)$_2$) was the difference between the amount of titrant oxidized by the enzyme and the amount of enzyme-FADH$_2$; and the concentration of the oxidized active site disulfide (enzyme-(SH)$_2$) was the difference between the total enzyme concentration and the enzyme-(SH)$_2$ concentration.

The profiles of enzyme-FADH$_2$ formed during anaerobic titrations by NADH are shown in Fig. 1 for pH values of 6.0 and 8.1. At pH 6.0, the $E_m$ values of the two FAD couples $E_m^I$ and $E_m^H$ are higher (less negative) than the $E_m^I$ values of the two disulfide couples $E_m^I$ and $E_m^H$, respectively, leading to greater concentrations of enzyme-FADH$_2$ relative to enzyme-(SH)$_2$ throughout the titration (e.g. approximately 70% FADH$_2$ and 30% dithiol at 1 equivalent of reduction). At pH 8.0, the enzyme-FADH$_2$ titration profile is a straight line, showing that the potentials of the two FAD couples $E_m^I$ and $E_m^H$ equal the potentials of the two disulfide couples $E_m^I$ and $E_m^H$, respectively. Since the $E_m^H$ of the FADH couple increases

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1 Portions of this paper (including "Materials and Methods" and Table I) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 83M-777, cite authors, and include a check or money order for $7.20 per set of photocopies. Full size photocopies are also included in the microfilm edition of the journal that is available from Waverly Press.

2 The abbreviations used are: PMA, phenylmercuric acetate; APAD$^+$, oxidized 3-aceetylpyridine adenine dinucleotide; APADH, reduced 3-aceetylpyridine adenine dinucleotide.
relative to the $E_m$ of the disulfide couple with decreasing pH, the disulfide couple must have a lower $H^+$ stoichiometry of reduction than the FAD couple.

The unequal values of $E_m$ for the FAD and disulfide couples at pH 6.0 afforded a unique opportunity to measure the rate of electron transfer among individual microforms. This is important since it is essential to the calculation of $E_m$ values that the oxidized and reduced enzyme species are at equilibrium when the absorbance measurements are made. The FAD of 1-equivalent reduced thioredoxin reductase is 70% reduced at pH 6.0. An anaerobic solution of oxidized enzyme was added to an equal amount of fully reduced enzyme (stoichiometric sodium dithionite) to give a solution initially of 50% enzyme-FADH$_2$, which increased to a concentration of 70% upon equilibrating to a mixture of the four enzyme microforms shown in Scheme I. The approach to equilibrium was monitored at 456 nm and found to occur with a half-life of about 8 min at pH 6.0. The addition of a 5-fold excess (over total enzyme) of PMA to the equilibrating mixture slowed the approach to equilibrium over 100-fold, implying that thiol-disulfide interchange mediates the equilibration of microforms I with microform IV to give microforms II and III. At pH values much above 6.0, the concentration of enzyme-FADH$_2$ is approximately equal to the concentration of enzyme-(SH)$_n$, thus precluding measurements of equilibration rates among these microforms. However, if electron transfer between microforms I and IV occurs via thiol-disulfide interchange, the rate of equilibration of these microforms should increase with increasing values of pH. The rate of equilibration between microforms II and III is an intramolecular electron transfer and a catalytic step and is therefore rapid on the time scale of the experiments in this study. To ensure that the criterion of equilibrium among enzyme microforms was fulfilled, absorbance measurements were not recorded until the absorbance changes had stabilized to the slow, steady changes due to the comproportionation of FAD and FADH$_2$ enzyme species to form semiquinone as described in the Miniprint.

Calculation of the apparent $E_m$ value for the macroscopic FAD/FADH$_2$ couple and for the macroscopic (SH)$_2$/(SH)$_n$ couple at each titration point showed their apparent $E_m$ values decreased steadily throughout the titration. For example, the calculations of the macroscopic FAD/FADH$_2$ couple of thioredoxin reductase at pH 7.0 shows 2 difference in $E_m$ of 0.007 V between the fractional FADH$_2$ levels of 0.38 and 0.78. The difference in $E_m$ for the macroscopic (SH)$_2$/(SH)$_n$ couple in the same experiment is 0.010 V between the fractional (SH)$_2$ levels of 0.19 and 0.53. Furthermore, for the enzyme-FADH$_2$ titration profiles at all pH values (e.g. Fig. 1 for pH 6.0 and 8.1), a line drawn through the first few data points intersects with a line drawn through the last few data points at 1.0 ± 0.1 Equivalents (see Miniprint for explanation of Equivalents). Thus, both of the macroscopic couples (FAD/FADH$_2$ and (SH)$_2$/(SH)$_n$) tend to have a slightly lower apparent value of $E_m$ throughout a titration. This may be explained by a small intramolecular negative interaction between the FAD and disulfide moieties of the enzyme. Specifically, in Scheme I, the $E_m$ value for the FAD couple where the disulfide is oxidized in these microforms is higher than the $E_m$ value for the FAD couple where the microforms contain a dithiol. In other words, reduction of the disulfide lowers the $E_m$ value of the FAD/FADH$_2$ couple and, conversely, reduction of the FAD lowers the $E_m$ value of the (SH)$_2$/(SH)$_n$ couple. In Scheme I, an interaction between the FAD and disulfide is described by the ratio $K_f/K_o = (K_f/K_o)$. This ratio is 1.0 for the case of no interaction between the FAD and disulfide and is about 4 for a negative interaction of the magnitude observed in this study. A non-Nernstian behavior could also be expected if the enzyme solution contained enzyme that was partially denatured. Two different preparations of thioredoxin reductase exhibited the same titration behavior, making this explanation less likely.

A full description of the stoichiometry of reduction of the FAD and disulfide centers of thioredoxin reductase requires separating the macroscopic measurements of FAD/FADH$_2$ and (SH)$_2$/(SH)$_n$ into the four microscopic enzyme forms of Scheme I. These quantities allow the $E_m$ values for the four microscopic enzyme couples to be calculated at different pH values and the $H^+$ stoichiometry of reduction of each microform to be obtained from the slope $\Delta E_m/\Delta pH$. Quantitation of the four enzyme microforms at 1 equivalent of reduction was accomplished in a separate experiment at each pH value using PMA as a probe of the equilibrium concentrations of enzyme microforms.

The organic mercurial $p$-chloromercuriphenylsulfonate binds tightly to the dithiol of reduced thioredoxin reductase (Zanetti and Williams, 1967). Addition of PMA to a partially reduced solution of thioredoxin reductase results in a rapid change in the absorbance at 456 nm. The absorbance change is due to PMA binding to microform II which pulls microform III into the microform II-PMA complex via the rapid intramolecular electron transfer equilibrium between microform III and microform II (catalytic step). Maximal absorbance changes upon adding PMA were obtained with a 1.6-fold excess of PMA over total enzyme. A 5-fold excess of PMA over total enzyme was used routinely in PMA addition experiments.

The concentration of each of the four enzyme microforms at any level of reduction before PMA addition can be calculated from a PMA addition experiment. These calculations are detailed in the Miniprint. Briefly, the concentration of microform III before PMA addition is calculated from the increase in absorbance at 456 nm upon adding PMA. The concentration of microform IV before PMA addition is equal to the residual FADH$_2$ after the addition of PMA, the only microform containing FADH$_2$ after PMA addition. The concentration of microform II before PMA addition is the product of the enzyme concentration and the number of equivalents added to reduce the enzyme minus the sum of the concentrations of microforms III and IV. The concentration of microform I before PMA addition is the difference between the total enzyme concentration and the sum of the concentrations of the other microforms.

Sodium dithionite is used to reduce thioredoxin reductase in PMA addition experiments since pyridine nucleotide catalyzes electron transfer between the microform IV-PMA complex and oxidized enzyme (microform I) to produce the microform II-PMA complex, resulting in anomalously high estimates for the concentration of microform III. Anaerobic reductions of thioredoxin reductase by sodium dithionite are not quantitative at pH values below 6.5. Hence, the number of sodium dithionite reducing equivalents in thioredoxin reductase before PMA addition is obtained by calculating the percentage of enzyme-FADH$_2$ from the absorbance at 456 nm and comparing this value with the enzyme-FADH$_2$ titration profile (Fig. 1) at the same pH value. Sodium dithionite reductions of thioredoxin reductase take about 1 h, ensuring that the enzyme forms are at equilibrium and validating the comparison with an NADH titration.

The equilibrium concentrations of the four enzyme microforms of Scheme I at one level of reduction allows the important ratios, $K_2/K_1$ and $K_4/K_3$ to be calculated. The ratio $K_2/K_1 = ([III]/[III])$ is a quantitative measure of the difference in
Enzyme microforms at each NADH titration point are calculated. The data for the FAD couple, as well as the concentrations of NAD+ and NADH in equilibrium, are given in Table I. It can be seen from Fig. 2 that, for maximal accuracy in measuring the concentrations of enzyme microforms, thioredoxin reductase was reduced by about 8.88 equivalents of dithionite before adding PMA. The variability of the disulfide and FAD ratios obtained from a PMA addition experiment allow simulation of the enzyme-FADH2 titration profile as shown by addition experiments at pH 6.0 and 8.1. The ratios $K_1/K_2$ and $K_1/K_4$ obtained from PMA experiments at the values of pH and concentrations of thioredoxin reductase used in this study are given in Table II. It can be seen from Fig. 2 that, for maximal accuracy in measuring the concentrations of enzyme microforms, thioredoxin reductase was reduced by about 1 Equivalents of dithionite before adding PMA. The variability in the ratio $K_1/K_4$, discussed in the Miniprint. It is apparent, however, that the FAD and disulfide have a negative interaction from the values of $K_1/K_4$ which are greater than unity at all pH values, and that the oxidation-reduction potentials of the disulfide and FAD ($K_1/K_4$) diverge as the pH is lowered.

The relative concentrations of each enzyme microform as well as the concentrations of NAD+ and NADH in equilibrium with them are required to calculate the microscopic $E_m$ values. Thus, two experiments are required at each pH value to calculate the microscopic $E_m$ values: 1) an NADH titration which gives the concentrations of NAD+ and NADH that are in equilibrium with the enzyme at each titration point, and 2) a PMA addition experiment that gives the two ratios $K_1/K_2$ and $K_1/K_4$ from which the relative concentrations of the four enzyme microforms at each NADH titration point are calculated. The data for the FAD couple, $E_m$, and the disulfide couple, $E_m'$, calculated in this manner are plotted according to the Nernst relationship in Fig. 3 for experiments at pH values of 6.0, 7.0, and 8.1. The solid lines drawn through the data points represent theoretical curves of $E_m$ versus fractional reduction calculated for a 2-electron reduction assuming the

### Table II

| pH  | [Enzyme] | $K_1/K_2$ | $K_1/K_4$ |
|-----|----------|-----------|-----------|
| 5.5 | 16.3     | 0.217     | 4.50      |
| 6.0 | 8.4      | 0.242     | 3.68      |
| 6.5 | 15.6     | 0.242     | 3.82      |
| 6.0 | 44.5     | 0.254     | 5.91      |
| 6.2 | 17.5     | 0.242     | 7.21      |
| 6.5 | 18.2     | 0.288     | 7.40      |
| 6.8 | 17.2     | 0.304     | 6.80      |
| 7.0 | 8.1      | 0.385     | 3.68      |
| 7.0 | 17.5     | 0.410     | 4.09      |
| 7.2 | 17.7     | 0.437     | 5.53      |
| 7.4 | 17.9     | 0.506     | 5.43      |
| 7.6 | 17.6     | 0.750     | 5.46      |
| 7.8 | 17.6     | 0.790     | 7.30      |
| 8.0 | 17.7     | 0.998     | 5.77      |
| 8.1 | 17.4     | 0.995     | 4.35      |
| 8.1 | 50.1     | 1.000     | 3.71      |
| 8.3 | 17.5     | 0.984     | 3.71      |
| 8.5 | 17.8     | 0.999     | 3.35      |

### Figure 2

Relationship of the distribution of the four oxidation-reduction microforms of thioredoxin reductase to the number of reducing equivalents incorporated into the enzyme. The ratios $K_1/K_2 = 0.242$, $K_1/K_4 = 3.82$ were determined in a PMA addition experiment using 15.6 μM enzyme in Buffer A at pH 6.0, 12 °C.

### Figure 3

Nernst curves of the FAD couple and the disulfide couple for NADH titrations combined with the results of PMA addition experiments at different concentrations of enzyme and at three different values of pH. Ratios ($K_1/K_2$ and $K_1/K_4$) appropriate to the pH and enzyme concentration were used (Table II). The open symbols refer to the FAD/FADH2 couple and the closed symbols to the disulfide couple. The different symbols represent separate titration experiments, pH 6.0: ○, 5.35 μM enzyme and 0.846 mM NAD'; ▲, 17.2 μM enzyme and 0.627 mM NAD'; ▼, 17.1 μM enzyme and 1.53 mM NAD'; ●, 42.5 μM enzyme and 1.72 mM NAD'; pH 7.0: ○, 8.47 μM enzyme and 0.246 mM NAD'; ▲, 16.16 μM enzyme and 0.94 mM NAD'; ▼, 17.3 μM enzyme and 0.48 mM NAD'; pH 8.1 (since the FAD and disulfide couples have equal potentials at pH 8.1, the open symbols are not visible): ●, 17.65 μM enzyme; ●, 44.1 μM enzyme. $E_m$ was calculated for each point as described in the Miniprint. The solid lines drawn through the data points represent theoretical curves calculated for a 2-electron reduction process.
with thioredoxin reductase. To test further for complexation, too low to measure until late in the titration. Thus, at these concentrations of enzyme (9-44 μM) and either NAD' or APAD' under the conditions employed, suggesting that neither NAD' nor APAD' forms a complex with reduced thioredoxin reductase (Zanetti and Williams, 1967). Charge transfers were observed between reduced thioredoxin reductase and one oxidation-reduction form of pyridine nucleotide containing an oxidized disulfide has a 2.1 - H' stoichiometry throughout the pH range studied. The slope of the disulfide couple ΔE₂/ΔpH is 0.052 V/pH from pH 5.5 to 8.5 employing a least squares analysis (correlation coefficient = 0.997). Thus, reduction of the disulfide in thioredoxin reductase containing an oxidized FAD is accompanied by a 1.8 - H' stoichiometry from pH 5.5 to 8.5.

The ΔE₄⁵/ΔpH and ΔE₅⁵/ΔpH profiles for the second equivalent of enzyme reduction (Fig. 4B) are similar to the profiles for the first equivalent of enzyme reduction (Fig. 4A) except that the values of E₄⁵ and E₅⁵ are lower than the E₁⁵ and E₂⁵ values by an average of 0.020 ± 0.011 V at all pH values.

The small differences in ΔE₄⁵/ΔpH profiles between a preliminary study (O'Donnell and Williams, 1981) and the ΔE₃⁵/ΔpH profiles of Fig. 4, A and B are due to the use of extinction coefficients of enzyme species determined at pH 7.0 for calculating values of E₃⁵ at all pH values and also for not making dilution corrections for the concentration of titrant NADH added. In addition, the E₃⁵ values at pH 7.0 of the pyridine nucleotide couples and the value of RT/NF were not corrected for temperature in the preliminary study.

E₄⁵ calculated from the data points. Only data points which were within 0.004 V of the average E₄⁵ of a titration were used in averaging the calculated E₄⁵ values. The values of E₄⁵, at pH 7.0 were: E₄⁵ = -0.254 V, E₄⁵ = -0.243 V, E₄⁵ = -0.260 V, and E₄⁵ = -0.271 V.

At pH 6.0 and 7.0, the equilibrium between pyridine nucleotide and enzyme greatly favors the oxidation of NADH, resulting in equilibrium concentrations of NADH which are too low to measure until late in the titration. Thus, at these pH values, NAD⁺ was added prior to the titration, allowing equilibrium measurements of NADH at lower values of fractional enzyme reduction.

The validity of E₄⁵ values calculated from measurements of enzyme species in equilibrium with a reference couple and titrant is negligible, or equal in strength for all enzyme microforms. A long wavelength charge transfer band is associated with complex formation between NADP⁺ and reduced thioredoxin reductase (Zanetti and Williams, 1967). Charge transfer is not observed between reduced thioredoxin reductase and either NAD⁺ or APAD⁺ under the conditions employed, suggesting that neither NAD⁺ nor APAD⁺ forms a complex with thioredoxin reductase. To test further for complexation, titrations of the enzyme with NADH were performed using different concentrations of enzyme. Oxidation-reduction equilibria are independent of concentration, whereas complexation equilibria are concentration dependent. Thus, doubling the concentration of enzyme will increase the extent of complexation 4-fold. If significant complexation occurred preferentially between one oxidation-reduction form of thioredoxin reductase and one oxidation-reduction form of pyridine nucleotide, the calculated midpoint potential should shift as the enzyme concentration is varied. The values of E₄⁵ for the four enzyme couples were determined using different concentrations of enzyme (9-44 μM) and were within 0.002 V. This difference is within the experimental error of the system. These data are shown in Fig. 3 for the FAD and disulfide couples (E₄⁵ and E₅⁵, respectively) at pH values of 6.0, 7.0, and 8.1.

The E₄⁵ values for the four microscopic couples of thioredoxin reductase were calculated from pyridine nucleotide titrations and PMA addition experiments at pH values spanning the range from 5.5 to 8.5. The results of the first equivalent of reduction for the FAD couple, E₄⁵, and the disulfide couple, E₅⁵, are shown in Fig. 4A. The results of the second equivalent of reduction for the FAD couple, E₅⁵, and the disulfide couple, E₆⁵, are shown in Fig. 4B. The variation of E₅⁵ with pH has a slope, ΔE₅⁵/ΔpH, of 0.060 V/pH and a correlation coefficient of 0.997 by a least squares analysis. The ΔE₅⁵/ΔpH for an oxidation-reduction couple with a 2-proton stoichiometry is, theoretically, 0.0566 V at 12°C (Clark, 1960, Appendix, Table E). Thus, the results of Fig. 4A show that the reduction of the FAD in thioredoxin reductase containing an oxidized disulfide has a 2.1 - H⁺ stoichiometry throughout the pH range studied. The slope of the disulfide couple ΔE₆⁵/ΔpH is 0.052 V/pH from pH 5.5 to 8.5 employing a least squares analysis (correlation coefficient = 0.997). Thus, reduction of the disulfide in thioredoxin reductase containing an oxidized FAD is accompanied by a 1.8 - H⁺ stoichiometry from pH 5.5 to 8.5.
The active center base in thioredoxin reductase is about 0.2 – H⁺/pH unit less than theoretical throughout the range of study. This suggests the presence of a base that ionization behavior of which is linked to the oxidation-reduction state of the disulfide. Such a base will alter the intramolecular equilibrium between the FAD and disulfide couples in 2-electron reduced enzyme as shown in Scheme 3. The ionizable group on the dithiol enzyme can be either the same ionizable amino acid side chain as in disulfide enzyme (except with a lowered pK on dithiol enzyme) or one of the nascent active site thiol having a low pK due to an interaction with the protonated base, i.e., a thiol-base ion pair. The formation of a thiol-base ion pair having a thiol anion with a low pK is predicted from the chemistry of thioredoxin reductase catalysis and has a precedent in a number of enzymes including the closely related flavoenzymes lipoamide dehydrogenase and glutathione reductase. The equilibrium constant between microforms II and III is the ratio $K_1/K_2$ (Scheme 1). The value of $K_1/K_2$ is obtained from the data of a PMA addition experiment. The intramolecular equilibrium constant $K_1/K_2$ (Table 11) and the inverse $K_2/K_1$ are plotted as a function of pH in Fig. 5. The interpretations of these plots are derived in the Miniprint. The pK value from the $K_1/K_2$ plot is that of a group on microform III (pKs, Scheme 3) and the pK value from the $K_2/K_1$ plot is that of a group on microform II (pKs, Scheme 3). The values of pH and pKs are 7.59 and 6.98, respectively. The values for the intramolecular equilibrium, microform II to microform III, for the fully protonated $(K_T)$ and deprotonated $(K_B)$ enzyme forms are obtained from the acidic and basic limbs of the theoretical fits to the data of Fig. 5.

Clark (1980) develops equations for situations similar to that just described in which the enzyme species that contain a disulfide (III) have an ionization with a pK greater than...
that of an ionization on enzyme species that contain a dithiol (II) (Clark, 1969, pp. 118-130). Following Clark, we have derived an equation relating $E_n$ to pH for this case in the Miniprint. If the two $pK$ values were more widely separated, the $E_n$ versus $pH$ curve would have a slope of $0.0566 \text{ V} \text{pH}$ above the acid $pK$, a slope of $0.0283 \text{ V} \text{pH}$ between the two $pK$ values, and a slope of $0.0566 \text{ V} \text{pH}$ above the alkaline $pK$. Since the two $pK$ values are separated by only 0.6 pH unit, the slopes merge as shown for an ideal case in Fig. 6. The actual value of the $E_n$ at a $pH$ equal to a $pK$ will be $0.0086 \text{ V}$ above or below the theoretical slopes (dashed lines) at 12°C (Clark, 1969, p. 128). Thus, for the case in point, the data will appear to define a straight line. Using the equation relating $E_n$ and pH (see Miniprint) and the $pK$ values determined in Fig. 5, the slope of the line between $pH$ 5.5 and $pH$ 8.5 is 0.050 V/$pH$. Thus, this model fits the data (with a slope of 0.052 V/$pH$) reasonably well, indicating that the decreased slope of the $E_n$ versus $pH$ is due to interaction of a base near the disulfide.

There is also a base near the FAD in oxidized thioredoxin reductase as indicated by the dependence of the FAD fluorescence on pH shown in Fig. 7. A theoretical fit to the data for a single ionization yields a $pK$ of 7.03, the line drawn through the data points of Fig. 7. Further evidence for a $pK$ on the oxidized enzyme comes from close examination of the absorbance spectra revealing a pH-dependent change in the ratio of extinction coefficient of the 380 nm peak to the 455 nm peak from 1.03 at $pH$ 7.6 to 0.99 at $pH$ 6.0.

**DISCUSSION**

The oxidation-reduction midpoint potentials ($E_m$) of the FAD and disulfide couples in thioredoxin reductase have been determined at pH values spanning the range 5.5—8.5. The proton stoichiometry of the disulfide couple (obtained from the slope of $E_m$ versus $pH$ plots) was 1.8 protons while the proton stoichiometry of the FAD couple was 2.1 protons. The proton stoichiometry of the disulfide couple compared to the FAD couple is reflected in the observation of an increased ratio of flavin reduction to disulfide reduction during titrations performed at low pH relative to titrations at high pH. The proton stoichiometry results disclose the presence of a basic amino acid side chain with an ionization behavior that is linked to the oxidation-reduction state of the disulfide.

The ionization of an enzyme base linked to the oxidation-reduction state of the disulfide is also found in the flavoproteins lipoamide dehydrogenase and glutathione reductase which contain an active center disulfide. In lipoamide dehydrogenase, an essential base on oxidized enzyme has a $pK$ of less than 5.5 (Matthews et al., 1977). Upon reduction of the active center disulfide in lipoamide dehydrogenase, a thiol-base ion pair forms in which the thiol has a $pK$ of about 4.8, and the $pK$ of the base is shifted to 7.8 (Matthews et al., 1977). Thiol-base ion pairs involving thiols of low $pK$ have also been demonstrated in papain (Polgar, 1973; Lewis et al., 1976), glyceraldehyde-3-phosphate dehydrogenase (Polgar, 1975), and glutathione reductase (Arscott et al., 1981).

The proton stoichiometry of the disulfide couple in thioredoxin reductase is consistent with the formation of a thiol-base ion pair upon reduction of the disulfide. The ion pair hypothesis for thioredoxin reductase is shown in Scheme 4 where the $pK$ of the base is assumed to be the same in enzyme containing a disulfide as in enzyme having a fully protonated dithiol. This assumption is reasonable since the disulfide and the dithiol are both uncharged. The data of the PMA addition experiments yield estimates for the $pK$ values of the group on disulfide enzyme of 7.59 and the group on dithiol enzyme of 6.98. These $pK$ values correspond to the ionization constants of the base ($K_b$) and the thiol ($K_a$) on enzyme containing a fully protonated dithiol (form B, Scheme 4). The $E_m$ versus $pH$ plots for the disulfide couples of thioredoxin reductase (Fig. 4) do not show a break in the slope up to $pH$ 8.5, indicating that more than one proton associates with the enzyme upon disulfide reduction at least up to $pH$ 8.5. Since the enzyme form having a deprotonated base and a thiol anion (form E, Scheme 4) has only one proton, this species must not exist in significant amounts below $pH$ 8.5. Thus, the $pK$ values of the ion pair base ($K_{10}$) and the thiol on enzyme with a deprotonated base ($K_a$) (forms C and D, respectively, Scheme 4) must be greater than 8.5. For the linked equilibria of Scheme 4, $pK_a + pK_b = pK_5 + pK_{10}$. Thus, for a value of $pK_5$ that is greater than 8.5, the value of $pK_{10}$ must be greater than 9.1. The intramolecular equilibrium constant for transfer of a proton from the thiol to the base is about 4.0 ($K_{5}/K_a$, Scheme 4) and, thus, dithiol enzyme exists mainly as an ion pair at physiological $pH$.

The ion pair is an attractive hypothesis because the ionization behavior of both the thiol and base of the ion pair fulfills needed functions predicted by the chemistry of thioredoxin reductase catalysis. Specifically, thiol-disulfide interchange reactions are known to be initiated via attack on the disulfide by a thiol anion (i.e. transfer of electrons between the thiol of thioredoxin reductase to the disulfide of thioredoxin). Since, in the cell, the direction of electron flow is from NADPH to thioredoxin, thioredoxin reductase must initiate the thiol-disulfide interchange reaction with thio-

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4 F. A. Johnson and C. H. Williams, Jr., unpublished.
doxin. The inherent nucleophilicity of a thiolate is dependent on the pK of the thiol. The higher the pK of the thiol, the greater the nucleophilicity of the thiol anion (Wilson et al., 1977; Shaked et al., 1980). However, since the reactive species is the thiol anion, the pK of the thiol must be low enough to yield a significant concentration of thiol anion at the pH of the reaction (Jencks, 1969). Hence, the value of 6.98 for the pK of the putative ion pair thiol of thioredoxin reductase yields a thiol which is largely in a deprotonated state at physiological pH. In addition, formation of the mixed disulfide between thioredoxin reductase and thioredoxin would be concerted with an increase in the acidity of the protonated base which could function as a proton donor to the nascent thiolate of thioredoxin. Thus, both a nucleophilic thiol anion and a protonated base are required for efficient catalysis as encompassed in the thiol-base ion pair model.

The ion pair of lipoamide dehydrogenase and the putative ion pair of thioredoxin reductase is expected in the hydrophobic milieu of thioredoxin reductase. The proton stoichiometry of about 2 for the reduction of the FAD to FADH₂ in thioredoxin reductase is consistent with the spectrum of the enzyme-FADH₂ (Ghisla et al., 1974) and the pH independence of the reduced spectrum. The resolved spectrum of the FAD in thioredoxin reductase reveals that the FAD is in a hydrophobic environment. Although free FADH₂ has a pK of 6.5, an elevation of this pK value is expected in the hydrophobic milieu of thioredoxin reductase.

The function of the slight negative interaction between the FAD and disulfide moieties is not clear but could be simply to increase the concentrations of microforms II and III relative to microforms I and I₁ (Scheme 1). This would be advantageous if a mixture of microforms II and III are more catalytically competent than microform IV.

The methods applied here to thioredoxin reductase will be applicable to any oxidation-reduction protein containing two reducible moieties provided that a unique property can be exploited to determine the concentration of any one of the oxidation-reduction species. In addition, the plot used in Fig. 5 gives a more accurate alternative to the classical method of Clark (1960) for the determination of pK values of bases where the pK is linked to the oxidation-reduction state.

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Active Center Base in Thioredoxin Reductase

Supplementary Material to: PROTON CHEMISTRY IN THE REDUCTION OF THE FAD AND DISHIBILITY OF STRIPOXIN-511 THIOREDOXIN REDUCTASE

Evidence for a base at the active site

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Materials and Methods

Thioredoxin reductase was purified from E. coli by modification of the procedure of Conley and Fight (1975) using 0.1 M saccharose in place of glucose to obtain the enzyme from the FAD-agar plate. Thioredoxin reductase (E.C. 1.8.1.2) leads to partial reduction of FAD (O'Donnell and Williams, unpublished). Sodium dithionite was purchased from Sigma Chemical Co. Thioredoxin reductase (E.C. 1.8.1.2) leads to partial reduction of FAD (O'Donnell and Williams, unpublished).

Experimental Data

The data were fitted to equations using a nonlinear least-squares program (Microsoft, version of June 1973; Levenberg, 1944) as adapted by the Statistical Research Laboratory, The University of Michigan. This program fits a specified function to data by means of stepwise Gauss-Seidel iterations on the parameters.

Assay of thioredoxin reductase was performed with the standard assay procedure (Williams et al., 1949). Each reaction mixture contained 0.01 M Tris-Cl, pH 7.8, 0.5 mM NADPH, and 100 nM thioredoxin in a final volume of 1 ml. After 30 min at room temperature, the reaction was stopped by the addition of 1 ml of 10% trichloroacetic acid. The mixture was aged at 25°C for 30 min and centrifuged at 10,000 g for 10 min. The supernatant was assayed for NADH by a standard assay procedure using the NADH-diphosphokinase. The absorbance at 340 nm was measured on a Beckman model 25 spectrophotometer set at 340 nm. The extinction coefficient of NADH at 340 nm was determined as described by Chance and Maehly (1955).

Titrations of thioredoxin reductase were made by adding aliquots of 0.1 M NaI/NaNO, to the reaction mixture containing thioredoxin reductase and NADH. At each reaction mixture, 0.01 M Tris-Cl was added to give a final concentration of 0.01 M Tris-Cl in the reaction mixture. The mixture was aged at 25°C for 30 min and centrifuged at 10,000 g for 10 min. The absorbance at 340 nm was measured on a Beckman model 25 spectrophotometer set at 340 nm. The extinction coefficient of NADH at 340 nm was determined as described by Chance and Maehly (1955).

The extinction coefficients of thioredoxin reductase were determined at 450 nm by measuring the absorbance at 450 nm of thioredoxin reductase containing FAD and NADH. The extinction coefficient of FAD in the reaction mixture was determined as described by Chance and Maehly (1955).

Below is a table showing the extinction coefficients of various FAD and NADH concentrations at 450 nm.

| [FAD] (mM) | [NADH] (mM) | [FAD] extinction coefficient | [NADH] extinction coefficient |
|-----------|-------------|-----------------------------|-----------------------------|
| 0.0        | 0.0         | 1.0                         | 1.0                         |
| 0.1        | 0.1         | 0.98                        | 0.98                        |
| 0.2        | 0.2         | 0.96                        | 0.96                        |
| 0.3        | 0.3         | 0.94                        | 0.94                        |
| 0.4        | 0.4         | 0.92                        | 0.92                        |
| 0.5        | 0.5         | 0.90                        | 0.90                        |

The extinction coefficients of thioredoxin reductase were determined at 450 nm by measuring the absorbance at 450 nm of thioredoxin reductase containing FAD and NADH. The extinction coefficient of FAD in the reaction mixture was determined as described by Chance and Maehly (1955).

**Table 1**

Extinction coefficients of various FAD and NADH concentrations at 450 nm.

A) The extinction coefficient at 450 nm of thioredoxin reductase containing FAD and NADH was determined as described by Chance and Maehly (1955).

B) The extinction coefficients of various FAD and NADH concentrations at 450 nm were determined as described by Chance and Maehly (1955).

**Figure 1**

The extinction coefficients of thioredoxin reductase at 450 nm were determined as described by Chance and Maehly (1955). The extinction coefficients of thioredoxin reductase at 450 nm were determined as described by Chance and Maehly (1955).

**Figure 2**

The extinction coefficients of thioredoxin reductase at 450 nm were determined as described by Chance and Maehly (1955). The extinction coefficients of thioredoxin reductase at 450 nm were determined as described by Chance and Maehly (1955).
The extinction coefficients of 560 at wavelengths other than 547 nm were obtained from a calculated spectrum of 560. To calculate the spectrum of 560, the absorbance of 560 was calculated for each wavelength using an extinction coefficient of 3200 \( \text{cm}^{-1} \text{M}^{-1} \). The absorbance at each wavelength was then calculated from this spectrum using the absorbance coefficients of 547 nm. The absorbance coefficients of 547 nm were obtained from a calculated spectrum of 547 nm using an extinction coefficient of 3200 \( \text{cm}^{-1} \text{M}^{-1} \). The absorbance at each wavelength was then calculated from this spectrum using the absorbance coefficients of 547 nm.

The results of this analysis are presented in Table 1. The extinction coefficients of 560 obtained from the calculated spectrum are given in Table 1. The values in Table 1 were used to calculate the absorbance at 547 nm for each sample. The absorbance at each wavelength was then calculated from this spectrum using the absorbance coefficients of 547 nm.

The absorbance coefficients of 560 were determined at 560 nm, 0.0, and 20.0 absorbance units. The absorbance at 547 nm was determined for each sample. The absorbance at each wavelength was then calculated from this spectrum using the absorbance coefficients of 547 nm.

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The absorbance coefficients of 560 were determined at 560 nm, 0.0, and 20.0 absorbance units. The absorbance at 547 nm was determined for each sample. The absorbance at each wavelength was then calculated from this spectrum using the absorbance coefficients of 547 nm.
Substituting the given kinetic constants into the previous equation of the derivation, 
(1)

\[ \text{E}_{2} = \frac{k_{2}}{k_{1}} \times \text{E}_{1} \]

Define \( k_{1} = [\text{E} \cdot \text{F}] \) and \( k_{2} = [\text{E} \cdot \text{F}] \) and substitute into the previous equation:
(2)

\[ \text{E}_{2} = \frac{k_{1}}{k_{2}} \times \frac{[\text{E} \cdot \text{F}]}{[\text{E} \cdot \text{F}]} = \frac{k_{1}}{k_{2}} \times \text{E}_{1} \]

**Calculation of the concentrations of enzyme microforms**

Note: Calculate the new value for \( \text{E}_{1} \cdot \text{F} \) from \( \text{E}_{1} \cdot \text{F} \) to obtain a second approximation of \( \text{E}_{1} \cdot \text{F} \) from the same equation and calculate the constant.
(3)

\[ \text{E}_{1} \cdot \text{F} = \frac{\text{E}_{1} \cdot \text{F}}{\text{E}_{1} \cdot \text{F}} \times \text{E}_{1} \]

**Calculation of the apparent equilibrium constant**

\[ K_{1} = \frac{[\text{E} \cdot \text{F}]}{[\text{E} \cdot \text{F}]} = \frac{[\text{E} \cdot \text{F}]}{[\text{E} \cdot \text{F}]} \]

It is important to note that the measurements of microforms 1 and 2 are the lowest steps because they are at low concentration at the equilibrium of reduction, and the calculation of microform 1 gives the appropriate sum of the sums of the calculation of all the other microforms. Since the calculation of \( k_{1} \) from equation (3) is required, and these microforms are mutants and the final variables around the equilibrium of reduction. These equations are determined by the expression and the data of Table 1. It is apparent however, that the \( \text{E}_{1} \cdot \text{F} \) and \( \text{E}_{1} \cdot \text{F} \) have a negative interaction from the values of \( k_{1} \) which are greater than unity at all pH values.

**Comparison of the NADH turnover with the TMA turnover**

The distribution of enzyme microforms throughout a titration is described by the four independent reactions shown below (1-4) which can be solved simultaneously for four of the eight variables:
(5)

\[ 3 \text{TMAD} + \text{E}_{1} \rightarrow \text{TMAD} \cdot \text{E}_{1} \]

\[ \text{E}_{1} \rightarrow \text{E}_{2} \]

\[ \text{E}_{2} \rightarrow \text{E}_{3} \]

\[ \text{E}_{3} \rightarrow \text{E}_{4} \]

Where \( \text{E}_{1} \) and \( \text{E}_{2} \) are the NADH turnover and the TMA turnover.

**Calculation of the apparent equilibrium constant**

\[ K_{1} = \frac{[\text{E} \cdot \text{F}]}{[\text{E} \cdot \text{F}]} = \frac{[\text{E} \cdot \text{F}]}{[\text{E} \cdot \text{F}]} \]

where \( K_{1} \) is the derivative of the total NADH turnover and \( [\text{E} \cdot \text{F}] \) and \( [\text{E} \cdot \text{F}] \) are the concentrations of all the active centers and the total active centers in TMA turnover. In at \( [\text{E} \cdot \text{F}] \) value of \( [\text{E} \cdot \text{F}] \) the concentration of the active center equals the concentration of total activity and the relationship between \( [\text{E} \cdot \text{F}] \) and \( [\text{E} \cdot \text{F}] \) is obtained following similar determinations in TMA turnover (1969) and it is shown below:
(6)

\[ [\text{E} \cdot \text{F}] = \frac{[\text{E} \cdot \text{F}]}{[\text{E} \cdot \text{F}]} \]

where \( K_{1} \) is the value of \( [\text{E} \cdot \text{F}] \) at pH 6 and the equilibrium constants are those described above.

Using the assignment of equilibrium constants given in Scheme I and the values of A and B in parentheses, respectively, the equilibrium constant, \( K_{1} \), for the pseudo equilibrium between microforms 1 and 2 is
(7)

\[ K_{1} = \frac{[\text{E} \cdot \text{F}]}{[\text{E} \cdot \text{F}]} \]

where \( K_{1} \) is the value of \( [\text{E} \cdot \text{F}] \) at pH 6 and the equilibrium constants are those described above.

The difference in pH values between the initial point of enzyme turnover and the final point is divided into the slopes of the plot and \( [\text{E} \cdot \text{F}] \) vs. [pH] and the amounts of the catalytic and buffer activity of the enzyme. The values of [pH] are determined at least 2 points of [pH] vs. [h] and at intervals between 0.04 and 0.06, as determined from the slopes of \( [\text{E} \cdot \text{F}] \) vs. [pH] and [h] vs. [pH] respectively.
Proton stoichiometry in the reduction of the FAD and disulfide of Escherichia coli thioredoxin reductase. Evidence for a base at the active site.
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