Lipoamide Dehydrogenase from *Escherichia coli* Lacking the Redox Active Disulfide: C44S and C49S. Redox Properties of the FAD and Interactions with Pyridine Nucleotides†

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ABSTRACT: The cysteines that comprise the active site disulfide in lipoamide dehydrogenase have been individually mutated to a serine residue to give the altered enzymes, C44S and C49S, making it possible to study the redox behavior of the FAD in the absence of the disulfide. The redox potential of the FAD in C44S and C49S was −379 and −345 mV, respectively, at pH 7.0, 25 °C. A plot of the redox potential as a function of pH for C49S gave slopes of 57 mV/pH from pH 5.0 to 7.9 and 10 mV/pH from pH 7.9 to 8.8. The plot of the redox potential as a function of pH for C44S gave slopes of 70 mV/pH from pH 5.0 to 7.9 and 4 mV/pH from pH 7.9 to 8.38. The change in the slope at pH 7.9 is associated with the ionization (pK_a) of the $FADH_2$ to $FADH^-$ in the reduced form of both enzymes. These determinations show that the redox potential of the FAD in C49S, in C44S, and in wild type enzyme is modulated by the electronegativity of its nearest neighbor, hydroxyl, thiolate, or disulfide, and that the flavin is bound more tightly to the oxidized forms of these enzymes than to the reduced forms. The redox potentials of these enzymes determined using NADH and NADPH at pH 7.6, 25 °C are as follows: C44S, −350 mV, −369 mV; C49S, −328 mV, −353 mV, respectively. Thus, pyridine nucleotide binding raises the redox potential of the flavin, showing that both substrates bind more tightly to the reduced form of the enzymes, as well as tighter binding of NADH to the enzymes than that of NADPH. K_d values for the binding of NADH and NADPH to oxidized C44S and C49S were determined in pre-steady-state kinetics at pH 7.6 and 25 °C, which were monophasic when NADPH was the reductant and biphasic with NADH. The binding constants for NADPH were 660 μM for C44S and 500 μM for C49S; using NADH, the binding constants were 137 μM for C44S and 23 μM for C49S. Fluorescence and absorbance spectrophotometry were used to determine the binding of NAD^+ to the oxidized forms of the enzymes as 275 μM and 270 μM for C44S and C49S, respectively.

The cysteines that comprise the redox active disulfide in lipoamide dehydrogenase have distinct functions; Cys^{44} interchanges with dihydrolipoamide, and Cys^{49} transfers electrons to the flavin (Thorpe & Williams, 1976a,b, 1981; Hopkins & Williams, 1995). Each of these residues in the *Escherichia coli* enzyme has been individually mutated to a serine residue (C44S and C49S), making it possible to study the redox behavior of the FAD in the absence of the disulfide and in the presence and absence of pyridine nucleotides (Allison et al., 1988; Russell et al., 1989).

Studies of the wild type enzyme have used indirect methods to determine the redox potential of the FAD. Two-electron-reduced lipoamide dehydrogenase (EH_2)[1] is an equilibrium mixture of several species (Scheme 1, Hopkins & Williams, 1995). In the pig heart enzyme, the charge-transfer complex (species IIb) predominates, so that the redox pair is effectively E_{ox} and the charge-transfer species. In the *E. coli* enzyme more than one EH_2 species is present in a redox equilibrium (Wilkinson & Williams, 1979a). In wild type enzyme, therefore, it is important not to associate the E_{ox}/EH_2 potential with the disulfide/dithiol potential or the EH_2/EH_4 potential with FAD/$FADH_2$ potential, except as a rough approximation, although it is a better approximation for the pig heart enzyme. In C44S and C49S, the disulfide is absent and the redox potential of the FAD can be measured and compared to the EH_2/EH_4 potential of wild type enzyme, keeping the caveat in mind.

Lipoamide dehydrogenase isolated from *E. coli* shows very strong inhibition by the product of the physiological reaction, NADH. At very high concentrations, NAD^+ also inhibits (Sahlman & Williams, 1989b; Williams, 1965). It has been shown that inhibition is caused by NADH binding to the two-electron-reduced enzyme and by NAD^+ binding to the oxidized enzyme in dead-end complexes (Wilkinson & Williams, 1981; Matthews et al., 1976, 1979). Lipoamide

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[1] Abbreviations: FAD, $FADH_2$, and $FADH^-$, oxidized, neutral reduced, and anionic reduced forms of flavin adenine dinucleotide; AAD^+, oxidized aminopyridine adenine dinucleotide; APADH, reduced acetylpyridine adenine dinucleotide; X/XO, xanthine/xanthine oxidase; E_{ox}, oxidized enzyme; EH_2, two-electron-reduced enzyme; EH_4, four-electron-reduced enzyme. The active site mutation of lipoamide dehydrogenase: C44S, $cysteine^{44}$ has been replaced by a serine, and C49S, $cysteine^{49}$ has been replaced by a serine.

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dehydrogenase catalyzes the reduction of NAD^+ and the oxidation of dihydrolipoamide by proceeding through several two-electron-reduced intermediates (EH_2). Since the enzyme possesses two redox centers, it is capable of accepting four electrons, but the four-electron-reduced species (EH_4) is unable to participate in catalysis. It has been shown that the product, NADH, can reduce the enzyme to its EH_4 state, which causes inhibition of catalysis. The ease of reduction of the *E. coli* enzyme to its catalytically inactive EH_4 species is a consequence of the redox potential of the flavin (Williams, 1992). The redox potentials for the E_{ox}/EH_2 and EH_2/EH_4 couples of *E. coli* lipoamide dehydrogenase are -264 and -314 mV at pH 7.0 and 25 °C, respectively (Wilkinson & Williams, 1979). As just discussed, these macroscopic redox potentials cannot be assigned to the disulfide/dithiol or the FAD/$FADH_2$ couples, since there are several possible states of the EH_2 form of the enzyme. It has been proposed that the interaction with pyridine nucleotides modulates the redox potential of the enzyme (Matthews et al., 1979, 1976). The influence of pyridine nucleotides on the redox potential of the FAD/$FADH_2$ couple is therefore of interest.

C44S and C49S are inactive in the physiological reaction, but they are capable of using NADH as an electron donor with artificial electron acceptors at catalytic efficiencies comparable to that of the wild type enzyme (Hopkins & Williams, 1995). The titration of C44S and C49S with NADH suggests that the redox potential of the FAD in C44S is considerably lower than that of the FAD in C49S. Redox potentials determined for these altered enzymes clearly are the redox potentials of the FAD/$FADH_2$ couple, since the disulfide is absent. In the present studies, the altered enzyme flavin redox potentials and the effect of pH on them have been determined. In addition, C44S and C49S have been used as models to better understand the interaction of the flavin in lipoamide dehydrogenase with pyridine nucleotides and the resultant effects on catalysis and the redox potentials.

MATERIALS AND METHODS

Reagents. Xanthine, benzyl viologen, methyl viologen, safranine O, and pyridine nucleotides were purchased from Sigma. Xanthine oxidase and 5-deazaflavin were generous gifts from Dr. Vincent Massey, University of Michigan. All buffer salts were reagent grade.

Enzyme Purification. The enzymes were isolated and purified as described previously (Hopkins & Williams, 1995).

Oxidation−Reduction Titrations. All buffers were 100 mM with 0.3 mM EDTA added. The following buffers were used at 25 °C: acetate, pH 4.99−5.90; phosphate, pH 6.23−7.6; Tris-HCl, pH 7.90−8.38; and glycine−NaOH, pH 8.82. The pH of the titration solution was determined after each titration. The concentration of the enzyme was determined by using an extinction coefficient at 444 nm of 9,955 M^{-1} cm^{-1} for C44S and 12,140 M^{-1} cm^{-1} for C49S (Hopkins et al., 1991; Hopkins & Williams, 1995). The concentration of safranine O in the stock solution was determined gravimetrically. The concentration of each viologen dye was determined using extinction coefficients of $\epsilon_{ox,257nm}$ 21 000 M^{-1} cm^{-1}, $\epsilon_{red,604nm}$ 13 600 M^{-1} cm^{-1} for methyl viologen, and $\epsilon_{ox,260nm}$ 27 000 M^{-1} cm^{-1}, $\epsilon_{red,560nm}$ 12 200 M^{-1} cm^{-1} for benzyl viologen (Mayhew, 1978; Michaelis & Hill, 1933; Mayhew & Muller, 1982; Tsukahara et al., 1987). The following wavelengths were monitored to determine reduction during the titration: increases at 604 and 560 nm for methyl and benzyl viologen, respectively; decreases at 444 nm for enzyme; decreases at 520 and 550 nm for safranine O.

Anaerobiosis and Titration with Dithionite. These methods have been described in Hopkins & Williams, 1995.

Photoreductions Using 5-Deazaflavin and EDTA. The enzyme (10−20 μM) and redox dye (10−20 μM) containing EDTA (1−10 mM) with 5-deazaflavin (1−2 μM) in a side arm were made anaerobic as described. The concentration of 5-deazaflavin was determined using an extinction coefficient of 12 000 M^{-1} cm^{-1} at 400 nm. The compound 3,10-dimethyl-5-deazaisoalloxazine (5-deazaflavin) is named as in the reference, but note that it lacks the 7- and 8-methyl groups (Massey & Hemmerich, 1977, 1978). The catalytic amounts of 5-deazaflavin used did not interfere with the spectrum of the enzyme and dye. The reduction was initiated by exposing the reaction mixture to a variable light source (20−50 s). The cuvette was placed in an ice water bath during light exposure.

Xanthine/Xanthine Oxidase Reduction. The enzyme (10−20 μM), redox dye (10−20 μM), and xanthine (100−600 μM) with xanthine oxidase (20−40 nM) in a side arm were made anaerobic. The concentration of xanthine oxidase used in the titration was determined using an extinction coefficient of 37 800 M^{-1} cm^{-1} at 450 nm (Massey et al., 1969). A Milton Roy Spectronic 3000 diode array spectrophotometer recorded 100 continuous spectra over 4−6 h. Since this process is catalytically driven, care must be taken to use the appropriate amount of xanthine and xanthine oxidase in order to achieve sufficient reduction at a rate that provides equilibration between the oxidized and reduced species of dye and enzyme (Massey, 1991).

Of the three methods of reduction used in the determination of the redox potentials, the xanthine/xanthine oxidase method was used for most of the data collection, and the other two methods, photoreduction and dithionite, were used to verify the results. The xanthine/xanthine oxidase method overcomes a number of problems inherent in the dithionite and photoreduction methods. For example, the time needed for the reaction mixture to reach equilibrium and the need to invert in order to mix between additions of dithionite increased the possibility of oxygen leakage. The reduced viologen dyes react more readily with oxygen than do the reduced enzymes and served as sensitive indicators of oxygen. The xanthine/xanthine oxidase method has an internal oxygen scrubbing system because the xanthine is in large molar excess and the other substrate for xanthine oxidase is oxygen (Green, 1934). Another advantage of this method is that the reaction cuvette is not moved after the initial tipping in of the xanthine oxidase to start the reduction. However, the photoreduction and dithionite methods were very useful, especially when used in parallel with the xanthine/xanthine oxidase method, where the chances for technical problems are much less. Finally, it should also be mentioned that the xanthine/xanthine oxidase method works well only when the system is optimized for the enzymes under study, C44S and C49S. The redox potentials determined using dithionite as the reductant agreed with those using xanthine/xanthine oxidase where safranine O was the redox indicator, in the pH range of 5−7. It should be noted that the viologen dyes were not used below pH 7.

Quantification of Oxidized and Reduced Enzymes and Dyes. Concentration of the Oxidized and Reduced Viologen Dyes. The initial total concentration of benzyl (or methyl) viologen was determined using the extinction coefficients given above. The sum of the concentrations of oxidized and reduced benzyl (or methyl) viologen was set equal to the initial total.

Concentration of Oxidized and Reduced Enzyme When Using the Viologens. The contribution of the reduced viologen to the absorbance of the enzyme containing solution was determined by first plotting the extinction at 560 or 604 nm versus the extinction at 444 nm over the course of viologen reduction in the absence of enzyme. This graph was linear, and the slope and intercept were used to determine ϵ_{444} from ϵ_{560} or ϵ_{604} at any level of viologen reduction in the presence of enzyme. The extinction at 444 nm due to reduced viologen was converted to absorbance and subtracted from the total observed absorbance to determine the total expected absorbance of the enzyme in the absence of the reduced viologen.

Both oxidized and reduced forms of the enzymes absorb at 444 nm; therefore, the $\Delta\epsilon_{444}$ for the oxidized and reduced enzymes was determined at each pH value. The absorbance spectra of the reduced enzymes are pH dependent. The conversion of oxidized enzyme (E_{ox}) to reduced enzyme (E_{red}), ΔA_{444}, can be determined from the difference in absorbance observed and expected. The concentration of oxidized enzyme was then determined by the difference.

Concentration of Oxidized and Reduced Safranine O. Safranine O in its oxidized form has maximal absorbance at 520 nm. The maximal wavelength was used with C49S, but C44S has substantial absorbance at this wavelength; therefore, 550 nm was used. The initial total amount of absorbance of safranine O was set equal to 100%. The amount of oxidized safranine O remaining was the fraction of A_{520} or A_{550} remaining. Safranine O has a pH dependent redox potential with two redox linked pK_a's at pH 4.71 and 5.75, which gives redox potentials of −198 and −282 mV at pH 5.00 and 7.00, respectively.

Concentration of Oxidized and Reduced Enzyme When Using Safranine O. Since the oxidized form of safranine O also obscures the flavin maximal absorbance at 444 nm, a graph was constructed of the percent oxidized safranine O at 520 and 550 nm versus the percent oxidized at 444 nm in the absence of enzyme. During the experiment the concentration of the enzyme was corrected for dilution and converted to absorbance. This absorbance was subtracted from the total initial absorbance at 444 nm to determine the initial dye contribution at 444 nm. The concentrations of E_{red} and E_{ox} were determined as described above.

Redox Potential Calculations (Clark, 1960). The redox potential difference between the dye and the enzyme was calculated by plotting the log $([ox]/[red])_e$ of the enzyme versus the log $([ox]/[red])_d$ of the dye according to the method of Minnaert (1965). The Nernst equations for the dye and enzyme are as follows:

$$E_h(e) = E_m(e) + (59/n_e)\log([ox]/[red])_e \quad \text{(for the enzyme)}$$

$$E_h(d) = E_m(d) + (59/n_d)\log([ox]/[red])_d \quad \text{(for the dye)}$$

At equilibrium the system potentials are equal, $E_h(e) = E_h(d)$:

$$E_m(e) + (59/n_e)\log([ox]/[red])_e = E_m(d) + (59/n_d)\log([ox]/[red])_d$$

and this equation is rearranged to give the working equation,

$$\log([ox]/[red])_e = (n_e/n_d)\log([ox]/[red])_d + (E_m(d) - E_m(e))(n_e/59)$$

where n_e is the number of electrons accepted by the enzyme and n_d is the number of electrons accepted by the dye. The slope will be 2 for a one electron accepting dye coupled with a two electron accepting enzyme, and the slope will be one if the enzyme and dye both accept two electrons. When the $\log([ox]/[red])_d$ (dye) is equal to zero, the y-intercept, $(E_m(d) - E_m(e))(n_e/59)$, will equal $\log([ox]/[red])_e$. Using this relationship and the known redox potential of the dye permits calculation of the redox potential of the enzyme.

Determination of the K_d Values for the Binding of NAD$^+$ and AAD$^+$ to the Oxidized Altered Enzymes at pH 7.6. Absorbance and fluorescence properties were used to determine the binding constants under aerobic conditions. All of these experiments were performed using 100 mM phosphate buffer, pH 7.6, containing 0.3 mM EDTA at 25 °C. The enzyme concentrations were 10−20 μM. The stock concentrations of pyridine nucleotides were determined using extinction coefficients of 9100 M^{-1} cm^{-1} at 331 nm (Fisher et al., 1973) and 17 800 M^{-1} cm^{-1} at 257 nm for AAD$^+$ and NAD$^+$, respectively. Typically, the spectrum of the oxidized enzyme was measured, and then aliquots of concentrated pyridine nucleotides (10 mM) were added and the spectrum was recorded. Absorbance was used with C44S because the addition of NAD$^+$ to the oxidized enzyme caused increased absorbance in the 530 nm region as well as diminution of the flavin peak. No observable absorbance changes were detected when the identical experiment was performed with C49S. However, addition of AAD$^+$ to both enzymes caused spectral changes that were observable using absorbance. Fluorescence was used to determine the binding constants of NAD$^+$ to C49S and C44S since it is known that the binding of the pyridine nucleotides could quench flavin fluorescence (Massey & Veeger, 1961; Murrel, 1961; Weber, 1950). The absorbance and fluorescence spectra of the oxidized enzymes were measured with no NAD$^+$ present prior to titration with 10 mM NAD$^+$ and recording of the fluorescence changes. The K_d values were determined by constructing a Benesi−Hildebrand (1949), Stockell (1959), and/or Stinson-Holbrook (1973) plot.

Static Reduction with NADH and NADPH at pH 7.6. The stock solutions of the reduced pyridine nucleotides (10 mM) were prepared in 20 mM unneutralized Tris, and the concentrations were determined using an extinction coefficient of 6220 M^{-1} cm^{-1} at 340 nm. These solutions were diluted to 5 and 1.8 mM in 100 mM phosphate, pH 7.6, containing 0.3 mM EDTA for NADPH and NADH titrations, respectively, and made anaerobic. The enzymes were made anaerobic; addition of titrant and determination of spectral changes were performed as described above. The amount of oxidized enzyme was determined by the difference between the total amount of enzyme present and the amount of reduced enzyme present. There was an isosbestic point for the oxidized and reduced forms of the enzyme at 320 nm for C44S and at 329 nm for C49S; the amount of

unreacted reduced pyridine nucleotide can be determined at these wavelengths once the absorbance due to enzyme was subtracted. The extinction coefficients for the reduced pyridine nucleotides were 5020 M^{-1} cm^{-1} at 320 nm and 6090 M^{-1} cm^{-1} at 329 nm. The amount of oxidized pyridine nucleotide was assumed from the reaction stoichiometry to be equal to the amount of E_{red} produced. Alternatively, the amount of NAD(P)H at equilibrium could be determined from the stoichiometry

Determination of Redox Potentials. The concentrations of the reduced and oxidized forms of the enzymes and pyridine nucleotides were used to determine the redox potentials of the FAD/$FADH_2$ couple in C44S and C49S at 25 °C using the following equations (Clark, 1960), where n = number of electrons:

$$E_h(e) = E_m(e) + (59/n_e) \log([E_{ox}]/[E_{red}]) \quad (1)$$

$$E_h(d) = E_m(d) + (59/n_d) \log([NAD(P)^+]/[NAD(P)H])$$

At equilibrium $E_h(e)$ is equal to $E_h(d)$ and

$$E_m(e) - E_m(d) = (59/n) \log\{[E_{red}][NAD(P)^+]/[E_{ox}][NAD(P)H]\}$$

$$E_m(e) - E_m(d) = (59/n) \log K_{eq}$$

Known redox potentials: NAD^+/NADH at 25 °C, pH 7.6, E_m = −330 mV; $NADP^+$/NADPH, 25 °C, pH 7.6 E_m = −346 mV (Krause et al., 1974; Engel & Dalziel, 1967) were used to calculate the enzyme potential.

Stopped-Flow Studies Using NADH and NADPH at pH 7.6, 25 °C. Both enzymes were prepared in 100 mM phosphate at pH 7.6 containing 0.3 mM EDTA at concentrations yielding 5−16 μM after mixing in the stopped flow instrument. The instrument used allowed full spectra to be recorded via a diode array detector and kinetics were recorded at a single wavelength via a photomultiplier tube (Hopkins & Williams, 1995). The enzyme was placed in a tonometer, and anaerobiosis was achieved as described above. NAD(P)H was prepared and characterized as described above. The diluted solutions of NAD(P)H were placed in 10 mL syringes, and anaerobiosis was achieved by bubbling nitrogen into the syringes. After mixing the enzyme and substrate, spectral changes were recorded at 444 nm for each of the enzymes.

Redox Titration in the Presence of AAD^+. The redox potentials of the altered enzymes were determined in the presence of the essentially nonreducible pyridine nucleotide analog, AAD^+, using the xanthine/xanthine oxidase method (Massey, 1991). The buffer used in this experiment was 100 mM phosphate, pH 7.6, containing 0.3 mM EDTA. A cuvette equipped with a side arm was used. The concentration of the enzymes was 10−20 μM, determined before and after the addition of AAD^+, since binding of AAD^+ to the enzymes causes spectral perturbations. An excess of AAD^+ was added to the enzyme solution, and a spectrum was recorded. Xanthine and methyl viologen (C44S) or benzyl viologen (C49S) were added to the solution, and a spectrum was recorded. The titration proceeded as described above. The determination of the concentration of the enzyme and dye, calculation of the redox potential difference between the dye and the enzyme, and the Nernst equations for the dye and enzyme were applied as described above.

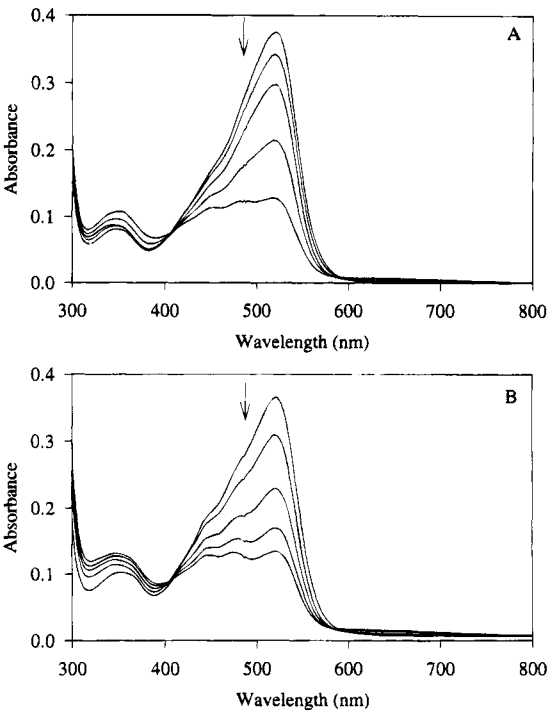

FIGURE 1: Redox titration of C44S and C49S in the presence of safranine O. The enzymes were in 100 mM actetate with 0.3 mM EDTA added at 25 °C. (A) C44S at pH 5.00 (9.42 μM) containing 200 μM xanthine and 32 nM xanthine oxidase. The A_{550} due to safranine O was 0.150 initially. (B) C49S at pH 5.13 (9.55 μM) containing 200 μM xanthine and 34 nM xanthine oxidase. The A_{520} due to safranine O was 0.361 initially. The arrows indicate the direction of the changes in absorbance during the titration.

RESULTS

Redox Titrations. The xanthine/xanthine oxidase method for reduction was used for most of the redox potential determinations. Several redox dyes were required to cover the pH range of interest.

Representative spectra for C44S and C49S using safranine O are shown in Figure 1. It can be seen that the contribution of the dye to the absorbance of the enzyme at 444 nm is significant, hence the need for correction. The contribution of C49S to the absorbance of the safranine O at 520 nm is almost insignificant, and corrections were not necessary (Figure 1B). The contribution of C44S to the absorbance of the safranine O at 520 nm, its maximum, is largely due to the long wavelength shoulder (530 nm). Therefore, the amount of oxidized and reduced dye was determined at 550 nm (Figure 1A).

Representative spectra for C44S and C49S using benzyl viologen at pH 7.06 are shown in Figure 2. While similar statements could be made about the separation of the enzyme and benzyl viologen maxima as were made regarding safranine O, it can be seen in Figure 2 that the separation is less in the case of benzyl viologen and the corrections were larger. The data using benzyl viologen were thus less reliable. With all three dye systems, the long wavelength shoulder of C44S made determinations of its potential less reliable than those of C49S.

Representative spectra for C44S and C49S using methyl viologen at pH 8.38 are depicted in Figure 3, and it can be

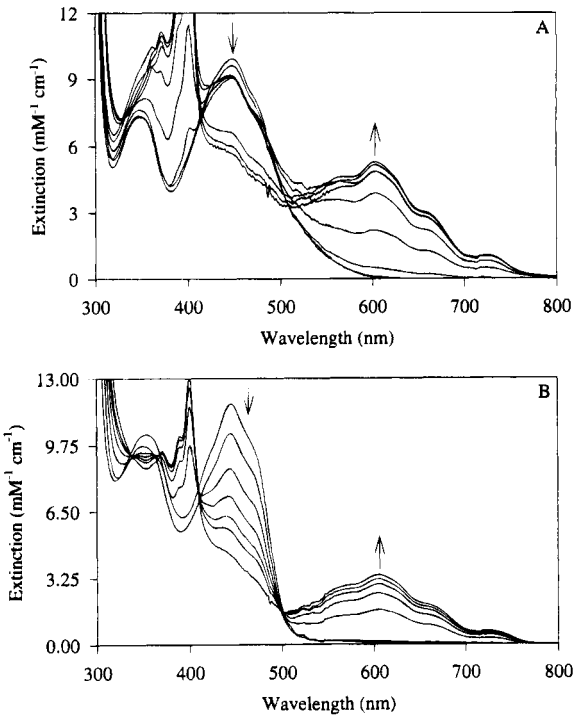

FIGURE 2: Redox titration of C44S and C49S in the presence of benzyl viologen. The enzymes were in 100 mM phosphate, pH 7.06, with 0.3 mM EDTA added at 25 °C. (A) C44S (10.25 μM) containing 7.71 μM benzyl viologen, 1 μM methyl viologen, 400 μM xanthine and 34 nM xanthine, oxidase. (B) C49S (10.79 μM) containing 8.24 μM benzyl viologen, 400 μM xanthine, and 8 nM xanthine oxidase. The arrows indicate the direction of the changes in absorbance during the titration.

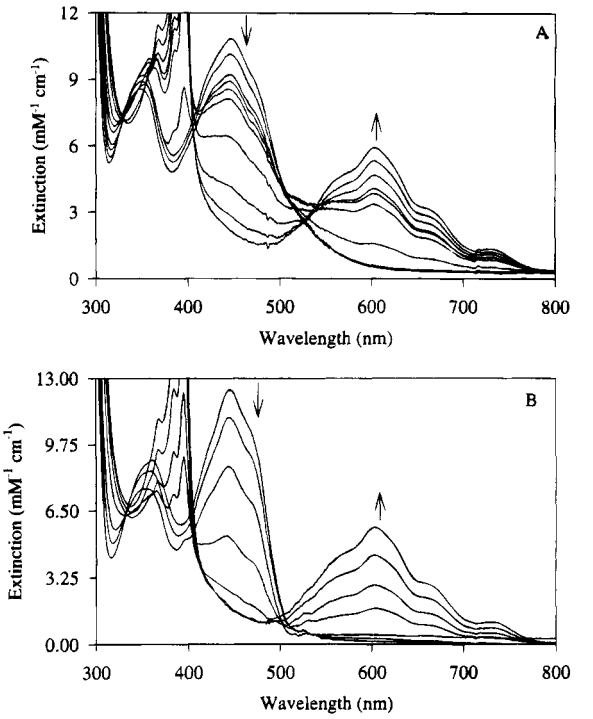

FIGURE 3: Redox titration of C44S and C49S in the presence of methyl viologen. The enzymes were in 100 mM Tris-HCl, pH 8.38, with 0.3 mM EDTA added at 25 °C. (A) C44S (9.84 μM) containing 9.80 μM methyl viologen, 100 μM xanthine, and 23 nM xanthine oxidase. (B) C49S (10.71 μM) containing 9.52 μM methyl viologen, 200 μM xanthine, and 24 nM xanthine oxidase. The arrows indicate the direction of the changes in absorbance during the titration.

seen that the separation of enzyme and dye absorbances is much better than with benzyl viologen. Even the very sharp absorbance at 395 nm due to the methyl viologen radical contributes little to the absorbance of the enzymes.

The redox potentials were calculated by plotting the log-($[E_{ox}]/[E_{red}]$) versus the log($[dye_{ox}]/[dye_{red}]$) as described in the Materials and Methods section. Such plots served as an indicator of equilibration between the oxidized and reduced species of the enzyme and dye. Lack of equilibration between the oxidized and reduced forms of the enzyme and dye is one cause of the data not fitting the theoretical slope.

Nernst plots (Figure 4) were constructed for C44S and C49S from the data at pH values 5.00 (C44S), 5.13 (C49S), 7.06, and 8.38, and the data were fitted to a theoretical slope of 29.5 mV for a two electron reduction. It can be seen that the data are satisfactory only over a limited portion of the titrations. In most cases the data diverged from ideal when large corrections were required or when the system potential was more than 50 mV different from the measured (enzyme) potential. Given the almost 200 mV span over which data were taken, the results are qualitatively satisfactory.

The redox potential data for C44S and C49S are plotted as a function of pH (Figure 5) in order to obtain a profile of the enzyme as it undergoes two electron reduction. The theoretical profile of a two electron reduction associated with two protons and one proton are 59 mV/pH and 29.5 mV/pH, respectively. The C44S enzyme plot (Figure 5A) gave slopes of 70 mV/pH from pH 5.00 to 7.9. While this slope is noticeably higher than the theoretical slope, it indicates that two protons are associated with two electrons in the reduction. The data from pH 7.9 to 8.38 gave a slope of 4

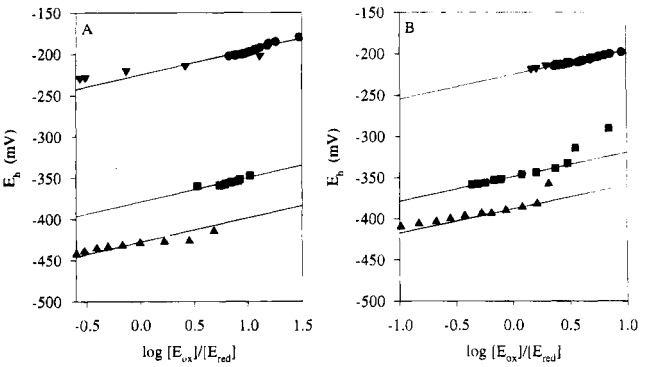

FIGURE 4: Representative Nernst plots of the redox titration of C44S and C49S as a function of pH. The system potentials, E_h, at three different pH values are plotted as a function of the log($[E_{ox}]/[E_{red}]$) at 25 °C. The solid lines represent the data fitted to a theoretical slope of 29.5 mV. (A) C44S: pH 5.00, safranine O, circles (X/XO), inverted triangles (dithionite); pH 7.06, benzyl viologen, squares (X/XO); and pH 8.38, methyl viologen, triangles. (B) C49S: pH 5.13, safranine O, circles (X/XO), inverted triangles (photoreduction); pH 7.06, benzyl viologen squares; and pH 8.38, methyl viologen, triangles.

mV/pH, and this slope is significantly lower than the theoretical slope of 29.5 mV/pH for one proton associated with a two electron reduction. Interpretation of the data (only two data points) at high pH is not clear. The slope break at pH 7.9 is in part associated with the ionization of the flavin in its reduced form from $FADH_2$ to $FADH^-$ (detected spectrally, data not shown).

The C49S enzyme plot (Figure 5B) gave slopes of 57 mV/pH from pH 5.0 to 7.9 and 10 mV/pH from pH 7.9 to 8.8, with a slope break at pH 7.9. The slope of 57 mV/pH is in agreement with the theoretical slope of 59 mV/pH for two

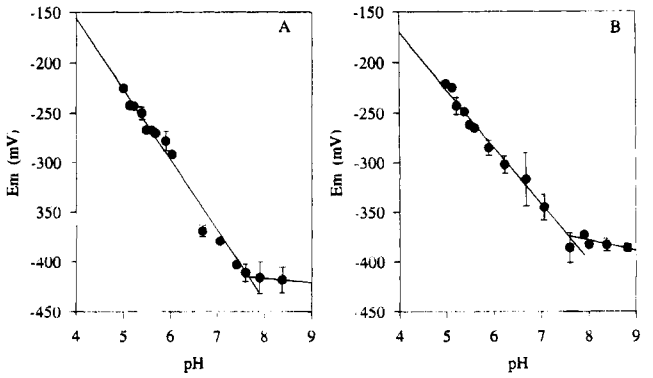

FIGURE 5: Redox potentials of C44S and C49S as a function of pH. The redox potential was measured over the pH range 5.00–8.8 using redox dyes and the following three reduction methods: xanthine/xanthine oxidase, photoreductions, and dithionite. Each point on the plot represents the average of at least two titrations at that pH value, and the error bars are given. Where there are no error bars, only two titrations were done. The redox dyes used were safranine O (pH 5.00–6.28, C44S; pH 5.00–6.68, C49S), benzyl viologen (pH 6.42–7.06, C44S; pH 6.68–7.06, C49S), and methyl viologen (pH 7.6–8.38, C44S; pH 7.6–8.8, C49S). (A) The solid lines are linear regressions of the experimental data which gave slopes of 70 mV/pH unit (pH 5.00–7.9) and 4 mV/pH unit (pH 7.9–8.38) with the slope break at pH 7.9 for C44S. (B) The solid lines are linear regressions of the experimental data which gave slopes of 57 mV/pH unit (pH 5.00–7.9) and 10 mV/pH unit (pH 7.9–8.8) with the slope break at pH 7.9 for C49S.

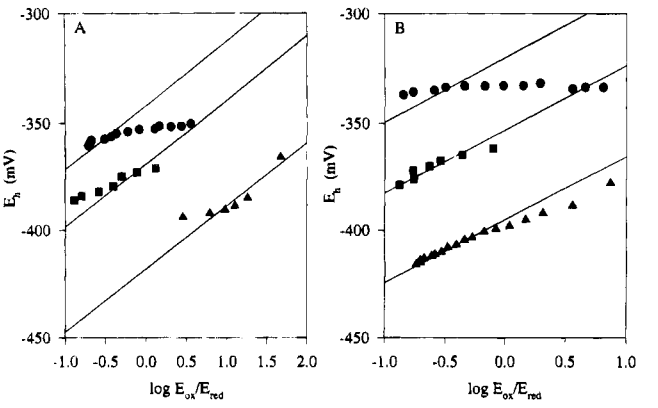

FIGURE 6: Nernst plots for (A) C44S and (B) C49S at pH 7.6 and 25 °C. The redox reference couples were as follows: circles, NADH; squares, NADPH; triangles, methyl viologen (xanthine/xanthine oxidase). The solid line represents the data fitted to a theortical slope of 29.5 mV.

Table 1: Pyridine Nucleotide Binding Constants for Oxidized Enzyme

	K_d (μM)	
	C44S	C49S
NAD^+	275, 310[a]	260[b]
AAD^+	39[c]	142[c]
NADPH	660	500
NADH	137	23

[a] Determined using absorbance and fluorescence. [b] Determined using fluorescence. [c] Determined using absorbance. All other values were determined from stopped flow data.

protons associated with a two electron reduction, while the slope of 10 mV/pH is noticeably lower than the theoretical slope of 29.5 mV/pH for one proton associated with a two electron reduction. Interpretation of the data at high pH is unclear. The slope break at pH 7.9 is again associated with the ionization of the flavin in its reduced state from $FADH_2$ to $FADH^-$ (data not shown).

Static Reduction with NADH and NADPH at pH 7.6. Neither C44S nor C49S were reduced completely by NADH or NADPH when the level of reduction by pyridine nucleotides is compared to the level of reduction achieved using dithionite. As shown in earlier experiments, C44S is more difficult to reduce than is C49S (Hopkins & Williams, 1995). These data indicate that the redox potentials of both altered enzymes are lower than those of the pyridine nucleotides. Nernst plots were constructed from the reduction data using NADH and NADPH, and they are shown in Figure 6 along with the data from the redox titration using the xanthine/xanthine oxidase method with methyl viologen as the redox couple. The redox potentials for C44S are −350, −369, and −411 mV using NADH, NADPH, and methyl viologen , respectively. The redox potentials for C49S are −328, −353, and −386 mV using NADH, NADPH, and methyl viologen, respectively. The non-Nernstian behavior required that the redox potentials be determined by fitting a line of slope 29.5 mV to the maximum number of points. These plots indicate that the interaction of the pyridine nucleotides with both enzymes has caused the redox potentials to be raised when compared to the methyl viologen data. The non-Nernstian behavior using NADH as the reductant will be addressed in the Discussion section.

Stopped-Flow Studies Using NADH and NADPH at pH 7.6. Both enzymes were reduced with several concentrations of NADH and NADPH in the stopped flow. The primary goals of these experiments were to determine constants for the binding of pyridine nucleotides to the oxidized enzymes and to determine the rate of flavin reduction. The reduction with NADPH was slow for both enzymes, with monophasic kinetics. Binding constants were determined for both enzymes from the concentration dependence of the rates (Table 1). The maximal rate of reduction was 0.57/min and 3.9/min for C44S and C49S, respectively.

Rapid reaction studies of both enzymes using NADH showed biphasic kinetics, and the data were fitted to two exponentials. The first phase of reduction was dependent on the concentration of NADH, and the second phase was concentration independent. K_d values derived from these data are given in Table 1. The maximal fast rate of reduction was 182/s and 278/s for C44S and C49S, respectively. Thus, C49S is reduced faster by both NADH and NADPH than is C44S. The rate of reduction with NADH is 320- and 70-fold faster than the rate of reduction with NADPH for C44S and C49S respectively. The rate of the slow phase of reduction by NADH was 1.93/s for C44S and 6.93/s for C49S and might reflect intermolecular electron transfer. The data from both rapid reaction studies using NADH and NADPH have shown that C49S has a faster rate of reduction than C44S as well as tighter binding of both pyridine nucleotides.

Determination of the K_d Values for NAD^+ and AAD^+ Binding to Oxidized C44S and C49S at pH 7.6. The binding constants determined using absorbance and fluorescence are shown in Table 1 for C44S and C49S. The K_d values determined for NAD^+ by absorbance (310 μM) and fluorescence (275 μM) for C44S are comparable by the two techniques. The K_d value for NAD^+ binding to C49S was determined by fluorescence. The raw data were used to determine the point of saturation by plotting the change in fluorescence emission upon binding of NAD^+ to both

Table 2: Redox Potentials of C44S and C49S at pH 7.6 and 25 °C

	E_m (mV)	
	C44S	C49S
NAD^+	−338	−325
NADH	−350	−328
NADPH	−369	−353
X/XO	−411	−386
X/XO(AAD^+)	−444	−375

[a] The redox potentials of the NAD^+/NADH and $NADP^+$/NADPH couples at pH 7.6 and 25 °C are −330 and −346 mV, respectively (Krause et al., 1974; Engel & Dalziel, 1967).

enzymes as the concentration of NAD^+ increases and fitting the data to a rectangular hyperbola. The K_d values were determined by a Stinson−Holbrook plot or a Stockell plot. Both proteins bind NAD^+ with similar affinity. However, the binding of AAD^+, a nonreducible pyridine nucleotide analog, to both altered proteins is tighter for both, and C44S has a 4-fold higher affinity for this ligand than C49S (Table 1). The binding of AAD^+ to C44S causes qualitatively similar, but larger, spectral perturbations than those seen when NAD^+ binds to the enzyme. Absorbance at the flavin peak decreases, and there is increased absorbance at the charge-transfer region of the spectrum (530 nm). Binding of AAD^+ to C49S causes spectral perturbations at long wavelengths, while there are no spectral perturbations observed when NAD^+ is the titrant. The binding of AAD^+ to C49S causes a diminution of the flavin peak, a slight shift of the peak to longer wavelengths, and the production of characterless long wavelength absorbance. These perturbations are not as large as those observed for C44S (data not shown).

Redox Titration in the Presence of AAD^+ at pH 7.6. The redox potential of C44S was determined in the presence of 200 μM AAD^+, and that of C49S was determined in the presence of 250 μM AAD^+. The redox potentials were estimated relative to methyl viologen. Unlike NAD^+, which is readily reduced, AAD^+ is an essentially nonreducible pyridine nucleotide analog. This experiment was performed to observe the effect of oxidized pyridine nucleotide binding on the redox potential of the enzymes. The estimated redox potentials were −444 and −375 mV for C44S and C49S, respectively. All of the redox potentials determined for C44S and C49S at pH 7.6 are summarized in Table 2. The redox potential for C44S with AAD^+ present was lower than the redox potentials determined using NADH, NADPH, and methyl viologen. The redox potential for C49S with AAD^+ present was lower than the redox potential determined using NADH and NADPH but about the same as the redox potential determined relative to methyl viologen. The potentials determined by titration of dithionite-reduced enzymes with NAD^+ agreed well (Table 2, line 1) with those measured by direct titration with NADH (Table 2, line 2).

DISCUSSION

C44S and C49S present the opportunity to study the behavior of the flavin in the absence of the disulfide. Previous studies with lipoamide dehydrogenase from pig heart determined a redox potential of −280 mV associated with the reduction of the enzyme by two electrons, E_{ox} to EH_2 (Matthews & Williams, 1976). It is known that the

Table 3: Redox Potentials of Lipoamide Dehydrogenase, FAD and NAD^+, pH 7.0, 25 °C

enzyme	E_m (mV)	enzyme	E_m (mV)
C44S	−379	*E. coli* LipDH EH_2/EH_4	−314[b]
C49S	−345	*E. coli* LipDH E_{ox}/EH_2	−264[b]
pig heart LipDH EH_2/EH_4	−346[a]	FAD	−219[c]
		dihydrolipoamide	−287[d]
pig heart LipDH E_{ox}/EH_2	−280[a]	NAD^+	−320[e]

[a] Matthews & Williams, 1976. [b] Value for the EH_2/EH_4 couple, E_1, was estimated from the data of Wilkinson and Williams (1979a), who found a potential of −264 mV for the E_{ox}/EH_2 couple, E_2. The equation: $E_1 = E_2 - 29.6 \log K$ was used, where K, the association constant for comproportionation, was 50. [c] Lowe & Clark, 1965. [d] Massey, 1960. [e] Burton & Wilson, 1953; Olson & Anfinsen, 1953.

pig heart enzyme cannot be reduced beyond the EH_2 stage either by dihydrolipoamide or by NADH at pH 7.6 (Massey, 1960). This indicates that the enzyme potential for the addition of the second electron pair is lower than the potentials of the substrate or product, dihydrolipoamide or NADH (Table 3). The spectrum of pig heart lipoamide dehydrogenase at the EH_2 stage with its high absorbance at 455 nm and prominent long wavelength shoulder has been interpreted as a thiolate−FAD charge-transfer complex (Ghisla & Massey, 1989). Thus, in the ground state at least, the potential of the disulfide/dithiol couple, must be higher than the potential of the FAD/$FADH_2$ couple. In spite of the fact that it is not rigorously correct to equate the macroscopic E_{ox}/EH_2 redox potential with the microscopic potential of the disulfide/dithiol couple, it can be useful to consider the comparison in order to further equate the EH_2/EH_4 couple with the FAD/$FADH_2$ couple. Matthews and Williams (1976) calculated the EH_2/EH_4 redox potential for the pig heart enzyme as −364 mV at pH 7.6 from the comproportionation constant for the $2EH_2 \rightleftharpoons E_{ox} + EH_4$ equilibrium, which establishes the potential difference between E_{ox}/EH_2 and EH_2/EH_4. Again, it is not rigorously correct to equate this macroscopic potential with the potential of the FAD/$FADH_2$ couple, but it can be useful.

The situation with the *E. coli* lipoamide dehydrogenase is more complicated, and it would be misleading to associate the flavin redox potential with the potential of the EH_2/EH_4 couple, since there are a number of species present at the EH_2 level of reduction (Wilkinson & Williams, 1979a). The middle line of Scheme 1 in Hopkins and Williams (1995) shows the several species present at the EH_2 level. Species IIb is the thiolate−FAD charge-transfer complex predominating in the reduced pig heart enzyme, and species IIa is its prototropic tautomer. The covalent intermediate, species IIc, is only observed under special conditions. Species IId is the $FADH_2$−NAD^+ charge-transfer complex. Wilkinson and Williams (1979a) found that species IIa, IIb, and IId were present at equilibrium in the ratio of 27:60:13 at pH 7.6. Thus, at the two-electron-reduced level, the flavin is already partially (13%) reduced. It can be seen by comparing the data in Table 3 for the *E. coli* and pig heart enzymes that the potentials of the E_{ox}/EH_2 and EH_2/EH_4 couples are higher and closer together in the *E. coli* enzyme compared with the pig heart enzyme.

These studies have provided a direct determination of the flavin redox potential and have allowed comparison of the redox potentials for C44S and C49S to that of the wild type enzyme as shown in Table 3. The fact that the redox

potentials for C44S and C49S are substantially lower than that of the *E. coli* wild type enzyme EH_2/EH_4 can be attributed to several factors. The flavin redox potential is modulated in part by the residues that are in close proximity to the flavin. In C44S, the thiolate anion enhances the electronegativity of the flavin, resulting in a lowering of the redox potential. C49S has a hydroxyl group in close contact with the flavin and will be expected to exert a smaller electronegative enhancement on the flavin. As discussed in the preceding paper (Hopkins & Williams, 1995), the milieu of Cys^{49} (or Ser^{49}) lowers the pK_a by approximately 6 pH units, to 2.7. This is significantly lower than the apparent pK_a associated with the charge transfer thiol in pig heart lipoamide dehydrogenase, 3.9–4.4 (Sahlman & Williams, 1989a). It could be argued that the charge-transfer thiol in C44S exerts a larger effect on the flavin potential than does the thiol in pig heart lipoamide dehydrogenase and this is reflected in the lower potential of C44S.

Studies of the redox potential of the pig heart enzyme EH_2/EH_4 couple over the pH range of 5.5–7.9 gave a slope of 60 mV/pH, with no change in the slope (Matthews & Williams, 1976). These redox potentials were calculated from measurements of the extent of dismutation of EH_2 to E_{ox} and EH_4 together with the E_{ox}/EH_2 redox potentials. The slopes determined for C49S and C44S from pH 5.0 to 7.9 of 57 mV/pH and 70 mV/pH, respectively, showed that C49S had behavior that was close to the theoretical slope of 59 mV/pH while that of C44S was noticeably higher than the theoretical slope. Slopes that do not conform to canonical expectations can often be attributed to the effects of other groups in the vicinity of the redox couple whose protonation state is linked to the redox change. Moreover, if two such groups are present and if their pK_a values are separated by less than one pH unit, their titration can obscure slope changes (Clark, 1960; O'Donnell & Williams, 1983). Both altered enzymes showed a change in the slope at pH 7.9. Thus, below this pH, two protons are associated with the two-electron reduction; that is, the product is $FADH_2$.

The lesser slopes at pH values greater than 7.9 indicate flavin ionization, i.e., the product of reduction is $FADH^-$, but they also suggest that other group(s) having pK_a values linked to the redox state of the flavin are present in both enzymes. These are in addition to flavin known to have a pK_a of 7.9 for the ionization of $FADH_2$ to $FADH^-$ (see below). Since the change in the slope for the altered enzymes occurs at pH 7.9, it is reasonable that no change in the slope was observed for the pig heart enzyme from pH 5.5 to 7.9. In fact, the data of Matthews and Williams (1976) for the E_{ox}/EH_2 potentials between pH 7.6 and 7.9 suggest an imminent change in the slope. These data indicate that the altered enzymes exhibit redox behavior similar to that of the pig heart enzyme.

The crystal structure of lipoamide dehydrogenase shows that besides the thiols (Cys^{44}, Cys^{49}) and the base ($His^{444'}$), a number of other ionizable residues are present in the active site (Schierbeek et al., 1989). These residues could be differentially protonated/deprotonated on either the oxidized or the reduced forms of the enzyme, and these interactions could alter the redox behavior.

The assignment of the change in the slope at pH 7.9 to deprotonation of $FADH_2$ to $FADH^-$ was based on the shape of the spectra of the reduced enzymes as a function of pH (data not shown). Dithionite titrations over a range of pH values showed that the $FADH_2$ of C44S has a pK_a of 7.9, and the pK_a of $FADH_2$ for C49S was between the pH values of 7.6 and 8.6. The uncertainty in the latter case was due to interference from excess dithionite and the reduced methyl viologen mediator. The pK_a of approximately 7.9 for the flavin ionization for both altered enzymes indicates that the environment surrounding the flavin affects not only the redox potential, but also the ionization of the reduced flavin. The reduced flavin ionization is also altered from pH 6.7 in free $FADH_2$ (Lowe & Clark, 1965) to pH 7.9 in both altered enzymes.

The flavin redox potential is modulated by interaction with specific amino acid residues as mentioned above. It is also modulated by the overall environment of the protein, since it has been shown that binding of the flavin to an enzyme alters the redox potential (Table 3). Nearly all flavoenzymes catalyze specific reactions whereby the flavin is used to transfer electrons from one substrate to another. In order for these reactions to occur, the electron transfer should be thermodynamically favorable, other factors such as product removal being equal. In the case of lipoamide dehydrogenase, it has been shown here that the altered enzymes lower the redox potential of the flavin upon binding to the protein (Table 3). This binding indicates that the flavin is bound more tightly to the oxidized form of the enzyme than to the reduced form of the enzyme.

With only these considerations, and the low redox potentials of the altered enzymes, it is difficult to understand how it would be possible for these enzymes to be reduced by NADH (redox potential of −320 mV at pH 7.0 and 25 °C) which it does readily. Since it is known that NAD^+ and NADH are capable of binding to the oxidized and reduced forms of lipoamide dehydrogenase (Wilkinson & Williams, 1981, 1979b; Matthews et al., 1979; Maeda-Yorita et al., 1991), it seems reasonable that their binding will somehow alter the redox potential of the flavin further in order to facilitate reduction by NADH.

Binding of pyridine nucleotides during reduction of either enzyme using NADH and NADPH causes the redox potential to be raised with respect to the redox potential determined relative to a redox dye (Figure 6). The redox potential of a system will be raised or lowered if a ligand binds with different affinities to the oxidized and reduced forms of the measured system. Tighter binding of a ligand to the reduced form will raise the redox potential of the measured system while tighter binding of a ligand to the oxidized form will lower the measured redox potential of the system. Several such systems have been studied, and a few examples are cited (D-amino acid oxidase, Van den Berghe-Snorek & Stankovich, 1985; *p*-hydroxybenzoate hydroxylase, Williamson et al., 1988; flavodoxin, Ludwig et al., 1990). It is important to note that the equations presented in Clark (1960) and the systems just mentioned are not directly applicable to the effects of ligands that are redox active. Analysis of the data presented here has shown that NADH, NAD^+, NADPH, and $NADP^+$ must bind more tightly to the reduced form of the enzymes, since the redox potentials of the enzymes are raised in the presence of these ligands.

The non-Nernstian behavior of the NADH data for both enzymes indicated that the binding of NAD^+ and NADH was affecting the redox potential. A close inspection of the Nernst plots for the NADH data (Figure 6, circles) indicates that the midpoint potential (E_m) of the enzymes must be

changing, and this is causing the system potential (E_h) to remain relatively constant.

Since these were equilibrium titrations (i.e., spectral data were not recorded until all changes ceased), it would appear that the binding of NAD^+ and NADH to E_{ox} and E_{red} is responsible for these changes in the redox potential. This non-Nernstian behavior also indicates that the ligands must bind more tightly to the reduced form than to the oxidized form of the enzyme, since the midpoint potential of the enzyme is raised as the reduction of the enzyme proceeds (Figure 6A). Indeed, it can be calculated using eq 1 that as the titration proceeds and the putative binding of pyridine nucleotide to the reduced enzyme becomes greater, the E_m rises from -365 mV at $\log(E_{ox}/E_{red}) = 0.5$, to -352 mV at $\log(E_{ox}/E_{red}) = 0$, to -342 mV at $\log(E_{ox}/E_{red}) = -0.5$. The effect on the redox potential when NADPH is used as a reductant for either enzyme is quite small. Unlike the NADH data, the NADPH data for both enzymes showed nearly Nernstian behavior, which is indicative of the vastly weaker binding affinity of NADPH compared with that for NADH. Nernstian behavior is observed for both pyridine nucleotides and for both enzymes in the later stages of the titration, when the enzyme is almost fully saturated with ligand, and these data yield a rough approximation of the redox potential in the presence of pyridine nucleotide.

Lambeth and Kamin (1976) have studied a system (adrenodoxin reductase) in which it was assumed that only $NADP^+$ served as a ligand and NADH (with vanishingly weak binding) was used as the reductant. Thus, only free enzyme was available for reduction. They showed that if the redox potentials in the absence and presence of ligand were known and the binding constant for the ligand to oxidized enzyme was known, the binding constant for the interaction of ligand with the reduced enzyme could be predicted. They derived the following equation, where the difference between the redox potentials determined in the presence and absence of ligand is ΔE_m:

$$K_r = K_o \times 10^{-(nF\Delta E_m/2.3RT)}$$

and where K_r is the dissociation constant for ligand binding to the reduced enzyme and K_o is the dissociation constant for the ligand binding to the oxidized enzyme. Such a treatment would not be appropriate for data from titrations of C44S and C49S, since in order for NADH to bind and reduce the enzyme, NAD^+ would have to dissociate.

Previous studies have provided spectral evidence for the binding of oxidized and reduced pyridine nucleotides to different forms of lipoamide dehydrogenase (Massey & Palmer, 1962; Williams, 1965; Matthews et al., 1976, 1979; Maeda-Yorita et al., 1991). These studies have demonstrated charge-transfer interactions with the reduced flavin as the donor and NAD^+ as the acceptor and with the pyridine nucleotide analog APADH as the donor and FAD as the acceptor. The binding constant for NADH to two-electron-reduced enzyme was 50 μM (Maeda-Yorita et al., 1991).

The dissociation constants for the binding of NADH and NADPH to both oxidized enzymes were determined. The results are summarized in Table 1. The very weak binding of NADPH to C44S and C49S compared with NADH, together with the observed effects of differential binding of pyridine nucleotides on the redox potential (Table 2), indicates that binding to the reduced enzyme is tighter. The redox data would predict that the binding constants for NADH would be tighter than those for NADPH since the redox potentials using NADH are higher than those using NADPH, and this was exactly the case as indicated above (Table 2).

Redox experiments were performed using AAD^+ which is a nonreducible analog of NAD^+. It was hypothesized that AAD^+ would mimic the interaction of NAD^+ and NADH with the enzymes, since while formally oxidized, it has an electron rich ring as in NADH. This similar interaction with the enzymes would result in redox potentials that would be representative of the redox potentials of the enzymes in the presence of NAD^+ and NADH. The K_d values for the binding of AAD^+ to the oxidized enzymes were tighter than those for NAD^+, and the spectral perturbations effected by the two pyridine nucleotides were noticeably different for both enzymes (Table 1). Redox titrations (methyl viologen) were performed in the presence of an excess of AAD^+. This system differs in a fundamental way from those using pyridine nucleotide as the reductant and ligand, because it was assumed that the reduced dye could react with the enzyme whether AAD^+ was bound or not. The assumption was based on analogy to the closely related enzyme glutathione reductase in which a safranine binding site distinct from the pyridine nucleotide site had been demonstrated (Karplus et al., 1989). Thus, AAD^+ did not act by removing part of the enzyme from the redox equilibrium but rather by its interaction with the isoalloxazine ring via their π clouds. The estimated redox potentials were -444 and -375 mV for C44S and C49S, respectively (Table 2). The estimated redox potential for C44S indicated that AAD^+ was bound more tightly to the oxidized enzyme than the reduced enzyme when compared to the redox potential in the absence of ligand (-411 mV). In contrast, the redox potential for C49S indicated that AAD^+ was bound more tightly to the reduced enzyme than the oxidized enzyme when compared to the redox potential in the absence of ligand (-386 mV), but the effect was small.

These redox potentials determined in the presence of AAD^+ are lower for both enzymes compared with those determined using NADH and NADPH (Table 2). Since the binding of AAD^+ to oxidized C44S and C49S was tighter than the binding of NAD^+ (Table 1), and the spectral perturbations effected by the two pyridine nucleotides were different, it does not seem unreasonable that the effect on the redox potentials would be different with AAD^+ compared with NAD^+. However, the results do not lend themselves to the simple interpretation predicted. These data support the hypothesis that the redox potentials determined in the presence of NADH and NADPH are complex redox potentials involving at least two binding constants. If this is the case, then it follows that the binding constants for NADH to the reduced enzymes must be very tight in order to account for the increase in the redox potential determined in the presence of NADH.

Determination of the K_d values for the binding of NAD(P)H to the reduced enzyme was not undertaken. The difference between the redox potentials determined in the absence and presence of pyridine nucleotide indicates that binding of NAD(P)H to the reduced enzymes would have to be several orders of magnitude tighter than the binding constants for the oxidized enzyme, i.e., in the micromolar

to submicromolar range. These determinations would be difficult since the enzyme concentration would need to be low, making spectral observation difficult and trace oxygen a significant problem. In addition, flavin dissociation would be a problem because the enzyme binds the reduced flavin much more loosely than the oxidized flavin. Estimates of the K_d values for the binding of NADH to the reduced enzyme have been obtained using the equation of Lambeth and Kamin with the caveat discussed above. This indicates K_d values for NADH of 2.4 μM for binding to reduced C44S and of 2.8 μM for binding to reduced C49S.

In conclusion, these studies have demonstrated the complexity of the interaction of pyridine nucleotides with the altered forms of lipoamide dehydrogenase, C44S and C49S. Simplification was achieved by using altered forms of the enzyme that lack the disulfide redox center. The data show that the pyridine nucleotides modulate the flavin redox potential. The redox potential of these enzymes was raised when NADH and NADPH were used as reductants. Previous studies with pig heart lipoamide dehydrogenase and adrenodoxin reductase have shown similar redox behavior, namely, increases in the enzyme redox potential, upon interaction with their physiological pyridine nucleotide substrates (Maeda-Yorita & Aki, 1988; Lambeth & Kamin, 1976). It would appear that the pyridine nucleotides bind in a manner that facilitates electron transfer to the enzyme-bound flavin (Matthews & Williams, 1976). If the redox potentials of these enzymes were not raised, a large excess of the pyridine nucleotides would be required in order to achieve required levels of reduction. What should also be noted are the differences in the redox potentials when NADH and NADPH are used. The natural substrate has a greater effect on the redox potential than the nonphysiological substrate, and this is a result of the tighter binding of NADH to the enzymes than NADPH.

Lipoamide dehydrogenase catalyzes an essentially irreversible reaction in the physiological direction where dihydrolipoamide must reduce NAD^+ (Table 3). The thermodynamic barrier is overcome in mitochondria (and perhaps in bacteria) by the very high NAD^+/NADH ratio. *In vitro*, the reaction in the physiological direction rapidly becomes product inhibited. The greater sensitivity of the bacterial enzyme to NADH compared with the mitochondrial enzyme suggested that the NAD^+/NADH ratio is a sensitive control mechanism in bacteria where control by phosphorylation/dephosphorylation is absent (Wilkinson & Williams, 1981). While the overall reaction in the opposite direction, reduction of lipoamide by NADH, is thermodynamically favorable, the very low redox potential of the FAD/$FADH_2$ couple is a potential barrier. This barrier is overcome by the modulation of the flavin potential by pyridine nucleotides.

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[2] In the distantly related enzyme NADH peroxidase, C42S is analogous to C49S in lipoamide dehydrogenase. The redox potential of the flavin is −219 mV (D. Parsonage and A. Claiborne, Wake Forest University, personal communication, submitted).

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