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

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

Michael E. O'Donnell† and Charles H. Williams, Jr.

*From the Veterans Administration Medical Center and the Department of Biological Chemistry, The University of Michigan, Ann Arbor, Michigan 48105*

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**Scheme 1. Use of the nonphysiological pyridine nucleotide**

*This research was supported by the Medical Research Service of the Veterans Administration and in part by Grant GM-21444 from the National Institute of General Medical Sciences, Public Health Service. This paper was taken from a dissertation submitted to the Graduate School of The University of Michigan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†Present address, Department of Biochemistry, Stanford University School of Medicine, Stanford, CA 94305.

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. This article must therefore be hereby marked.

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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 $E_m$ 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 ($\lambda_{\text{iso}} = 347$ 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$^+$; and the concentration of the oxidized active site disulfide (enzyme-(S)$_2$) 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 $E_L$ and $E_R$ are higher (less negative) than the $E_m$ values of the two disulfide couples $E_L$ and $E_R$, 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 $E_L$ and $E_R$ equal the potentials of the two disulfide couples $E_L$ and $E_R$, respectively. Since the $E_m$ of the FAD couple increases

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### MATERIALS AND METHODS

#### RESULTS

The FAD and disulfide centers in thioredoxin reductase are reduced in parallel, generating significant quantities of both microforms II and III during a titration (Scheme 1). The stoichiometry of reduction of individual microforms may differ. Thus, a full description of the reduction of thioredoxin reductase requires determining the concentrations of each enzyme microform at the different levels of reduction in a titration, as well as the concentrations of NAD$^+$ and NADH.

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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-acylpyridine adenine dinucleotide; APADH, reduced 3-acylpyridine adenine dinucleotide.

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### Fig. 1. Relationship of the percentage of enzyme FADH$_2$ to the number of NADH equivalents reacted with thioredoxin reductase during two NADH titrations.

- **Conditions:**
  - 42.5 μM enzyme containing 1.72 mM NAD$^+$ in Buffer A at pH 6.0, 12 °C.
  - 17.7 μM enzyme in Buffer A at pH 8.0, 12 °C.

- **Graph Overview:**
  - **X-axis:** Equivalents of NADH.
  - **Y-axis:** Percent FADH$_2$.
  - **Lines:** Solid lines calculated from the ratios of $K_f/K_c$ and $K_f/K_c$ obtained from PMA addition experiments.

- **Graph Data Points:**
  - pH 6.0: $K_f/K_c = 0.254$, $K_f/K_c = 5.91$.
  - pH 8.1: $K_f/K_c = 0.995$, $K_f/K_c = 4.25$. 

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relative to the $E_n$ 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 $E_n$ 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 $E_n$ 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 1. 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 thioldisulfide interchange mediates the equilibration of microform I with microform IV to give a 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)$_4$, thus precluding measurements of equilibration rates among these microforms. However, if electron transfer between microforms I and IV occurs via thioldisulfide 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 $E_n$ value for the macroscopic FAD/FADH$_2$ couple and for the macroscopic (S)$_2$/(SH)$_2$ couple at each titration point showed their apparent $E_n$ 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 a difference in $E_n$ of 0.007 V between the fractional FADH$_2$ levels of 0.38 and 0.78. The difference in $E_n$ for the macroscopic (S)$_2$/(SH)$_2$ 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 (S)$_2$/(SH)$_2$) tend to have a slightly lower apparent value of $E_n$ 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 1, the $E_n$ value for the FAD couple where the disulfide is oxidized in these microforms is higher than the $E_n$ value for the FAD couple where the microforms contain a dithiol. In other words, reduction of the disulfide lowers the $E_n$ value of the FAD/FADH$_2$ couple and, conversely, reduction of the FAD lowers the $E_n$ value of the (S)$_2$/(SH)$_2$ couple. In Scheme 1, an interaction between the FAD and disulfide is described by the ratio $K_f/K_s (= K_f/K_s)$. 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 (S)$_2$/(SH)$_2$ into the four microscopic enzyme forms of Scheme 1. These quantities allow the $E_n$ 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 $\Delta E_n/\Delta pH$. 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 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 used 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 1 at one level of reduction allows the important ratios, $K_f/K_s$ and $K_f/K_s$, to be calculated. The ratio $K_f/K_s (= [III]/[III])$ is a quantitative measure of the difference in
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 $E'_c$ versus fractional reduction calculated for a 2-electron reduction assuming the

![Fig. 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.](image)

Table II

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The validity of $E_m$ values calculated from measurements of enzyme species in equilibrium with a reference couple and the condition that complex formation between enzyme of a titration were used in averaging the calculated $E_m$ values. The values of $E_m$ at pH 7.0 were: $E_m^1 = -0.254$ V, $E_m^2 = -0.243$ V, $E_m^3 = -0.260$ V, and $E_m^4 = -0.271$ 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.

These data are shown in Fig. 3 for the FAD and disulfide couples ($E_m^2$ and $E_m^3$, respectively) at pH values of 6.0, 7.0, and 8.1.

The $E_m$ 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, $E_m^2$, and the disulfide couple, $E_m^3$, are shown in Fig. 4A. The results of the second equivalent of reduction for the FAD couple, $E_m^2$, and the disulfide couple, $E_m^3$, are shown in Fig. 4B. The variation of $E_m^2$ with pH has a slope, $\Delta E_m^2/\Delta pH$, of 0.060 V/pH and a correlation coefficient of 0.997 by a least squares analysis. The $\Delta E_m^2/\Delta pH$ 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 $\Delta E_m^3/\Delta pH$ 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 $\Delta E_m^3/\Delta pH$ 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 $E_m^3$ and $E_m^4$ are lower than the $E_m^2$ and $E_m^3$ values by an average of 0.020 ± 0.011 V at all pH values.

3The small differences in $\Delta E_m/\Delta pH$ profiles between a preliminary study (O'Donnell and Williams, 1981) and the $\Delta E_m/\Delta pH$ 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 $E_m$ at all pH values and also for not making dilution corrections for the concentration of titrant NADH added. In addition, the $E_m$ values at pH 7.0 of the pyridine nucleotide couples and the value of $RT/NA$ were not corrected for temperature in the preliminary study.
Values for the $E_m^\alpha$ and $E_m^\beta$ couples are calculated from the ratio $K_1/K_4$ and the $E_m^\alpha$ and $E_m^\beta$ values (explained in the Miniprint). The $\Delta E_m^\alpha/\Delta pH$ (Fig. 4B) is 0.060 V between pH 5.5 and 8.5 and has a correlation coefficient of 0.996 by a least squares analysis. Thus, reduction of the FAD in thioredoxin reductase containing a reduced disulfide has an approximately 2.1 $-$ H$^+$ stoichiometry throughout the pH range studied. The $\Delta E_m^\beta/\Delta pH$ is 0.052 V between pH 5.5 and 8.5 by a least squares analysis (correlation coefficient = 0.996). Thus, reduction of the disulfide in thioredoxin reductase containing a reduced FAD has an approximately 1.8 $-$ H$^+$ stoichiometry between pH 5.5 and 8.5.

The slopes $\Delta E_m^\alpha/\Delta pH$ for the disulfide couples of thioredoxin reductase show that the proton stoichiometry in the reduction of the disulfide is about 0.2 $-$ H$^+$/$\Delta pH$ unit less than theoretical throughout the range of study. This suggests the presence of a base the 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 thiols 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 II) 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 pKs and pKs are 7.59 and 6.98, respectively. The values for the intramolecular equilibrium, microform II to microform III, for the fully protonated ($K_2$) and deprotonated ($K_1$) 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 \( E_m \) to pH for this case in the Miniprint. If the two pK values were more widely separated, the \( E_m \) versus pH curve would have a slope of 0.0566 V below the acid pK, a slope of 0.0283 V/pH between the two pK values, and a slope of 0.0666 V/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 \( E_m \), at a pH equal to a pK will be 0.0086 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 \( E_m \) 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 \( E_m \) 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 (Arscol 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 (\( K_s \)) 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 (\( K_{bs} \)) and the thiol on enzyme with a deprotonated base (\( K_s \)) (forms C and D, respectively, Scheme 4) must be greater than 8.5. For the linked equilibria of Scheme 4, \( pK_{bs} + pK_b = pK_s = pK_{10} \). Thus, for a value of \( pK_b \) 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 (\( K_{bs} / K_s \), 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 fulfill 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 dithiol 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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*F. A. Johnson and C. H. Williams, Jr., unpublished.*
doxin. The inherent nucleophilecty of a thiolate is dependent on the pH of the thiol. The higher the pK of the thiol, the greater the nucleophilecty 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 thioi-base ion pair model.

The ion pair of lipoamide dehydrogenase and the putative ion pair of thioredoxin reductase differ in that the thiol anion and base in lipoamide dehydrogenase have pK values that are about 1.5 pH units lower than the corresponding pK values of the putative ion pair thiol anion and base in thioredoxin reductase. Thioredoxin reductase also differs from lipoamide dehydrogenase and glutathione reductase in that the putative thiolate ion in thioredoxin reductase does not charge transfer to the FAD. The lack of charge transfer in thioredoxin reductase could be due to an incorrect juxtaposition of the thiolate relative to the FAD or a suboptimal ionization potential of the thiol.

The proton stoichiometry of about 2 for the reduction of the FAD to FADH$_2$ in thioredoxin reductase is consistent with the spectrum of the enzyme-FADH$_2$ (Ghisla et al., 1974) and the pK independence of the reduced spectrum. The resolved pK value of the FAD in thioredoxin reductase reveals that the FAD is in a hydophic environment. Although free FADH$_2$ has a pK of 6.5, an elevation of this pK value is expected in the hydophic 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 IV (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.

Acknowledgment—The authors are grateful to Dr. Jules Shafer of this department for many helpful discussions.

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Thorpe, C., and Williams, C. H., Jr. (1976b) J. Biol. Chem. 251, 7726-7728
Supplementary Material to
PROTON STOCHASTICITY IN THE REDUCTION OF THE
FAD AND DISULFIDE OF THIOREDOXIN REDUCTASE

Active Center Base in Thioredoxin Reductase

Michael E. O'Donnell and Charles E. Williams, Jr.
Veterans Administration Medical Center and Department of
Biological Chemistry, The University of Michigan, Ann Arbor, Michigan 48106

RESULTS AND METHODS

Thioredoxin reductase was purified from E. coli by a modification of the procedure of
Cotler and Platt (1974) using 2.8 M NaCl in place of SO2 to obtain the enzyme from the 2.8 M NaCl
supernatant. The concentration of thioredoxin reductase was determined by determining the
absorbance at 250 nm of purified enzyme modified as described previously (O'Donnell and
Williams, unpublished). Sodium dodecylsulfate was purchased from Sigma. All reagents for phosphorus
were from E. B. E. (Chicago, IL).

Experimental data were fitted to equations by using a nonlinear least-squares program
SUPPLEMENT, version of June 1973 (Lawenh and Sopran, 1968) as adapted by the
Biostatic Research Laboratory, The University of Michigan. This program fits a specified function to
data by means of simplex Gaussian-Newton iterations on the parameters.

Ammonium citrate was prepared with an all glass titration apparatus (Williams et al.,
1970). Each reaction tube was made with five 15 mm glass tubes. The reagents used were added with a
syringe to the 3 ml reaction mixture. Reactions were performed at 37°C in a shaking water bath.


table

<table>
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<th>pH</th>
<th>Extinction Coefficient (ε)</th>
<th>λ (nm)</th>
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</tr>
<tr>
<td>6.5</td>
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</tr>
<tr>
<td>8.3</td>
<td>1.0000</td>
<td>340</td>
</tr>
</tbody>
</table>

The extinction coefficient of the enzyme-FAD complex at 450 nm was determined by
measuring the absorbance of the reaction mixture at 450 nm and comparing it to a standard
solution of FAD in water at 450 nm. The extinction coefficient of the enzyme-FAD complex
was calculated from the following equation:

\[
ε = \frac{A}{[E]_{FAD}}
\]

where \( A \) is the absorbance at 450 nm and \([E]_{FAD}\) is the concentration of FAD in the standard
solution.

ATOMIC ABSORPTION

The extinction coefficients of thioredoxin reductase were determined by
measuring the absorbance of the reaction mixture at 450 nm and comparing it to a standard
solution of FAD in water at 450 nm. The extinction coefficient of the enzyme-FAD complex
was calculated from the following equation:

\[
ε = \frac{A}{[E]_{FAD}}
\]

where \( A \) is the absorbance at 450 nm and \([E]_{FAD}\) is the concentration of FAD in the standard
solution.
The restrictive coefficients of 561 and 3170 were obtained from a calculated spectrum of 561. To calculate the spectrum of 561, the absorption of 517 at 330 was used, and the absorption of 3170 at 3040 was used. The absorption of 517 was used to calculate the absorption of 3170, and the absorption of 3170 was used to calculate the absorption of 561. The absorption of 517 was calculated using an extinction coefficient of 2.4 for the protein at 3170. The absorption of 3170 was calculated using an extinction coefficient of 1.5 for the protein at 3040.

The extinction coefficients of 561 and 3170 were calculated using the absorption of 517 at 330 and the absorption of 3170 at 3040. The absorption of 517 was calculated using an extinction coefficient of 2.4 for the protein at 3170. The absorption of 3170 was calculated using an extinction coefficient of 1.5 for the protein at 3040.

The extinction coefficients of 561 and 3170 were calculated using the absorption of 517 at 330 and the absorption of 3170 at 3040. The absorption of 517 was calculated using an extinction coefficient of 2.4 for the protein at 3170. The absorption of 3170 was calculated using an extinction coefficient of 1.5 for the protein at 3040.

The extinction coefficients of 561 and 3170 were calculated using the absorption of 517 at 330 and the absorption of 3170 at 3040. The absorption of 517 was calculated using an extinction coefficient of 2.4 for the protein at 3170. The absorption of 3170 was calculated using an extinction coefficient of 1.5 for the protein at 3040.

The extinction coefficients of 561 and 3170 were calculated using the absorption of 517 at 330 and the absorption of 3170 at 3040. The absorption of 517 was calculated using an extinction coefficient of 2.4 for the protein at 3170. The absorption of 3170 was calculated using an extinction coefficient of 1.5 for the protein at 3040.

The extinction coefficients of 561 and 3170 were calculated using the absorption of 517 at 330 and the absorption of 3170 at 3040. The absorption of 517 was calculated using an extinction coefficient of 2.4 for the protein at 3170. The absorption of 3170 was calculated using an extinction coefficient of 1.5 for the protein at 3040.

The extinction coefficients of 561 and 3170 were calculated using the absorption of 517 at 330 and the absorption of 3170 at 3040. The absorption of 517 was calculated using an extinction coefficient of 2.4 for the protein at 3170. The absorption of 3170 was calculated using an extinction coefficient of 1.5 for the protein at 3040.

The extinction coefficients of 561 and 3170 were calculated using the absorption of 517 at 330 and the absorption of 3170 at 3040. The absorption of 517 was calculated using an extinction coefficient of 2.4 for the protein at 3170. The absorption of 3170 was calculated using an extinction coefficient of 1.5 for the protein at 3040.

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The extinction coefficients of 561 and 3170 were calculated using the absorption of 517 at 330 and the absorption of 3170 at 3040. The absorption of 517 was calculated using an extinction coefficient of 2.4 for the protein at 3170. The absorption of 3170 was calculated using an extinction coefficient of 1.5 for the protein at 3040.

The extinction coefficients of 561 and 3170 were calculated using the absorption of 517 at 330 and the absorption of 3170 at 3040. The absorption of 517 was calculated using an extinction coefficient of 2.4 for the protein at 3170. The absorption of 3170 was calculated using an extinction coefficient of 1.5 for the protein at 3040.
Active Center Base in Thioredoxin Reductase

Substituting the equilibrium constants of Scheme III and the protein concentrations for enzyme species yields:

$$E_2 = \frac{[E]_2}{1 + [E]/K_{12}}$$

The values of $[E]_2$, $[E]_3$, and $[E]_4$ can be obtained from a plot of $E_2$ versus $[E]$ (Figure 5A). At high $[E]$ values, $[E]_2$ is constant and equation 5 reduces to $E_2 = [E]_2$, indicating the identity $E_2 = [E]_2$. At high $[E]$ values, the value of $[E]_2$ is obtained from the intercept of the ordinate of Figure 5A. When $[E]_2/[E]_3$ reaches 1, $E_2$ reaches $[E]_2$; therefore, the ordinate value of $E_2$ at $[E]_2/[E]_3 = 1$ is the intercept. The value of $[E]_2$ can be obtained from the midpoint of a plot of $E_2$ versus $[E]$.

From equation 5:

$$E_2 = [E]_2$$

At $[E]_2/[E]_3 = 1$, equation 5 reduces to the ordinate value of $[E]_2$. The values of the equilibrium constants were obtained from the data by using non-linear equations 2 and 3 to equations 4 and 5. From which converge, and fitting equations 4 and 5 in the data using the non-linear least squares computer program, NUMALS.

$$E_2 = \frac{[E]_2}{[E]_3 + [E]_4 + [E]_5}$$

Equation of the $E_2$ value in disulfide enzyme and oxidized enzyme

The protein stoichiometry is the reduction of the disulfide couple in thioredoxin reductase to describe below (see text):

$$[E]_2 = \frac{[E]_2}{[E]_3 + [E]_4 + [E]_5}$$

where $K_{12}$ is the sum of the protein acid and alkali of [disulfide enzyme] and $[E]_2$ [mM] and $[E]_3$ [mM] are concentrations of the protein oxidized and reduced (disulfide) of protein. At $[E]_2$ equal to $[E]_2$, the concentration of disulfide enzyme equals the concentration of reduced enzyme and the relationship between $[E]_2$ and $[E]_3$ and $[E]_4$ (NADH) concentration is obtained following similar directions in Table 1 and 2 and it is shown below:

$$E_2 = \frac{[E]_2}{[E]_3 + [E]_4 + [E]_5}$$

where $E_2$ is the value of $E_2$ at $[E]_2 = 0$ and the equilibrium constants are those defined above. The difference in $pH$ values between the two proteins (oxidized or reduced enzyme) is directly related to the slope of the $E_2$ versus $[E]$ plot. The values of $K_{12}$ and $K_{12}^*$ were obtained by fitting equations 4 and 5 with $K_{12}$ and $K_{12}^*$ to the protein solid state x-ray analyser. The $K_{12}$ value indicates that the protein stoichiometry of $E_2$ at least $pH$ 9.0. Values of $K_{12}$ and $K_{12}^*$ were obtained using the two protein solid state x-ray analyser. The $K_{12}$ value indicates that the difference in $pH$ of disulfide enzyme and protein of approximately 0.4-0.6 pH units determined from the plots of $E_2/[E]_3$ and $E_2/[E]_4$ vs. $E_2$.
Proton stoichiometry in the reduction of the FAD and disulfide of Escherichia coli thioredoxin reductase. Evidence for a base at the active site.
M E O'Donnell and C H Williams, Jr


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