

Reconstitution of *Escherichia coli* Thioredoxin Reductase with 1-DeazaFAD

EVIDENCE FOR 1-DeazaFAD C-4a ADDUCT FORMATION LINKED TO THE IONIZATION OF AN ACTIVE SITE BASE*

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The flavin prosthetic group (FAD) of thioredoxin reductase has been replaced by 1-deazaFAD (carbon substituted for nitrogen at position 1). Reduction of 1-deazaFAD-thioredoxin reductase by four electrons proceeds in two stages having midpoint potentials that are separated by 0.063 V. Two-electron reduced 1-deazaFAD-thioredoxin reductase (EH_2) has spectral characteristics that are different from both the fully oxidized and fully reduced enzyme. The fluorescence of the 2-electron reduced enzyme shows a mixture of two EH_2 species. The spectrum of one EH_2 species has a single absorption peak (λ_{max} , 414 nm; ϵ_{414} , 8750 M⁻¹ cm⁻¹) which is similar to the spectrum of 1-deazaFAD-C-4a adducts (referred to as the 414-nm absorbing species). In the other EH_2 species the electrons are in the dithiol, and it has an oxidized 1-deazaFAD spectrum (referred to as the 550-nm EH_2 species). The equilibrium between the two EH_2 species of 1-deazaFAD-thioredoxin reductase is pH dependent, forming more of the 414-nm absorbing species as the pH is lowered. The pH dependence suggests the presence of an active center base having a pK of 7.41 on the 414-nm EH_2 species and a thiol of pK 6.73 on the 550-nm EH_2 species. These pK values are similar to the pK values determined for native enzyme having a disulfide or a dithiol (7.59 and 6.98, respectively). Thus, the pH dependence of the equilibrium between the two EH_2 species of 1-deazaFAD-thioredoxin reductase is further evidence for an active site base with an ionization behavior that is linked to the chemical state of the active site disulfide moiety. The nature of the linked ionization is consistent with a thiol base ion pair formed upon disulfide reduction.

Thioredoxin reductase from *Escherichia coli* catalyzes the reversible transfer of electrons between NADPH and the disulfide of thioredoxin, a small protein (Moore *et al.*, 1964; Zanetti and Williams, 1967). The active center of thioredoxin reductase contains an FAD and an oxidation-reduction active

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disulfide (Zanetti and Williams, 1967; Thelander, 1968; Moore *et al.*, 1964; Ronchi and Williams, 1972). It is thought that the electrons flow from NADPH to the FAD, from the FAD to the disulfide, and from the dithiol to the disulfide of thioredoxin. Thioredoxin reductase is analogous to lipoamide dehydrogenase and glutathione reductase in having an FAD and active center disulfide and in transferring electrons between pyridine nucleotide and disulfide substrates (Williams, 1976; Holmgren, 1980). The active center disulfide sequences of glutathione reductase from yeast (Jones and Williams, 1975) and erythrocyte (Krohne-Ehrich *et al.*, 1977) are highly homologous to the disulfide sequences of lipoamide dehydrogenase from *E. coli* (Burleigh and Williams, 1972) and pig heart (Matthews *et al.*, 1974; Brown and Perham 1972, 1974). However, the active center disulfide sequence of *E. coli* thioredoxin reductase (Ronchi and Williams, 1972; Thelander, 1970) shows no homology to the disulfide sequences of lipoamide dehydrogenase and glutathione reductase. A further difference between lipoamide dehydrogenase and glutathione reductase and thioredoxin reductase is in the separation of the oxidation-reduction midpoint potentials (E_m) of the FAD and disulfide couples. The FAD couple of both lipoamide dehydrogenase and glutathione reductase has a much lower E_m value than the disulfide couple (ΔE_m , approximately 0.066 V from pH 5.5 to 7.6 in pig heart lipoamide dehydrogenase, Matthews and Williams, 1976) leading to a two-stage reduction; first the disulfide is reduced and then the FAD is reduced. The 2-electron reduced forms of lipoamide dehydrogenase and glutathione reductase are spectrally distinct enzyme species having a 530-nm absorbance band interpreted as a charge transfer complex between oxidized FAD as the acceptor and a thiol anion donor (Kosower, 1966; Searls and Sanadi, 1961; Massey and Ghisla, 1974). Studies from this laboratory suggest that the thiol anion charge transfer donor is stabilized in an ion-pair interaction with an essential base at the EH_2 ¹ level in lipoamide dehydrogenase (Matthews and Williams, 1976; Matthews *et al.*, 1977) and in glutathione reductase (Arscott *et al.*, 1981).

The E_m values of the FAD and disulfide couples of thioredoxin reductase are approximately equal (O'Donnell and Williams 1982, 1983). Thus, reduction of thioredoxin reductase appears to be a single stage process showing only a gradual bleaching of the FAD absorbance (isosbestic wavelength, 347 nm) throughout a 4-electron titration (Zanetti and Williams,

¹ The abbreviations used are: EH_2 , 2-electron reduced 1-deazaFAD-thioredoxin reductase; EH_4 , 4-electron reduced 1-deazaFAD-thioredoxin reductase; E_1 , oxidation-reduction potential for the couple EH_2/EH_4 ; E_2 , the oxidation-reduction potential for the couple oxidized enzyme/ EH_2 .

1967). No charge transfer band is detected during the reduction of thioredoxin reductase even though studies demonstrate a thiol anion at the active site (O'Donnell and Williams 1982, 1983).

The present paper is a study of a derivative of thioredoxin reductase in which the FAD is replaced by 1-deazaFAD, an analogue of FAD having a more negative E_m (Walsh *et al.*, 1978). The flavin replacement effects a separation of E_m between the flavin and disulfide couples which is similar to the separation determined for lipoamide dehydrogenase.

MATERIALS AND METHODS

Thioredoxin reductase was purified by a modification of the method of Pigiet and Conley (1977) using 1.0 M NaCl in place of NADPH to elute the enzyme from the 2',5'-ADP affinity column since aerobic turnover of NADPH leads to peroxide-mediated protein modification.² NAD⁺ and NADH were purchased from Sigma, sodium dithionite was from Tridom Chemical Corp., and activated charcoal was from Atlas Chemical Industries. 1-DeazaFAD at the riboflavin level was a generous gift of the synthetic chemistry group (Ashton *et al.*, 1977) of Merck, Sharpe and Dohme Research Laboratories to Dr. Christopher Walsh of Massachusetts Institute of Technology. The adenine dinucleotide, 1-deazaFAD, was a gift from Dr. Vincent Massey (University of Michigan) and was prepared using the partially purified flavokinase and FAD synthetase from *Brevibacterium ammoniagenes* following the general procedures of Spencer *et al.* (1976).

Absorbance measurements were performed in a Cary 118C recording spectrophotometer interfaced to a PDP/8E computer (Williams *et al.*, 1979) at 12 °C. Fluorescence measurements were performed in a Perkin-Elmer model 44B recording ratio spectrofluorimeter at 12 °C and were not corrected for monochromator artifacts.

The FAD was resolved from thioredoxin reductase by adding 5.17 ml of 8.0 M guanidinium chloride, 0.1 M K₂HPO₄-NaH₂PO₄, 0.3 mM EDTA, pH 7.6, to 3.1 ml of 42.1 μM thioredoxin reductase in 0.1 M K₂HPO₄-NaH₂PO₄, 0.3 mM EDTA, pH 7.6 (Buffer A) at 5 °C. The visible absorbance spectrum indicated resolution of the FAD from the enzyme within the time of mixing. The FAD was removed from solution by direct addition of defined activated charcoal in 0.1 ml of Buffer A. Additions of charcoal were repeated (four additions) until no detectable FAD absorbance remained. The supernatant was dialyzed against Buffer A to remove the guanidinium chloride. The concentration of apothioredoxin reductase was quantitated by amino acid analysis.

Apothioredoxin reductase was reconstituted with FAD by the addition of excess FAD to apothioredoxin reductase in Buffer A followed by extensive dialysis to remove unbound FAD. The concentration of reconstituted enzyme was quantitated by the absorbance at 455 nm using an extinction coefficient of 11,300 M⁻¹ cm⁻¹ (Williams *et al.*, 1967). Apothioredoxin reductase was reconstituted with 1-deazaFAD by adding aliquots of 1-deazaFAD and monitoring the appearance of fluorescence (excitation, 552 nm; emission, 637 nm). When the fluorescence ceased to increase upon further additions of 1-deazaFAD, the reconstituted enzyme was dialyzed against Buffer A to remove unbound 1-deazaFAD. The extinction coefficient of 1-deazaFAD-thioredoxin reductase at the visible wavelength maximum (552 nm) is approximately 6800 M⁻¹ cm⁻¹ determined by dithionite titration. The native and reconstituted enzymes were assayed using the 5,5'-dithiobis-(2-nitrobenzoic acid) coupled assay (Moore *et al.*, 1964).

Calculation of the Semiquinone Spectrum—The semiquinone spectrum was calculated from spectra obtained during an anaerobic sodium dithionite titration of 23 μM 1-deazaFAD-thioredoxin reductase at pH 7.6 (spectra not shown but the following argument can be followed by reference to Fig. 1). The spectrum prior to the final addition of dithionite contained the EH_2 and EH_4 forms of enzyme as well as a substantial amount of the 1-deazaFAD semiquinone form as judged from the absorbance at 700 nm. This spectrum will be referred to as Spectrum A. The spectrum of semiquinone was calculated by correcting Spectrum A for the absorbance contributions of the EH_2 and EH_4 species. Spectrum A contained 54% EH_4 based on the fluorescence change upon the final addition of dithionite (excitation, 380 nm; emission, 490 nm). Spectrum A also contained 10.1% of the fluorescence of the oxidized 1-deazaFAD in EH_2 (excitation, 552 nm; emission, 660 nm). The final addition of dithionite resulted

in a complete loss of the absorbance at 700 nm, a loss of the EH_2 fluorescence, an increase in the EH_4 fluorescence, and produced an absorption spectrum of fully reduced 1-deazaFAD-thioredoxin reductase, EH_4 . Thus, two changes occur upon the last addition of dithionite: 1) reduction of EH_2 to EH_4 and 2) reduction of semiquinone to EH_4 .

Although EH_2 is an equilibrium mixture of at least two enzyme forms (*i.e.* oxidized 1-deazaFAD-dithiol and a C-4a thiol-1-deazaFAD adduct) it is a spectrally distinct mixture at a given pH. The absorbance contribution of EH_2 to Spectrum A was calculated as follows. Two other spectra in the second phase of reduction (1.25 and 1.5 mol of dithionite/mol of 1-deazaFAD) had the same absorbance at 700 nm and presumably the same concentration of semiquinone. Thus, their difference spectrum, referred to as Spectrum B, yields the absorbance changes for EH_2 to EH_4 . The fluorescence of EH_2 decreased to 46% of the value before this dithionite addition. Based on the loss of EH_2 fluorescence, the reduction of EH_2 to EH_4 in the final addition of dithionite (Spectrum A to EH_4) resulted in absorbance changes equivalent to 22% of Spectrum B. Thus, 22% of Spectrum B was subtracted from Spectrum A to give a difference spectrum referred to as Spectrum C. Spectrum C is the calculated spectrum for a mixture of EH_4 and semiquinone. The contribution of EH_4 to Spectrum C is the amount of EH_4 in Spectrum A (54%) plus the 10% of EH_4 introduced by the subtraction of 22% of Spectrum B from Spectrum A ($0.22 \times 0.46 = 0.1$). Thus, 64% of the absorbance of reduced 1-deazaFAD-thioredoxin reductase was subtracted from Spectrum B, and the resultant spectrum was normalized for enzyme concentration to yield the spectrum of semiquinone shown in Fig. 2. Since the reduction state of the disulfide/dithiol couple in the 1-deazaFAD-semiquinone cannot be determined it is assumed that the reduction state of the disulfide/dithiol couple does not significantly affect the semiquinone spectrum.

Correction of Spectra for Semiquinone at pH 7.6—The absorbance spectra were corrected for semiquinone by calculating the concentration of semiquinone from the absorbance at 700 nm using the extinction coefficient of 1774 M⁻¹ cm⁻¹ and subtracting the absorbance spectrum of the appropriate concentration of semiquinone using the calculated spectrum of semiquinone at pH 7.6 (Fig. 2). The resulting spectrum is scaled for the concentration of enzyme as semiquinone.

Calculation of the Spectrum of EH_2 —During the first phase of an anaerobic sodium dithionite titration at pH 7.6, the amount of absorbance at 700 nm was identical for two spectra that were separated by 0.57 eq of reduction. Thus, the concentration of semiquinone is identical at the two levels of reduction, and the difference spectrum gives the absorbance changes for 57% E to 57% EH_2 . The difference spectrum was extrapolated to 100% absorbance change for the first stage of reduction, $EH_2 - E$, and the spectrum of E (starting spectrum) was added to the extrapolated difference spectrum to yield the absorbance spectrum of EH_2 at pH 7.6 (Williams *et al.*, 1979).

Estimation of the Spectrum of the 414-nm EH_2 Species—The spectrum of the 414-nm EH_2 species was estimated as follows. The absorbance changes for the conversion of the 550-nm EH_2 species to the 414-nm EH_2 species were the difference between Spectrum 9 and Spectrum 2 in the experiment of Fig. 7 after correcting each for an 11% contribution of semiquinone. The extent of the shift of the 550-nm EH_2 to the 414-nm EH_2 was calculated to be 28% assuming an extinction coefficient at 550 nm of the 550-nm EH_2 and the 414-nm EH_2 to be 6800 M⁻¹ cm⁻¹ and 600 M⁻¹ cm⁻¹, respectively. Since native thioredoxin reductase which has electrons trapped on the disulfide via complexation with phenylmercuric acetate (O'Donnell and Williams, 1983) has an extinction coefficient of the FAD close to that of native enzyme, the value of 6800 M⁻¹ cm⁻¹ was assumed for the 550-nm EH_2 species. An extinction coefficient of 600 M⁻¹ cm⁻¹ for the 414-nm EH_2 species at 550 nm was assumed on the basis of the slight absorbance at 550 nm for the C-4a 1-deazaFAD adduct of the reaction between α -hydroxybutanoate and lactate oxidase (Entsch *et al.*, 1980). This is consistent with the studies presented here which show that the 414-nm EH_2 species has little or no absorbance at 550 nm (see Fig. 5 and text). Using these extinction coefficients, the spectrum of EH_2 at pH 7.6 (see above) is calculated to consist of 80.7% of the 550-nm EH_2 species and 19.3% of the 414-nm EH_2 species. Thus, the spectrum of 100% 414-nm EH_2 species was obtained by adding the appropriate amount of the difference spectrum (Spectrum 2–Spectrum 9, Fig. 7) to the spectrum of EH_2 at pH 7.6. The spectrum of the 414-nm EH_2 species calculated by this method is shown as the dotted spectrum in Fig. 7. The use of different extinction coefficients at 550 nm for the two EH_2 species changes the extinction coefficient

² M. E. O'Donnell and C. H. Williams, Jr., unpublished data.

at 414 nm, but does not alter its general form.

Measurements of Oxidation-Reduction Midpoint Potentials—The E_m values for the two phases of reduction of 1-deazaFAD-thioredoxin reductase were calculated as described in the Miniprint of O'Donnell and Williams (1983). Briefly, the E_m of the first phase, E_2 , was calculated from absorbance measurements during an anaerobic NADH titration at pH 7.6, 12 °C. The equilibrium concentrations of E and EH_2 were calculated from the absorbance at 552 nm using extinction coefficients of $6800 \text{ M}^{-1} \text{ cm}^{-1}$ and $5500 \text{ M}^{-1} \text{ cm}^{-1}$ (from the calculated EH_2 spectrum), respectively. The equilibrium concentration of NADH was calculated from the residual absorbance at 340 nm after subtracting the absorbance contributions at 340 nm of E and EH_2 using their extinction coefficients of 3100 and $3400 \text{ M}^{-1} \text{ cm}^{-1}$, respectively. The equilibrium concentration of NAD^+ was the difference between the amount of titrant added and the equilibrium concentration of NADH. Semiquinone concentration was calculated from the absorbance at 700 nm using an extinction coefficient of $1775 \text{ M}^{-1} \text{ cm}^{-1}$. The absorbance measurements at 340 and 552 nm were corrected for semiquinone using the extinction coefficients of $3000 \text{ M}^{-1} \text{ cm}^{-1}$ and $300 \text{ M}^{-1} \text{ cm}^{-1}$, respectively. The E_m of the first stage of reduction, E_2 , was calculated using the Nernst relationship: $E_2 = E_m^{\text{NAD}^+/\text{NADH}} + 0.0283 \log([\text{NAD}^+][\text{EH}_2]/[\text{NADH}][\text{E}])$, where the value of $E_m^{\text{NAD}^+/\text{NADH}}$ was -0.315 at 25 °C and pH 7.0 as given in Clark (1960) and was also -0.315 at 12 °C and pH 7.6 as the corrections cancel (O'Donnell and Williams, 1983).

The E_m of the second phase, E_1 , was determined from a titration of fully reduced 1-deazaFAD-thioredoxin reductase (sodium dithionite) with NAD^+ . The NAD^+ titrant solution was standardized as described in O'Donnell and Williams (1983). The equilibrium concentrations of EH_2 and EH_4 were calculated from the absorbance at 552 nm using an extinction coefficient at 552 nm for EH_4 of $174 \text{ M}^{-1} \text{ cm}^{-1}$. The equilibrium concentration of NADH was calculated from the residual absorbance at 340 nm (ϵ_{340} , $6220 \text{ M}^{-1} \text{ cm}^{-1}$) after subtracting the contributions of EH_2 and EH_4 using an extinction coefficient for EH_4 at 340 nm of $3550 \text{ M}^{-1} \text{ cm}^{-1}$. The equilibrium concentration of NAD^+ was the difference between total NAD^+ added and the equilibrium concentration of NADH. The absorbance measurements were corrected for semiquinone as in the calculations of E_2 .

RESULTS

Preparation of 1-DeazaFAD-Thioredoxin Reductase—The FAD has been resolved from thioredoxin reductase using 5.0 M guanidine HCl and removed from solution by direct addition of activated charcoal. An amino acid analysis of the dialyzed apoprotein showed a 30% loss of total protein presumably due to adsorption of apoprotein to the activated charcoal. The dialyzed apoprotein contained no detectable FAD absorbance and was 0.7% active with respect to native enzyme. Apothioredoxin reductase was 87% reconstitutable with FAD. The absorbance spectrum of the FAD-reconstituted enzyme was indistinguishable from that of native enzyme, and the reconstituted enzyme had 99% of the activity of native enzyme.

The binding of 1-deazaFAD to apothioredoxin reductase is accompanied by vibronic resolution, an approximately 20-nm shift of the wavelength maximum of the visible band (552 nm), and an increased extinction coefficient of the ultraviolet band relative to the visible band. These spectral changes are the same in nature as those of FAD bound to thioredoxin reductase. Reconstitution of apothioredoxin reductase with 1-deazaFAD was 90% complete based on an extinction coefficient at 552 nm of $6800 \text{ M}^{-1} \text{ cm}^{-1}$ (dithionite titration). 1-DeazaFAD-thioredoxin reductase had 6.8% the activity of native enzyme.

Reduction of 1-DeazaFAD-Thioredoxin Reductase Occurs in Two Stages—An anaerobic sodium dithionite titration of 1-deazaFAD-thioredoxin reductase at pH 6.0 is shown in Fig. 1, A and B. 1-DeazaFAD-thioredoxin reductase is fully reduced by reaction with 2 eq of sodium dithionite (4 electrons). The reduction appears to occur in two stages producing an intermediate 2-electron reduced spectrum that has characteristics which differ from either oxidized or fully reduced en-

zyme. The first stage of reduction shows a decrease in absorbance at 552 nm, an increase at 414 nm, and an isobestic wavelength of 457 nm (Fig. 1A). The second stage of reduction shows a further decrease in absorbance at 552 nm including a decrease above 400 nm, a region which showed increasing absorbance during the first stage. The first three spectra of the second stage are isobestic at 397 nm. A plot of the absorbance changes at 440 nm, a wavelength between the isobestic wavelengths of the individual stages shows about 1 eq of reduction in each phase (inset to Fig. 1B).

The reduction of 1-deazaFAD-thioredoxin reductase is accompanied by a long wavelength-absorbing species. A long wavelength-absorbing species was observed by Spencer *et al.* (1977a) in reactions of reduced 1-deazariboflavin with oxygen and was attributed to the neutral form of the 1-electron reduced species of 1-deazariboflavin. The spectrum of the long wavelength species was calculated from spectra during a sodium dithionite titration at pH 7.6 (see under "Materials and Methods") and is shown in Fig. 2. The spectrum of the long wavelength species closely resembles the spectrum of the neutral radical of 1-deazaFAD-flavodoxin (Entsch *et al.*, 1980). Thus, we assign the 700-nm species as being the neutral semiquinone of 1-deazaFAD-thioredoxin reductase. This assignment is consistent with the substantial quantity of neutral semiquinone that is formed during dithionite reduction of native thioredoxin reductase, especially at pH 6.0 (O'Donnell and Williams, 1983).

The lack of exact isobestic points in reduction is probably due to the variable levels of semiquinone that are formed throughout the titration. This is suggested by Spectra 3–5 of the first stage of reduction which have similar absorbance at 700 nm and are isobestic (Fig. 1A) and by Spectra 10 and 11 of Fig. 1B which show the largest change in absorbance at 700 nm and corresponding large changes in the isobestic wavelength. Spectra of a dithionite titration of 1-deazaFAD-thioredoxin reductase at pH 7.6 (similar in profile and 700-nm absorbance to those at pH 6.0) were corrected for the semiquinone by subtraction of the calculated semiquinone spectrum at pH 7.6 (see under "Materials and Methods") and are shown in Fig. 3. The corrected spectra are isobestic (first stage, 475 nm; second stage, 426 nm), further evidence for the reduction occurring in two discrete stages and for variable levels of semiquinone produced throughout the titration.

The two-stage reduction of 1-deazaFAD-thioredoxin reductase is analogous to the reduction of lipoamide dehydrogenase and glutathione dehydrogenase except that the charge transfer band at 530 nm, maximal in the first stage of reduction in lipoamide dehydrogenase and glutathione dehydrogenase, is replaced by an absorbance band at 414 nm which is maximal after the first stage of 1-deazaFAD-thioredoxin reductase reduction. Thus, a spectrally distinct intermediate species of 2-electron reduced 1-deazaFAD-thioredoxin reductase is formed during reduction and will be referred to as EH_2 by analogy to the intermediate charge transfer species of lipoamide dehydrogenase and glutathione dehydrogenase.

The spectrum of 1-deazaFAD in fully reduced thioredoxin reductase (Spectrum 11, Fig. 1B; Spectrum 8, Fig. 3) has an absorbance maximum at 375 nm (ϵ_{375} , $5800 \text{ M}^{-1} \text{ cm}^{-1}$). The spectrum of the neutral dihydro-1-deazaFAD in free solution has an absorbance maximum at 480 nm (ϵ_{480} , $2000 \text{ M}^{-1} \text{ cm}^{-1}$), and the anion has no distinct peak above 330 nm (Spencer *et al.*, 1977a). Thus, thioredoxin reductase must stabilize a different structural form of reduced 1-deazaFADH₂. The several relevant tautomers and resonance forms of dihydro-1-deazaflavins are discussed in Spencer *et al.* (1977a). The pH independence of the reduced spectrum (pH 7.6 to pH 6.0; Figs. 1B

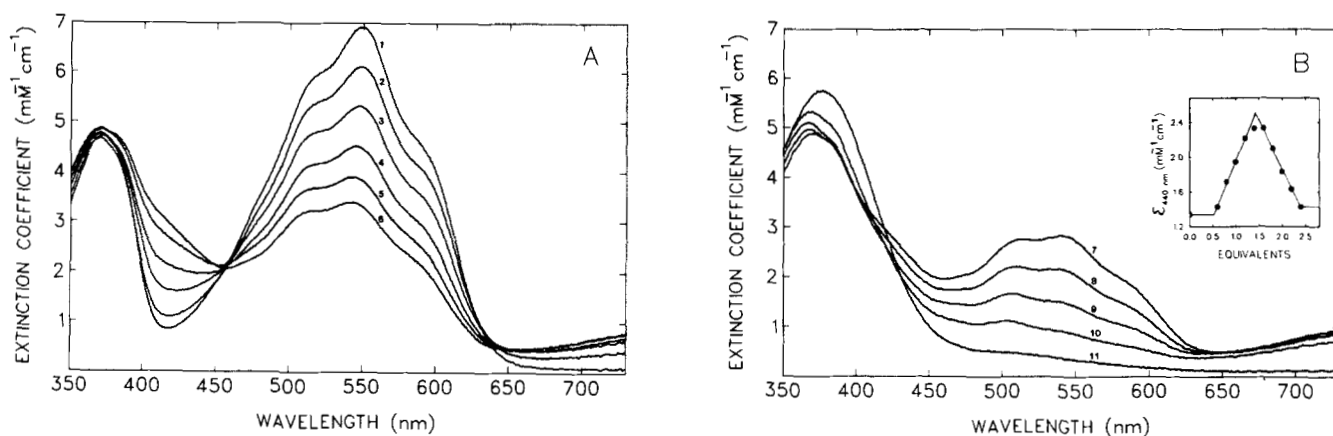


FIG. 1. Spectra observed during an anaerobic dithionite titration of 1-deazaFAD-thioredoxin reductase at pH 6.0. A shows the absorption spectra recorded in the first stage of an anaerobic dithionite titration of 18.9 μM 1-deazaFAD-thioredoxin reductase in 1.2 ml of 0.1 M $\text{NaH}_2\text{PO}_4\text{-K}_2\text{HPO}_4$, 0.3 mM EDTA, pH 6.0, 12 $^\circ\text{C}$. 1, oxidized enzyme; 2, 0.6 eq; 3, 0.8 eq; 4, 1.0 eq; 5, 1.2 eq; 6, 1.4 eq of dithionite/1-deazaFAD. B shows the absorbance changes observed in the second phase of the same dithionite titration. 7, 1.6 eq; 8, 1.8 eq; 9, 2.0 eq; 10, 2.2 eq; 11, 2.4 eq of dithionite/1-deazaFAD. Inset, relationship of the extinction coefficient at 440 nm to equivalents of dithionite added. Residual oxygen accounts for 0.5 mol of dithionite/mol of 1-deazaFAD.

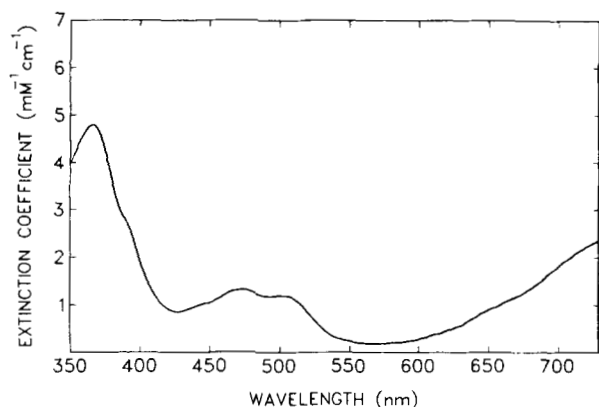


FIG. 2. Spectrum of the species having absorbance at 700 nm. The absorbance spectrum of the 700-nm absorbing species calculated from spectra recorded during a dithionite titration of 23.6 μM 1-deazaFAD-thioredoxin reductase in 1.2 ml of 0.1 M $\text{NaH}_2\text{PO}_4\text{-K}_2\text{HPO}_4$, 0.3 mM EDTA, pH 7.6, 12 $^\circ\text{C}$. Details are described under "Materials and Methods."

and 3, respectively) suggests a neutral dihydro-1-deazaFAD species in thioredoxin reductase. These results are consistent with those of native thioredoxin reductase which show the formation of neutral dihydro-FAD upon reduction over the pH range 5.5–8.5 (O'Donnell and Williams, 1983).

Oxidation-Reduction Midpoint Potentials—The E_m of the FAD and disulfide couples of native thioredoxin reductase were determined previously by measuring the equilibrium concentrations of oxidized and reduced species of pyridine nucleotide and enzyme during an NADH titration (O'Donnell and Williams, 1983). The E_m of the E/EH_2 and EH_2/EH_4 couples of 1-deazaFAD-thioredoxin reductase were determined from equilibrium measurements during titrations with NADH by the method used with native enzyme (see under "Materials and Methods"). The E_m value of the E/EH_2 couple (E_2) was -0.299 V at pH 7.6, 12 $^\circ\text{C}$. NADH does not reduce 1-deazaFAD-thioredoxin reductase past the EH_2 level at pH 7.6. This is consistent with the low potential of 1-deazaFAD in free solution. Thus, the E_m value of the EH_2/EH_4 couple (E_1) of 1-deazaFAD-thioredoxin reductase was estimated by first reducing the enzyme to EH_4 with dithionite and back

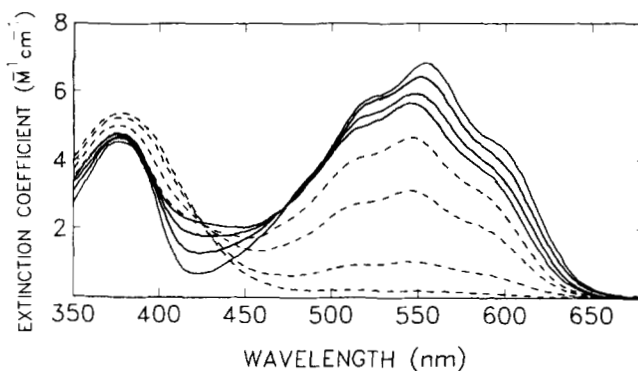


FIG. 3. Spectra observed during a dithionite titration of 1-deazaFAD-thioredoxin reductase at pH 7.6 corrected for absorbance due to the 700-nm species. Absorption spectra recorded after additions of dithionite to 23.6 μM 1-deazaFAD-thioredoxin reductase in 1.2 ml of 0.1 M $\text{NaH}_2\text{PO}_4\text{-K}_2\text{HPO}_4$, 0.3 mM EDTA, pH 7.6, 12 $^\circ\text{C}$. The spectra were corrected for the absorbance of semiquinone species as detailed under "Materials and Methods." Methyl viologen (0.5 μM) was added to facilitate reduction. The solid spectra indicate reduction up to 1 mol of dithionite/mol of 1-deazaFAD. The dashed spectra indicate reduction by more than 1 mol of dithionite/mol of 1-deazaFAD. From top to bottom, oxidized enzyme, 0.30 eq, 0.62 eq, 0.81 eq, 1.25 eq, 1.50 eq, 1.86 eq, 2.0 eq of dithionite/mol of 1-deazaFAD.

titration with NAD^+ . The value of E_1 was -0.362 V. The difference between E_1 and E_2 of 0.063 V predicts an equilibrium constant of 170 for comproportionation. In lipoamide dehydrogenase, the values of E_2 and E_1 were -0.316 and -0.382 V, respectively (pH 7.6, 25 $^\circ\text{C}$), giving a difference of 0.066 V (Matthews and Williams, 1976). Thus, the E_m values for the couples of native lipoamide dehydrogenase are about 0.020 V more negative than those of 1-deazaFAD-thioredoxin reductase, but the difference, $E_2 - E_1$, is very similar for the two enzymes.

The E_m value of 1-deazaFAD at pH 7.0, 25 $^\circ\text{C}$, in free solution is 0.061 V more negative than the E_m of free FAD (Walsh *et al.*, 1978). E_1 , the midpoint potential of 1-deazaFAD bound to thioredoxin reductase, is approximately 0.063 V more negative than the E_m of the FAD in the dithiol form of thioredoxin reductase (O'Donnell and Williams, 1983). Thus,

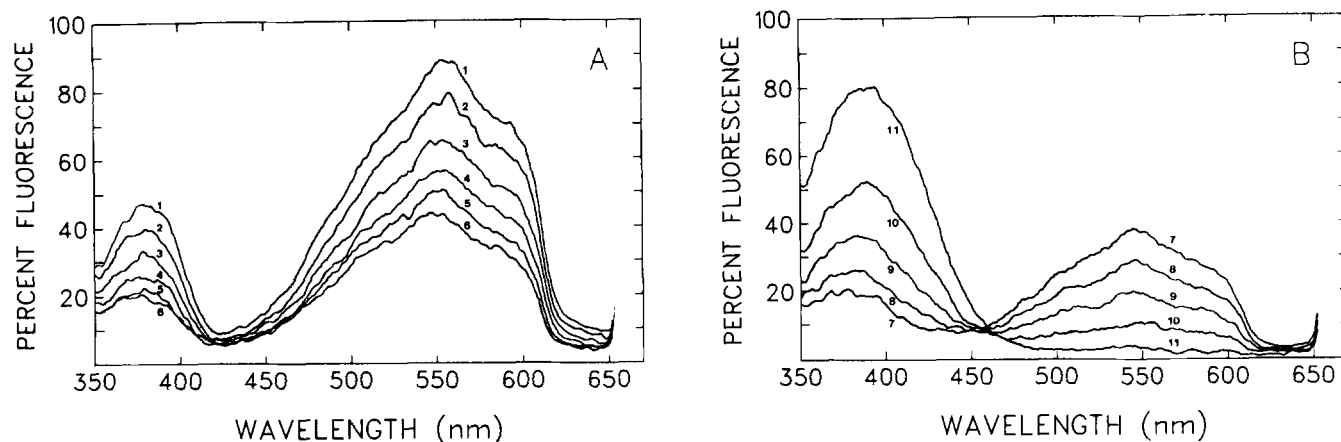


FIG. 4. Fluorescence excitation spectra observed during an anaerobic dithionite titration of 1-deazaFAD-thioredoxin reductase at pH 6.0, 12 °C. Excitation spectra were recorded with emission at 660 nm. The fluorescence spectra were collected during the dithionite titration of Fig. 1. A, fluorescence excitation spectra for reduction up to 1 mol of dithionite/mol of 1-deazaFAD. B, fluorescence excitation spectra for reduction by more than 1 mol of dithionite/mol of 1-deazaFAD.

the E_m values of both FAD and 1-deazaFAD are lowered to approximately the same extent upon binding to apothioredoxin reductase indicating that any interactions between the protein and the N-1 position of the FAD do not significantly affect the E_m of the flavin.

Nature of the EH_2 Fluorescence; Evidence for Two Species—In contrast to the lack of fluorescence of 1-deazaFAD in free solution and bound to enzymes (Spencer *et al.*, 1977b), both the oxidized and fully reduced forms of 1-deazaFAD-thioredoxin reductase are fluorescent. The oxidized 1-deazaFAD-thioredoxin reductase has 0.05% the fluorescence of FMN (pH 6.0; excitation maximum, 552 nm; emission maximum, 635 nm). The reduced form of 1-deazaFAD-thioredoxin reductase has 3.3% the fluorescence of FMN (excitation maximum, 392 nm; emission maximum, 575 nm), 66 times the fluorescence of oxidized 1-deazaFAD-thioredoxin reductase.

The fluorescence excitation spectra of 1-deazaFAD-thioredoxin reductase, shown in Fig. 4, were recorded during the dithionite titration shown in Fig. 1. The quantum yields of the oxidized and reduced forms of 1-deazaFAD-thioredoxin reductase are approximately equal at an emission wavelength of 660 nm. Therefore, the excitation spectra obtained monitoring at this wavelength will be related to the absorbance spectra both in shape and in magnitude. Two features of the fluorescence results are important to note. At 414 nm, where EH_2 has more absorbance than E , the fluorescence excitation spectra show decreasing intensity throughout the first stage of the titration. This shows that the absorbance at 414 nm is caused by a nonfluorescent 2-electron reduced form of 1-deazaFAD-thioredoxin reductase. After reduction by 1 eq of dithionite, the 2-electron reduced enzyme is still quite fluorescent having an excitation spectrum very similar to that of E except for a blue shift of approximately 4 nm. Thus, the fluorophores at the E and EH_2 levels are similar. These observations indicate that there are at least two spectrally distinct species at the EH_2 level, a fluorescent species having a maximum at approximately 550 nm, which will be referred to as the 550-nm EH_2 species, and a nonfluorescent species having an absorption maximum at 414 nm which will be referred to as the 414-nm EH_2 species. The relationship between the 552-nm fluorescence and the absorbance at 552 nm is shown in Fig. 5 for the experiment of Figs. 1 and 4. The plot is a straight line that extrapolates near to the origin showing that neither the 414-nm EH_2 species nor the EH_4

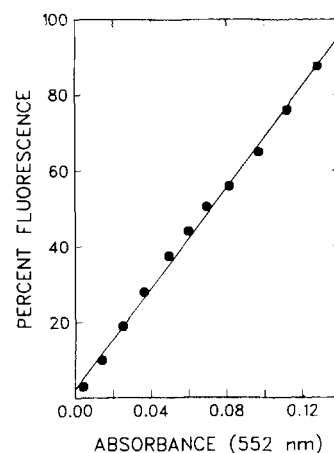


FIG. 5. Relationship of fluorescence to the absorbance at 552 nm of 1-deazaFAD-thioredoxin reductase during a dithionite titration at pH 6.0. The fluorescence was recorded with excitation at 552 nm and emission at 660 nm. The fluorescence and absorbance measurements are the experiment of Fig. 1. The details are given in the legend to Fig. 1. The solid line is a linear regression analysis of the data points.

species have appreciable absorbance at 552 nm.

Effect of Phenylmercuric Acetate on EH_2 ; the EH_2 Species Are in Rapid Equilibrium—The organic mercurial, phenylmercuric acetate, binds tightly to the active center dithiol of reduced thioredoxin reductase (O'Donnell and Williams, 1983). The addition of phenylmercuric acetate to 1-deazaFAD-thioredoxin reductase in the first stage of reduction caused a rapid loss of the 414-nm absorbance and a concomitant rise in the 552-nm absorbance in the time of mixing, about 15 s (Fig. 6). This shows that the 414-nm species is an EH_2 species and that the 550-nm EH_2 species and the 414-nm EH_2 species are in rapid equilibrium. The 700-nm absorbance is essentially unchanged by phenylmercuric acetate addition, consistent with semiquinone as the 700-nm absorbing species.

Native thioredoxin reductase also has two 2-electron reduced enzyme species (O'Donnell and Williams, 1983). These species are enzyme forms in which the electrons reside on either the disulfide or the FAD and will be designated here as $FADH_2$ /disulfide and FAD/dithiol species. The FAD/dithiol

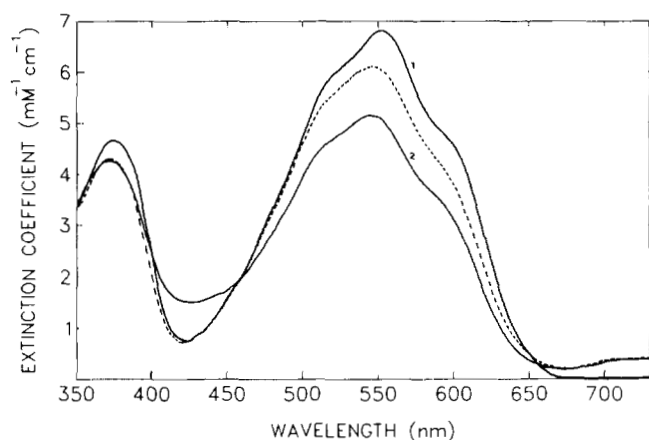


FIG. 6. Effect of phenylmercuric acetate on the absorbance spectrum of partially reduced 1-deazaFAD-thioredoxin reductase, pH 7.6. Enzyme was 13.6 μM in 1.0 ml of 0.1 M NaH_2PO_4 - K_2HPO_4 , 0.3 mM EDTA, pH 7.6, 12 °C. 1, oxidized enzyme; 2, 1.1 mol of dithionite/mol of 1-deazaFAD. The dashed spectrum was recorded after the addition of a 5-fold excess of phenylmercuric acetate (over enzyme) to the partially reduced enzyme.

EH_2 species is fluorescent² while the FADH_2 /disulfide EH_2 species is not fluorescent. The intramolecular equilibrium between the FADH_2 /disulfide and FAD /dithiol species is rapidly shifted upon addition of phenylmercuric acetate toward the FAD /dithiol-phenylmercuric acetate complex.

The fluorescence excitation spectrum of the 550-nm EH_2 species shows that it is a 1-deazaFAD/dithiol species analogous to the FAD /dithiol species of native enzyme. However, several lines of evidence argue against the 414-nm EH_2 species as a 1-deazaFAD H_2 /disulfide species. In native enzyme, the spectra of oxidized and reduced FAD are not altered by the oxidation-reduction state of the disulfide, whereas in 1-deazaFAD-enzyme, the absorbance and fluorescence properties of the 414-nm EH_2 species are very different from the spectral properties of fully oxidized enzyme or fully reduced enzyme, i.e. the spectrum of the 414-nm EH_2 relative to EH_4 is red shifted 40 nm (see below) and has no detectable fluorescence. The E_m values of the FAD and disulfide couples of fully oxidized native enzyme are approximately equal at pH 7.6 (-0.278 V and -0.282 V, respectively) (O'Donnell and Williams, 1983). Substantial amounts of both 1-deazaFAD- EH_2 species exist at the 2-electron reduced level. Thus, if the 414-nm EH_2 species contains 1-deazaFAD H_2 , the E_m of bound 1-deazaFAD would not be very different from the E_m of the disulfide. This is not consistent with the low potential of 1-deazaFAD bound to thioredoxin reductase (see above).

The Equilibrium between the Two EH_2 Species Is pH-dependent—Lipoamide dehydrogenase from both pig heart and *E. coli* have a fluorescent EH_2 species and a nonfluorescent charge transfer EH_2 species (Wilkinson and Williams, 1979).³ The electrons in these two spectrally distinct species of EH_2 in lipoamide dehydrogenase reside on the dithiol. The difference in the EH_2 species lies in their state of protonation. The nonfluorescent charge transfer EH_2 species has a thiol anion that forms a charge transfer complex with the FAD resulting in a new absorbance band at 530 nm (Kosower, 1966; Massey and Ghisla, 1974; Matthews and Williams, 1976; Wilkinson and Williams, 1979). The fluorescent EH_2 species contains a protonated thiol and is similar to oxidized enzyme in its absorbance and fluorescence properties. A 530-nm EH_2 species is also observed in glutathione reductase (Arscott *et al.*,

1981). The extinction coefficient at 530 nm of the charge transfer EH_2 species of lipoamide dehydrogenase and glutathione reductase is decreased as the pH is lowered, due to protonation of the thiol anion donor of the charge transfer complex (Matthews and Williams, 1976; Arscott *et al.*, 1981). If the 414-nm EH_2 species of 1-deazaFAD-thioredoxin reductase is a charge transfer species analogous to lipoamide dehydrogenase and glutathione reductase, the 414-nm absorbance of partially reduced 1-deazaFAD-thioredoxin reductase should decrease as the pH is lowered.

The pH dependence of the 414-nm absorbance of partially reduced 1-deazaFAD-thioredoxin reductase was measured by reducing the enzyme with slightly greater than 1 eq of dithionite in 0.010 M K_2HPO_4 - NaH_2PO_4 , 0.3 mM EDTA, pH 8.22, and the pH was lowered by 2- μl additions of 0.5 M acetic acid. The pH of the anaerobic enzyme solution was determined in an aerobic control experiment by measuring the pH of the same volume of buffer titrated with 0.5 M acetic acid. The results are shown in Fig. 7. As the pH was lowered, the 414-nm absorbance increased, and the 550-nm absorbance decreased. The observed spectral changes are opposite to those expected for a charge transfer EH_2 species. Thus, the pH dependence of the 414-nm absorbance shows that the 414-nm EH_2 species is not a thiolate-to-1-deazaFAD charge transfer species.

The pH dependence of the equilibrium between the 550-nm EH_2 species and 414-nm EH_2 species of 1-deazaFAD-thioredoxin reductase allows the spectrum of the 414-nm EH_2 species to be calculated (see under "Materials and Methods") and is shown as the dashed spectrum in Fig. 7. The characteristic twin maxima of oxidized 1-deazaFAD is replaced by a single peak in the 414-nm EH_2 species (λ_{max} , 414 nm; ϵ_{414} , 8750 $\text{M}^{-1} \text{cm}^{-1}$).

Model studies suggest that the transfer of electrons between flavin and disulfide proceeds via the covalent addition of a thiol at the C-4a position of the flavin. Thus, it was of interest to compare the calculated spectrum of the 414 nm EH_2 species with a known C-4a adduct of 1-deazaFAD. The reduced enzyme-substrate complex of 1-deazaFAD-*p*-hydroxyben-

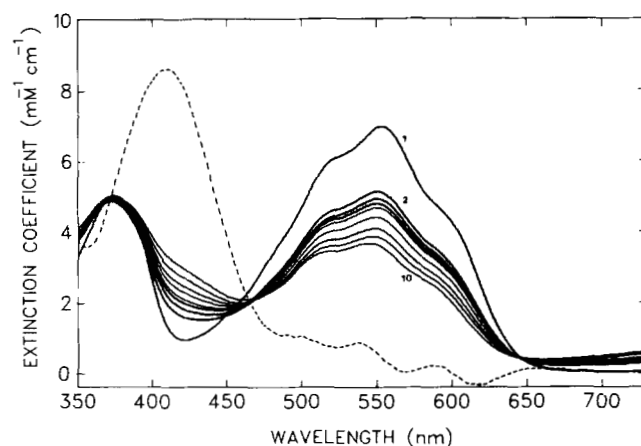


FIG. 7. Effect of pH on partially reduced 1-deazaFAD-thioredoxin reductase. Enzyme was 26.2 μM in 1.0 ml of 0.01 M NaH_2PO_4 - K_2HPO_4 , 0.3 mM EDTA, pH 8.22, 12 °C. The enzyme was partially reduced by dithionite. 1, oxidized enzyme; 2, 1.26 mol of dithionite/mol of 1-deazaFAD. The enzyme was then titrated anaerobically with 0.5 M acetic acid, pH 3.0. Spectra were recorded at the following values of pH. Curves 3 through 10: 3, pH 7.79; 4, pH 7.55; 5, pH 7.37; 6, pH 7.21; 7, pH 6.92; 8, pH 6.64; 9, pH 6.28; 10, pH 5.79. The dashed spectrum is an estimate of the 414-nm absorbing 1-deazaFAD-thioredoxin reductase species (see under "Materials and Methods").

³ F. A. Johnson and C. H. Williams, Jr., unpublished data.

zoate hydroxylase reacts rapidly with oxygen to form a structure proposed to be a C-4a-1-deazaFAD hydroperoxide (Entsch *et al.*, 1980) and also has a spectrum (λ_{\max} , 395 nm; ϵ_{395} , 9500 M⁻¹ cm⁻¹) similar to that of the 414-nm EH_2 species. Furthermore, the product of the reaction between 1-deazaFAD-lactate oxidase and 1-hydroxy-3-butynoate is proposed to be a C-4a/N-5 bridged adduct (Entsch *et al.*, 1980) and results in a spectrum (λ_{\max} , 382 nm; ϵ_{382} , 8300 M⁻¹ cm⁻¹) which is similar to that of the 414-nm EH_2 species.

The spectrum of the 414-nm EH_2 species is compatible with formation of a thiol C-4a adduct of 1-deazaFAD exhibiting spectral characteristics similar to analogous adducts involving substitution of carbon or oxygen moieties at that position. All such adducts lead to a tetrahedral carbon atom at the C-4a position. The flavin-binding pocket in thioredoxin reductase is quite apolar as judged by the vibronic resolution of the absorbance spectrum of both FAD and 1-deazaFAD bound to the enzyme. Thus, the 19- to 32-nm red shifted position of the absorbance maximum of the putative 1-deazaFAD C-4a adduct in thioredoxin reductase relative to that for the 1-deazaFAD C-4a adducts of *p*-hydroxybenzoate hydroxylase and lactate oxidase could be the result of solvent effects.

A thiol-to-flavin C-4a adduct has also been observed in a derivative of lipoamide dehydrogenase wherein one of the active site thiols is alkylated by iodoacetamide (Thorpe and Williams, 1976a). This enzyme derivative is induced to form a flavin C-4a adduct upon binding NAD⁺ (Thorpe and Williams, 1976b; Thorpe and Williams, 1981). Since NAD⁺ induces the FAD C-4a adduct in iodoacetamide-modified lipoamide dehydrogenase, NADP⁺ was added in a pH titration of partially reduced 1-deazaFAD-thioredoxin reductase analogous to the experiment of Fig. 7. The results were essentially the same as those of Fig. 7 indicating no significant induction effect of NADP⁺ on formation of the putative 1-deazaFAD C-4a adduct.

The respective concentrations of the 550-nm EH_2 species and 414-nm EH_2 species in the experiment of Fig. 7 were calculated from the 550-nm absorbance assuming extinction coefficients at 552 nm for the 550-nm EH_2 species and 414-nm EH_2 species of 6800 M⁻¹ cm⁻¹ and 600 M⁻¹ cm⁻¹, respectively, and correcting for semiquinone assuming extinction coefficients of 300 M⁻¹ and 1774 M⁻¹ cm⁻¹ at 552 and 700 nm,

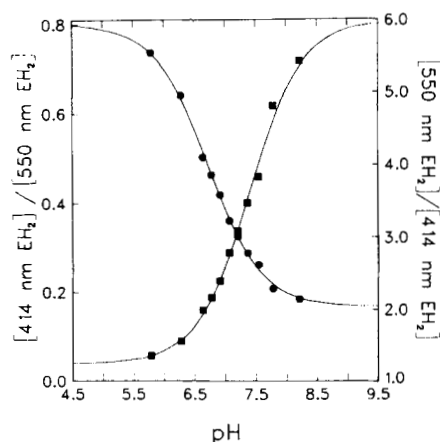


FIG. 8. Relationship of the equilibrium between the 414-nm EH_2 species and the 550-nm EH_2 species and the pH. The equilibrium constants for the equilibrium between the EH_2 species at different pH values were calculated from the absorbance measurements at 552 nm in the experiment of Fig. 7 (see text). ●, [414-nm EH_2]/[550-nm EH_2]; ■, [550-nm EH_2]/[414-nm EH_2]. The solid lines are theoretical fits to the data (see under "Materials and Methods").

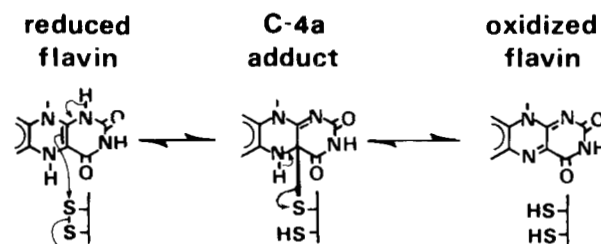
respectively. The equilibrium constant, 550-nm EH_2 /414-nm EH_2 , and the equilibrium constant in the opposite direction are plotted as a function of pH in Fig. 8. The derivation and interpretation of the plot of Fig. 8 is described in O'Donnell and Williams (1983). The theoretical fits to the data yield pK values of 7.41 and 6.73 which are the values for ionizations on the 414-nm EH_2 species and 550-nm EH_2 species, respectively. The values for the internal equilibrium between the 414-nm EH_2 species and the 550-nm EH_2 species at the basic and acidic limbs of the theoretical fits to the data are 0.167 and 0.80, respectively. Thus, the 414-nm EH_2 species has an ionization with a higher pK than that of a group on the 550-nm EH_2 species leading to a shift in equilibrium toward the 414-nm EH_2 species as the pH is lowered.

DISCUSSION

The reduction of 1-deazaFAD-thioredoxin reductase occurs in two stages that are separated in E_m by 0.063 V. Two electrons react with the enzyme in each stage. The two-stage nature of the reduction is predicted from the low potential of 1-deazaFAD relative to FAD in solution (ΔE_m , 0.063 V). Thus, in native enzyme, the FAD and disulfide couples have approximately equal E_m values and are reduced together whereas in 1-deazaFAD-thioredoxin reductase the disulfide is reduced (constituting the first stage, $E_2 = -0.299$ V, pH 7.6, 12 °C) and then the 1-deazaFAD is reduced (second stage, $E_1 = -0.362$ V, pH 7.6, 12 °C).

Lipoamide dehydrogenase and glutathione reductase show a two-stage reduction and a 2-electron reduced species having increased absorbance at 530 nm relative to both the oxidized and fully reduced enzymes. The 530-nm absorbance band is interpreted to be a thiolate-to-FAD charge transfer species. Two-electron reduced 1-deazaFAD-thioredoxin reductase, EH_2 , has increased absorbance at 414 nm relative to both the oxidized and fully reduced enzyme. The 414-nm absorbance of the EH_2 species of 1-deazaFAD-thioredoxin reductase is not caused by a thiolate-to-1-deazaFAD charge transfer interaction since protonation of 1-deazaFAD-thioredoxin reductase EH_2 leads to increased amounts of the 414-nm absorbance.

The fluorescence excitation spectra of 1-deazaFAD-thioredoxin reductase in the course of a dithionite titration show that EH_2 is a mixture of at least two spectrally distinct species, a fluorescent EH_2 species that absorbs maximally at 550 nm and a nonfluorescent EH_2 species that absorbs maximally at 414 nm. The 414-nm EH_2 species has a spectrum with a single absorbance peak (λ_{\max} , 414 nm; ϵ_{414} , 8750 M⁻¹ cm⁻¹) suggestive of a 1-deazaFAD C-4a adduct. Since the putative C-4a adduct is formed upon reduction of the disulfide, the presumed C-4a substituent is one of the active site thiols. Since FAD and 1-deazaFAD have similar chemical properties (Spencer *et al.*, 1977a) these results suggest an intermediate C-4a species in the transfer of electrons from FADH₂ to the disulfide in native enzyme as shown in Scheme 1. The C-4a of reduced flavin



SCHEME 1

adds to the disulfide to form a thiol-to-flavin C-4a adduct and a free thiol. The electron transfer is completed concurrent with the deprotonation of the flavin N-5 to form oxidized flavin and a dithiol.

An intermediate C-4a adduct species in the transfer of electrons between the FAD and thiols is consistent with model studies (Hemmerich, 1968; Hamilton, 1971; Gascoigne and Radda, 1967; Loechler and Hollocher, 1975; Yokoe and Bruce, 1975). A thiol-to-flavin C-4a adduct has also been observed in a derivative of lipoamide dehydrogenase wherein one of the active site thiols is alkylated by iodoacetamide (Thorpe and Williams, 1976a). This enzyme derivative is induced to form a C-4a adduct upon binding NAD^+ (Thorpe and Williams, 1976b; Thorpe and Williams, 1981).

Measurements of the proton stoichiometry of reduction of the disulfide in native thioredoxin reductase indicate a base at the active center with an ionization behavior that is linked to the oxidation-reduction state of the disulfide (O'Donnell and Williams, 1983). The proton stoichiometry results were best fit by a model wherein enzyme with an oxidized disulfide had a group with a pK of approximately 7.59 and enzyme containing a dithiol had a group with a pK of approximately 6.98. The pH dependence of the equilibrium between the two EH_2 species in 1-deazaFAD-thioredoxin reductase shows that the 414-nm EH_2 species (which does not have a dithiol) has an ionization with a pK of approximately 7.41, and an ionization of a group on the 550-nm EH_2 species (having a dithiol) has a pK of about 6.73. Thus, the pH dependence of the equilibrium between the EH_2 species is further evidence for the ionization of an active site base linked to the chemical state of the disulfide moiety.

Studies on lipoamide dehydrogenase suggest that a base on oxidized enzyme has a pK value below 5.5 and is shifted up to 7.8 upon reduction of the disulfide. The shift in the pK of the base is explained by the formation of a thiol-base ion pair upon disulfide reduction. The linkage of the pK of a base to the chemical state of the disulfide (oxidized, reduced, or putative C-4a) in both 1-deazaFAD-thioredoxin reductase and native enzyme is consistent with a thiol-base ion pair as shown in Scheme 2. We have indicated the pK 7.41 in Scheme 2 to be the nascent thiol which is in an ion pair interaction with the base, since the ion pair is favored in the apolar active site.

An 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, a thiolate is known to initiate

thiol-disulfide interchange reactions (*i.e.* transfer of electrons between the dithiol of thioredoxin reductase to the disulfide of thioredoxin). In addition, formation of the mixed disulfide between thioredoxin reductase and thioredoxin would be concerted with an increased acidity of the protonated base which could function as a proton donor to the free thiol of thioredoxin. Thus, both a nucleophilic thiol anion and a protonated base are required for efficient catalysis and are encompassed in the thiol-base ion pair model.

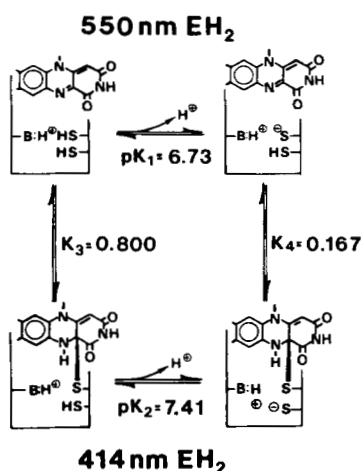
The results presented here on the mechanism of thioredoxin reductase suggest further similarities to the mechanisms of lipoamide dehydrogenase and glutathione reductase. Studies on iodoacetamide-modified lipoamide dehydrogenase suggest that native lipoamide dehydrogenase transfers electrons between the FAD and dithiol via a sulfur-to-flavin C-4a species. Characterization of 1-deazaFAD-thioredoxin reductase suggests a thiol-to-flavin intermediate in native thioredoxin reductase. Lipoamide dehydrogenase has a base whose pK is linked to the oxidation-reduction state of the disulfide and is the partner to a thiolate in an ion-pair interaction. The proton stoichiometry in the reduction of the disulfide of native thioredoxin reductase and the pH dependence of the equilibrium between the EH_2 species of 1-deazaFAD-thioredoxin reductase disclose the presence of a base in thioredoxin reductase with an ionization behavior that is linked to the oxidation-reduction state of the disulfide. The linked ionization is consistent with a thiol-base ion pair at the active site of thioredoxin reductase.

In lipoamide dehydrogenase, the ion pair thiol has a pK of about 4.8,³ and studies suggest that the base has a pK of less than 5.5 on oxidized enzyme (Matthews *et al.*, 1977). This may indicate a thiol anion in thioredoxin reductase that has a greater intrinsic nucleophilicity than the thiol in lipoamide dehydrogenase. Thioredoxin reductase differs from lipoamide dehydrogenase and glutathione reductase in that the putative thiolate in thioredoxin reductase does not charge transfer to the FAD. This may be due to an incorrect juxtaposition of the thiolate relative to the FAD or a suboptimal ionization potential of the thiolate.

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Reconstitution of *Escherichia coli* thioredoxin reductase with 1-deazaFAD. Evidence for 1-deazaFAD C-4a adduct formation linked to the ionization of an active site base.

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