Characterization of Lipoamide Dehydrogenase from *Escherichia coli* Lacking the Redox Active Disulfide: C44S and C49S†

Nancy Hopkins and Charles H. Williams, Jr.*

Department of Biological Chemistry, University of Michigan, and Department of Veterans Affairs Medical Center, Ann Arbor, Michigan 48105

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ABSTRACT: Lipoamide dehydrogenase from *Escherichia coli*, a dimeric flavoprotein in the pyridine nucleotide−disulfide oxidoreductase family of enzymes, catalyzes the reduction of NAD^+ by dihydrolipoamide. The two electrons are transferred via a redox active disulfide and FAD. Cys^{44} and Cys^{49} comprise the redox active disulfide, Cys^{44} interchanging with dihydrolipoamide and Cys^{49} interacting with the flavin. Each of these residues has been mutated to serine (C44S, C49S). The altered enzymes showed minute amounts of activity, 0.003% for C44S and 0.012% for C49S using the physiological substrates dihydrolipoamide and NAD^+. These very low activities were expected, since the disulfide was no longer present in C44S and C49S, making dithiol−disulfide interchange impossible. However, the enzymes were capable of catalyzing reactions using NADH as the electron donor and alternate electron acceptors: $K_3Fe(CN)_6$, thio-NAD^+, DCIP, and O_2. These activities with NADH indicated that interaction of C44S and C49S with pyridine nucleotides was not affected greatly by the mutation. The pH dependence of the charge-transfer absorbance of C44S gives pK_a values of 2.7, associated with titration of Cys^{49}, and 9.5, associated with titration of the acid−base catalyst, $His^{444'}$. A pK_a of 5.1 was estimated for Cys^{44} in C49S from the pH dependence of its reactivity with methyl methanethiosulfonate. The fluorescence of the FAD in oxidized wild type lipoamide dehydrogenase is markedly temperature dependent, while the remaining fluorescence of two-electron-reduced enzyme is independent of temperature. The fluorescence of the FAD in C44S and in C49S is likewise independent of temperature. The FAD of C44S and C49S is stoichiometrically titrated by 1 equiv of sodium dithionite. However, the FAD of C44S is markedly less completely reduced by 1 equiv of NADH than is the FAD of C49S. Ferricyanide stoichiometrically reoxidizes the $FADH_2$ of both altered forms of the enzyme.

Lipoamide dehydrogenase catalyzes the terminal reaction in the α-ketoglutarate and pyruvate dehydrogenase multienzyme complexes. This homodimeric flavoprotein of the pyridine nucleotide−disulfide oxidoreductase family catalyzes the reduction of NAD^+ by dihydrolipoamide. It has a subunit molecular mass of 50 kDa with two redox centers, the FAD and a disulfide, which are each capable of accepting two electrons. The oxidized enzyme proceeds through catalysis by passing two electrons from the reduced substrate, dihydrolipoamide, to the disulfide, from the dithiol to the FAD, and from reduced flavin to the ultimate electron acceptor, NAD^+. The species of reduced enzyme in catalysis are referred to as EH_2.[1] The kinetic mechanism has been shown to be ping-pong (Reed, 1973). The enzyme can be fully reduced by an additional two electrons to give four-electron-reduced enzyme (EH_4), where both the disulfide and the flavin are in the reduced state, but this enzyme form is not catalytically active (Wilkinson & Williams, 1981). Although the enzyme isolated from different sources has essentially the same catalytic mechanism, there are some distinctive quantitative differences between the enzyme isolated from pig heart and that from *Escherichia coli*. Certain mechanistic characteristics are best studied in the *E. coli* enzyme.

The *E. coli* enzyme is partially reduced to the four electron level with excess NADH or dihydrolipoamide, while the pig heart enzyme is only reduced to the two electron reduced level using excess substrate (Koike et al., 1960; Massey et al., 1960; Massey & Veeger., 1961; Williams, 1965). These differences reflect the redox potentials for E_{ox}/EH_2 (−264 mV, −280 mV) and EH_2/EH_4 (−317 mV, −346 mV) for the *E. coli* and pig heart enzymes, respectively, at pH 7.0, 25 °C (Matthews & Williams, 1976; Wilkinson & Williams, 1979). The redox potentials for the *E. coli* enzyme are more positive than those of the pig heart enzyme, explaining why the *E. coli* enzyme can be more easily over-reduced to the EH_4 state. The *E. coli* enzyme also exhibits less thiolate−FAD charge transfer than the pig heart enzyme when they are reduced to the EH_2 level. It has been shown that three spectrally distinct forms are in equilibrium, at neutral pH: the thiolate−FAD charge-transfer species, which is non-

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* To whom correspondence should be addressed, VA Medical Center (151), 2215 Fuller Rd., Ann Arbor, MI 48105. Tel (313) 769-7100, ext. 5610; FAX (313) 761-7693.

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[1] Abbreviations: APADH and $APAD^+$, reduced and oxidized forms of 3-acetylpyridine adenine dinucleotide, respectively; thio-NAD^+, thionicotinamide adenine dinucleotide; DCIP, dichlorophenolindophenol; MMTS, methylmethane thiosulfonate; site-directed mutation of lipoamide dehydrogenase: C44S, $cysteine^{44}$ replaced with a serine residue and C49S, $cysteine^{49}$ replaced with a serine residue; E_{ox}, oxidized enzyme; EH_2, two-electron-reduced enzyme; EH_4, four-electron-reduced enzyme (see Scheme 1).

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fluorescent and favored at high pH; the prototropic tautomer of the charge-transfer complex that has 68% of the fluorescence of the oxidized enzyme and is favored at low pH; and a form of EH_2 that has reduced flavin and the disulfide (Wilkinson & Williams, 1979). The presence of two additional species at the EH_2 level provides an explanation for the observation of less thiolate−FAD charge-transfer complex in the reduction of the *E. coli* enzyme in comparison to the pig heart enzyme. This difference in the electronic distribution for the *E. coli* enzyme at the EH_2 level has led to studies to achieve a better understanding of these EH_2 species.

The redox active disulfide is composed of two cysteine residues (Cys^{44} and Cys^{49}) that have distinct functions. Cys^{44} participates in dithiol−disulfide interchange with the reduced substrate, and Cys^{49} participates in a thiolate−FAD charge-transfer interaction with the flavin (Thorpe & Williams, 1976a,b, 1981), which is detected spectrally by increased absorbance in the 530 nm region of the flavin spectrum during reduction to the two-electron-reduced species. Both of the cysteines have been mutated to serines to give two altered enzymes (C44S and C49S), each having one thiol remaining (Allison et al., 1988; Russell et al., 1989; Hopkins et al., 1991). C44S and C49S have been used to study the properties of the remaining cysteine residue and the behavior of the flavin in the absence of the disulfide. In all of these studies, the altered forms have been compared to the wild type enzyme as well as to the other enzymes in this family.

MATERIALS AND METHODS

Reagents. Oxidized and reduced pyridine nucleotides and MMTS were purchased from Sigma Chemical Co. Sodium dithionite was purchased from Matheson, Coleman and Bell. The Q-Sepharose chromatographic support was purchased from Pharmacia-LKB. Hydroxylapatite was purchased from Bio-Rad Labs. FAD was generously provided by Dr. Vincent Massey of the University of Michigan. Dihydrolipoamide was synthesized from lipoamide. Lumiflavin 3-acetic acid was a generous gift from the late Dr. Peter Hemmerich of the University of Konstanz. All buffer salts and growth media components were of the highest quality.

Mutagenesis and Cell Growth. *E. coli* lipoamide dehydrogenase was altered by site-directed mutagenesis as previously described (Allison et al., 1988; Russell et al., 1989; Williams et al., 1989). The cell growth was a modification of the method described by Maeda-Yorita et al. (1991). The cells were harvested and stored at −20 °C.

Enzyme Purification. The altered and wild type enzymes were purified in a similar manner. The cells were homogenized in 20 mM Tris buffer, pH 7.6, containing 0.3 mM EDTA, with the addition of 4.5 mg/mL of PMSF (phenylmethanesulfonyl fluoride) to prevent proteolysis. The cell suspension was sonicated in a Branson Sonifer, Model 200, for five bursts of 3 min each using an ice and NaCl mixture to keep the temperature below 0 °C. Subsequent procedures were at 4 °C unless otherwise specified. Streptomycin sulfate (2% w/v) was added to the solution, which was allowed to stand for 10 min in order to remove nucleic acids. The suspension was centrifuged at 20 000 rpm for 20 min and 45 000 rpm for 1 h in a Beckman Model L8-70M ultracentrifuge using a VTi-50 rotor to remove cell debris and nucleic acids. The supernatant was decanted, precipitated with 35% saturated ammonium sulfate, and allowed to stand for 30 min. The ammonium sulfate suspension was centrifuged at 10000*g* for 30 min, and the pellet was discarded. The supernatant was precipitated with 85% saturated ammonium sulfate and allowed to stand for 1 h. The ammonium sulfate suspension was centrifuged at 15000*g* for 1 h. The supernatant was discarded, and the pellet was resuspended in 20 mM Tris buffer, pH 7.6, containing 0.3 mM EDTA. The protein suspension was dialyzed overnight against the same buffer. The protein solution was centrifuged at 15000*g* for 30 min to remove precipitated protein and applied to a Q-Sepharose anion exchange column attached to a Waters Fast Protein Purification System at 10 mL/min with 20 mM Tris buffer, pH 7.6, containing 0.3 mM EDTA at 25 °C. The enzymes were eluted with 20 mM Tris buffer, pH 7.6, with 0.3 mM EDTA containing 0.2 M NaCl. The eluant containing the enzyme was precipitated with 0-85% ammonium sulfate at 4 °C. The pellet was resuspended in 100 mM phosphate buffer, pH 7.6, containing 0.3 mM EDTA (standard buffer) and dialyzed overnight against the same buffer. The enzyme was applied to a hydroxylapatite column and eluted with standard buffer containing 0.3 M ammonium sulfate. The pure enzyme was precipitated with 0−85% ammonium sulfate. The enzyme was resuspended in standard buffer and dialyzed overnight against standard buffer. The concentrated enzymes were stored at −20 °C in 1.2 mL Eppendorf tubes. Approximately 50−100 mg of mutant enzyme or 200 mg of wild type enzyme was obtained from 50 g (wet weight) of cells. Enzyme purity was determined by sodium dodecyl sulfate−polyacrylamide gel electrophoresis according to the method of Laemmli (1970). Spectral ratios between 280 nm and the FAD peak position were determined (5.8 for wild type enzyme, 5.5 for C44S and C49S).

Spectral Analysis. The spectra were taken using either a Cary 118-C spectrophotometer interfaced with a computer, or a Perkin-Elmer Lambda 4A spectrophotometer. Typically, enzyme solutions (10−50 μM) at 25 °C were scanned from 700 to 250 nm. Fluorescence emission and excitation spectra were taken with a Perkin-Elmer Model MPF-44B fluorescence spectrometer, measuring emission at 560 nm for excitation spectra and measuring excitation at 360 nm for emission spectra. The fluorescence was measured with 10−20 μM enzyme at 5 °C.

Fluorescence as a Function of Temperature. The fluorescence excitation and emission spectra of wild type enzyme, oxidized and two-electron-reduced, C44S and C49S, were measured at 35, 25, 15, and 5 °C. The two-electron-reduced wild type enzyme was generated by adding 1 equiv of anaerobic sodium dithionite to an anaerobic solution of oxidized enzyme. Upon stabilization of the EH_2 species, the visible spectrum was recorded and the fluorescence spectrum was measured at 35 °C. After all the temperature changes were completed, the visible spectrum was recorded to be certain that the extent of disproportionation was not significant.

Determination of Extinction Coefficients. The extinction coefficient of the protein-bound FAD at 444 nm for C44S and C49S was determined by removing the FAD from the protein and quantitating the free FAD. The wild type enzyme was included for comparison. Determinations were performed in triplicate. All calculations were adjusted for

volume changes when necessary. The three methods used for removing the FAD were as follows: 4.5 M guanidinium chloride (O'Donnell & Williams, 1984), 0.2% sodium dodecyl sulfate (Zanetti & Williams, 1967), and 5% trichloroacetic acid.

The spectra of the enzymes were recorded, at a concentration of 8−10 μM in 10 mM phosphate, pH 7.6, containing 0.3 mM EDTA. Guanidinium chloride (8 M) was added together with buffer to give a total volume of 1 mL and a final concentration of 4.5 M guanidinium chloride. The extinction coefficient of free FAD is enhanced in 4.5 M guanidinium chloride to a value of 11 800 M^{-1} cm^{-1} as determined separately, and this value was used in the calculations to determine the extinction coefficients of FAD bound to the enzymes.

When sodium dodecyl sulfate was used to remove the flavin, the enzymes (8−11 μM) were prepared in 1 mL of 10 mM Tris-sodium/phosphate, pH 7.6, containing 0.3 mM EDTA, and a spectrum was recorded. A volume of 20 μL of 10% sodium dodecyl sulfate was added and the spectrum again recorded. The FAD was released from the altered enzymes, but it remained bound to the wild type enzyme until the solution was heated at 45 °C for 60 min in a sealed tube.

When trichloroacetic acid was used to remove the flavin, the enzymes were prepared in 10 mM phosphate, pH 7.6, containing 0.3 mM EDTA in a total volume of 0.90 mL, and the spectrum was recorded. A volume of 100 μL of ice cold 50% TCA was added, and the enzyme solutions were allowed to sit on ice for 10 min. The samples were centrifuged for 15 min, and the supernatant was decanted. The pellet was dissolved in 1 mL of 8 M guanidinium chloride, and a spectrum was recorded to determine if any flavin remained. Solid sodium bicarbonate was added to the supernatant to give an approximate pH of 7.0, and the spectrum was recorded.

Enzyme Assays. There were six assays used to characterize the altered enzymes. All assays were performed at 25 °C. The assays can be divided in two classes. The first class involves the use of dihydrolipoamide, the natural electron donor, and NAD^+, the natural substrate, or $APAD^+$, a pyridine nucleotide analog, to determine activity in the physiological direction. The other class involves NADH as the donor, and various electron acceptors, O_2, $K_3Fe(CN)_6$, thio-NAD^+, and DCIP. All assay reactions were measured in a Beckman DU at the appropriate wavelength. The turnover number is defined as (μmol of substrate oxidized or reduced)/(min·μmol of enzyme FAD). The physiological assays required enzyme concentrations in the nanomolar range for the wild type enzyme and micromolar amounts for the mutant enzymes. The assay required 80 μM dihydrolipoamide and 400 μM NAD^+ or $APAD^+$ at pH 7.6 in standard buffer, and the increase in the absorbance at 340 or 363 nm was monitored for the reduction of NAD^+ and $APAD^+$, respectively. Extinction coefficients of 6220 M^{-1} cm^{-1} and 9100 M^{-1} cm^{-1} were used for NAD^+ and $APAD^+$ reduction, respectively. The assays involving NADH as the donor required nanomolar quantities of all the enzymes. The NADH oxidase activity was measured with 50 μM NADH at pH 7.6 in air saturated standard buffer.

The NADH/$K_3Fe(CN)_6$ activity was measured with 50 μM NADH and 0.67 mM $K_3Fe(CN)_6$ at pH 7.90 in 100 mM Tris-HCl containing 0.3 mM EDTA by following the decrease in absorbance at 420 nm for the reduction of $K_3Fe(CN)_6$. An extinction coefficient of 1036 M^{-1} cm^{-1} was used (Massey, 1960). The pH optima were determined using buffers as follows: 100 mM buffers containing 0.3 mM EDTA were used; pH 4.98−5.50, acetate; pH 6.04−7.94, phosphate; pH 8.49−9.00, Tris-HCl; and pH 9.50, glycine−NaOH. All assays were performed in triplicate.

The NADH/thio-NAD^+ transhydrogenase activity was measured with 25 μM NADH and 25 μM thio-NAD^+ at pH 7.6 in standard buffer. The increase in absorbance at 395 nm indicative of the reduction of thio-NAD^+ used an extinction coefficient of 11 300 M^{-1} cm^{-1} (Stein et al., 1963).

The NADH/DCIP diaphorase activity was measured with 200 μM NADH and 40 μM DCIP at pH 7.2 in 100 mM phosphate buffer, pH 7.6, containing 0.3 mM EDTA. The reaction was followed by observing the decrease in absorbance at 600 nm using an extinction coefficient of 21 000 M^{-1} cm^{-1} (Steyn-Parve & Beinert, 1958).

NADH/$K_3Fe(CN)_6$ Spectral Studies at pH 7.6. C44S and C49S were treated with 1 equiv of NADH and then 1 equiv of the one electron acceptor $K_3Fe(CN)_6$ to test for the presence of flavin semiquinone. The enzymes (20 μM), NADH, and $K_3Fe(CN)_6$ were prepared in standard buffer and made anaerobic. Upon completion of anaerobiosis, 1 equiv of NADH was added to the enzyme with a syringe, and the reduction was monitored by observing the decrease in absorbance at the flavin peak. When the spectral changes ceased, the spectrum was recorded. The NADH syringe was removed under positive pressure, and a syringe containing $K_3Fe(CN)_6$ was attached. The oxidation was monitored by observing the increase in absorbance at the flavin peak upon addition of 1 and then 2 equiv of $K_3Fe(CN)_6$.

Dithionite Titrations. Dithionite was prepared according to a method described previously (Burleigh et al., 1969). The enzymes (10 μM) were prepared in standard buffer and placed in an all glass anaerobic cuvette equipped with a port for the addition of anaerobic dithionite from an airtight syringe (Burleigh et al., 1969; Lambeth et al., 1973) capable of delivering 2 μL additions. A small amount of methyl viologen (2−5% of enzyme concentration) was added to the enzyme solution before anaerobiosis as a mediator. The absorbance and fluorescence spectra of the enzymes were recorded before anaerobiosis, and the concentration of the enzymes was determined using the extinction coefficient for the enzyme-bound FAD. The enzyme solution was made anaerobic with 10 alternating cycles of purified nitrogen and vacuum. After the completion of anaerobiosis, the spectrum of the enzyme solution was recorded and the concentration was determined again, accounting for any volume loss. Dithionite (2−4 μL) was added, and the decrease at the flavin peak indicating reduction was observed. A spectrum was recorded after each addition upon completion of any changes in the flavin peak absorbance. The equilibration time needed between additions was 10−40 min.

Determination of the pK_a of the Remaining Thiol. C44S has the charge-transfer (Cys^{49}) thiol remaining, and C49S has the interchange thiol (Cys^{44}) remaining. The pK_a values were determined aerobically.

The charge-transfer absorbance in C44S at 530 nm is taken as an indication of the interaction of the thiol in its anionic form with the flavin, and the protonation/deprotonation of this thiol (Cys^{49}) can be monitored spectrally at 530 nm as the pH is changed. Enzyme (47−55 μM in 10 mM

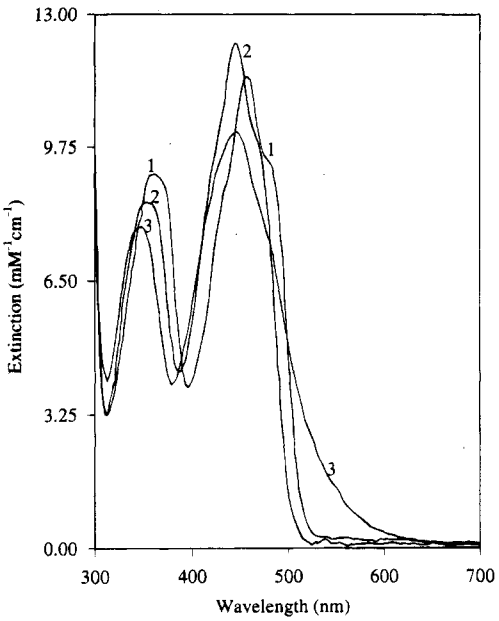


FIGURE 1: Absorbance spectra of *E. coli* lipoamide dehydrogenase enzymes. (1) Wild type, 10.26 μM; (2) C49S, 9.60 μM; and (3) C44S, 10.1 μM. The enzymes were prepared in 100 mM sodium/potassium phosphate, pH 7.6, containing 0.3 mM EDTA.

phosphate, pH 7.6, containing 0.3 mM EDTA) was mixed with buffers having a constant ionic strength of 0.5 M at 4 °C in the stopped-flow spectrophotometer, and the spectrum was recorded. The following buffers were used: glycine hydrochloride; pH 1.8−2.22; citrate−phosphate, pH 2.87−7.17; Tris−phosphate, pH 7.11−9.36; glycine−NaOH, pH 9.31−10.7; and arginine−NaOH, pH 10.78−12.1. In control experiments, the buffer without enzyme was mixed 1:1 with the constant ionic strength buffers to ensure that the desired pH jump was achievable. The detector used in this experiment was a Tracor-Northern spectrophotometer with a diode array detector capable of taking a full spectrum in 5.42 ms.

C49S lacks a spectral indicator of the protonation state of the thiol. The pK_a value associated with protonation/deprotonation of the interchange thiol remaining in C49S was determined by treating the enzyme with methyl methanethiosulfonate (MMTS) (Smith et al., 1975; Roberts et al., 1986). The reaction of MMTS with the Cys^{44} in C49S is associated with a change in the enzyme spectrum best detected in the difference spectrum between untreated and treated enzyme, subtracting the absorbance at 488 nm from that at 412 nm. Control experiments showed that this change was unique to C49S and was absent in wild type enzyme and in C44S. The enzymes (30−33 μM) in 10 mM phosphate, pH 7.6, containing 0.3 mM EDTA were mixed with the appropriate buffer containing a 10-fold excess of MMTS, aerobically, in the stopped-flow instrument, and the spectra were recorded using the diode array detector. Difference spectra were generated between unreacted and fully reacted enzyme at each pH value over the pH range of 3.61−8.38 at 4 °C to determine the pK_a of the interchange thiol in C49S. The pH jump buffers were 100 mM at the desired pH containing MMTS, 5% acetonitrile (to increase the solubility of MMTS in aqueous solutions), and 0.3 mM EDTA. The final pH of the mixture was determined in a control experiment by mixing the buffer without enzyme with the pH jump buffer in a 1:1 ratio in the stopped flow.

Table 1: Extinction Coefficients for C44S and C49S

enzyme	ϵ_{530} (mM^{-1} cm^{-1})	ϵ_{444} (mM^{-1} cm^{-1})
C44S	1.97 ± 0.084	9.95 ± 0.11
C49S		12.14 ± 0.21

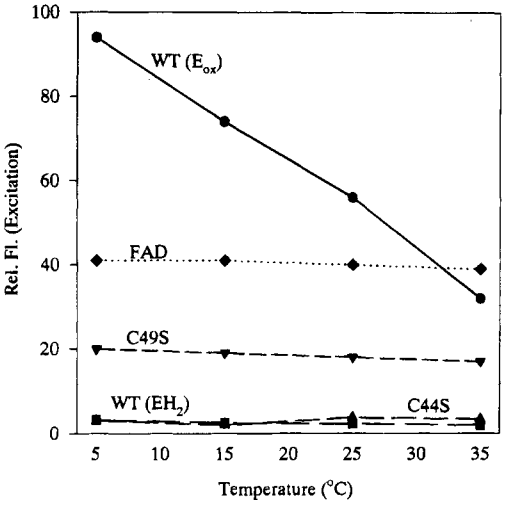


FIGURE 2: Relative fluorescence excitation as a function of temperature. The readings were measured at the maximal wavelength for each sample: free FAD, 450 nm; C44S and C49S, 444 nm; and wild type E_{ox} and EH_2, 455 nm. The wild type EH_2 enzyme was prepared by reducing oxidized enzyme anaerobically with 0.9 equiv of sodium dithionite.

RESULTS

Spectral Characterization of C44S and C49S. C49S is bright yellow like the wild type enzyme, and their spectra are compared in Figure 1. The main flavin band is blue shifted 11 nm, the trough at 390 nm is higher, and the shoulder at 470 nm is less pronounced, indicative of a more hydrophilic environment around the flavin (Harbury et al., 1959). C44S has an orange-red color with a spectrum (Figure 1, spectrum 3) resembling that of the two-electron-reduced wild type enzyme, with the thiolate−FAD charge transfer absorbance in the 550 nm range and an 11 nm blue shifted flavin peak. Table 1 shows the extinction coefficients for both enzymes at 444 nm and for the C44S enzyme at 530 nm.

When the fluorescence of C44S, C49S, free FAD, wild type oxidized, and two-electron-reduced enzymes was monitored as a function of temperature (Figure 2), only the wild type oxidized enzyme showed a large dependence on temperature, with the fluorescence increasing as the temperature is lowered. This behavior was also noted for the enzyme from pig heart, but its significance is not clear (Massey, 1963). However, given the magnitude of the effect, temperature must be strictly controlled when measuring the fluorescence of lipoamide dehydrogenase. The excitation spectra of wild type enzyme and of C49S were essentially identical to their respective absorbance spectra. On the other hand, the excitation spectra of C44S and two-electron-reduced wild type enzymes resembled the absorbance spectrum of oxidized wild type enzyme, demonstrating that the fluorophore in all cases is enzyme-bound FAD. It has been shown that the thiolate−FAD charge transfer absorbance at 530 nm does not contribute to the fluorescence (Wilkinson & Williams, 1979).

Table 2: Enzyme Activities for C44S and C49S[a]

enzyme	C44S	C49S
$Lip(SH)_2NH_2/NAD^+$	0.169 (0.003)	0.622 (0.01)
$Lip(SH)_2NH_2/APAD^+$	0.379 (0.1)	0.029 (0.01)
NADH/DCIP	0.593 (56)	0.598 (57)
$NADH/O_2$	0.285 (33)	0.542 (62)
$NADH/thio\text{-}NAD^+$	27.4 (400)	11.1 (150)
$NADH/K_3Fe(CN)_6$	297 (10)	1740 (55)
V_{max} (min^{-1})	320 (5)	2350 (35)

[a] All values are turnover numbers [(mol of substrate oxidized or reduced)/(min·mol of enzyme-bound FAD)]. The pH was 7.6, except in the ferricyanide assay where it was 7.9. The values in parentheses are comparison with wild type enzyme and are given in units of percent (%). The substrate concentrations are given in the Materials and Methods section. The turnover number under apparent V_{max} conditions for the NADH/ferricyanide reaction is for 50 μM NADH.

Enzyme Activities. The enzyme activities were determined using six assays, and the values are summarized in Table 2. Results shown in the top two lines involved the forward reaction of the enzyme using dihydrolipoamide as the donor and the natural substrate NAD^+ or $APAD^+$ as the acceptor. Both enzymes are essentially inactive. When measuring activities as low as these, the inherent oxidase activities must be subtracted, and this correction is of the same order of magnitude as the measured activity. Results shown in the next four lines involved NADH as the donor in the reverse direction and artificial electron acceptors, DCIP, $K_3Fe(CN)_6$, O_2, and $thio\text{-}NAD^+$. These activities, which are presumed to involve only the FAD, are similar to those of the wild type enzyme.

C44S and C49S exhibited severe NADH substrate inhibition regardless of the acceptor, as is the case with wild type enzyme (Wilkinson & Williams, 1981). Therefore, it was not possible to determine steady-state parameters for the enzymes when NADH was the substrate. However, a turnover number was measured for the $NADH/K_3Fe(CN)_6$ reaction under apparent V_{max} conditions using 50 μM NADH.

It was thought that the enzymes might go through a semiquinone state in the reduction of $K_3Fe(CN)_6$ since it is a one electron acceptor. C44S and C49S were reduced anaerobically with 1 equiv (two electrons) of NADH and then reoxidized in two stages with 1 equiv (one electron) each of $K_3Fe(CN)_6$. The spectra after each addition are shown in Figure 3. It is apparent from the spectra that any semiquinone formed by C44S or C49S upon addition of $K_3Fe(CN)_6$ was not detectable. However, it was possible that the semiquinone formed and either disproportionated or further oxidized before the spectra could be measured. There was a major difference in the reaction of C44S and C49S during this experiment. The two enzymes showed different levels of reduction by NADH, 15% and 50% for C44S (Figure 3A) and C49S (Figure 3B), respectively. Both enzymes and the remaining NADH were fully reoxidized when 2 equiv of $K_3Fe(CN)_6$ was added.

pK_a of the Remaining Thiol. The remaining thiol in C44S is the charge-transfer thiol, while that in C49S is the interchange thiol. Macroscopic pK_a values have been determined for these thiols in lipoamide dehydrogenase, but definitive assignments of the individual microscopic pK_a values are difficult where both thiols are present (Sahlman & Williams, 1989a,b; Williams, 1992). The absence of the second thiol in C44S and C49S makes the assignments feasible.

The pK_a of the charge-transfer thiol remaining in C44S was determined by monitoring the absorbance at 530 nm due to thiolate−FAD charge transfer as a function of pH. Limited stability of the enzyme at the more acidic and basic pH values necessitated the use of the stopped flow to record a spectrum before flavin dissociation was detected. The extinction at 530 nm as a function of pH is shown in Figure 4 , and the solid line is a theoretical curve fitted to three pK_a values. The pK_a at 2.7 was assigned to the charge-transfer thiol, that at 9.5 was assigned to $His^{444'}$, the active site base, and the apparent pK_a at 5.8 was unassigned. The basis for these assignments is addressed in the Discussion.

The pK_a of the interchange thiol remaining in C49S was determined by following the reaction of MMTS with C49S as a function of pH. The extent of the reaction was indicated by a spectral red shift that was not observed when this reagent was added in excess to the wild type enzyme or to C44S. Thus, it appears to result from a specific interaction of the thiomethyl substituent with the flavin. The absorbance difference between a positive peak at 488 nm and negative peak at 412 nm provided a sensitive measurement of the reaction (Figure 5). The solid line is a theoretical curve fitted to a pK_a of 5.1, assigned to the interchange thiol.

Dithionite Titrations. C44S and C49S were reduced anaerobically with dithionite to determine the number of equivalents needed to achieve full reduction. The wild type enzyme requires 2 equiv to achieve full reduction since it has two redox centers, the disulfide and the flavin, and each is capable of accepting two electrons (Williams, 1992). In contrast, the altered enzymes have only one thiol, which has replaced the disulfide, and therefore, full reduction requires 1 equiv of dithionite, as shown in Figure 6.

DISCUSSION

The use of site-directed mutagenesis has made it possible to confirm that the functional roles of the nascent thiols of the two-electron-reduced species in lipoamide dehydrogenase from *E. coli* are equivalent to those in the pig heart enzyme which were determined from chemical modification studies (Thorpe & Williams, 1976a,b, 1981). C44S exhibited behavior comparable to the two-electron-reduced wild type enzyme at pH 7.6, demonstrating interaction of the remaining thiol, Cys^{49}, with the FAD. Each altered enzyme required only two electrons in the dithionite titrations, to reduce the FAD, as expected, and the final spectra had the appearance of fully reduced enzyme (Figure 6). Moreover, the C49S (Figure 6B) showed no indication of a thiolate−FAD charge-transfer spectrum during reduction since the remaining thiol, Cys^{44}, cannot interact with the FAD.

Reduction of wild type enzyme by dithionite proceeds in two stages, the first 2 electrons being taken up primarily by the disulfide to form the thiolate−FAD charge-transfer complex and the second two electrons primarily reducing the FAD. The 2-electron reduced state is actually a mixture of three spectrally distinct species, as shown in Scheme 1. This mixture is best observed in the *E. coli* enzyme, since the thiolate−FAD charge-transfer complex predominates at equilibrium in the pig heart enzyme. The distribution of species in lipoamide dehydrogenase from *E. coli* is pH dependent; at pH 7.6 the thiolate−FAD charge-transfer complex, species IIb, constitutes 60%; its prototropic tautomer, species IIa, 27%; and the $FADH_2-NAD^+$ charge-

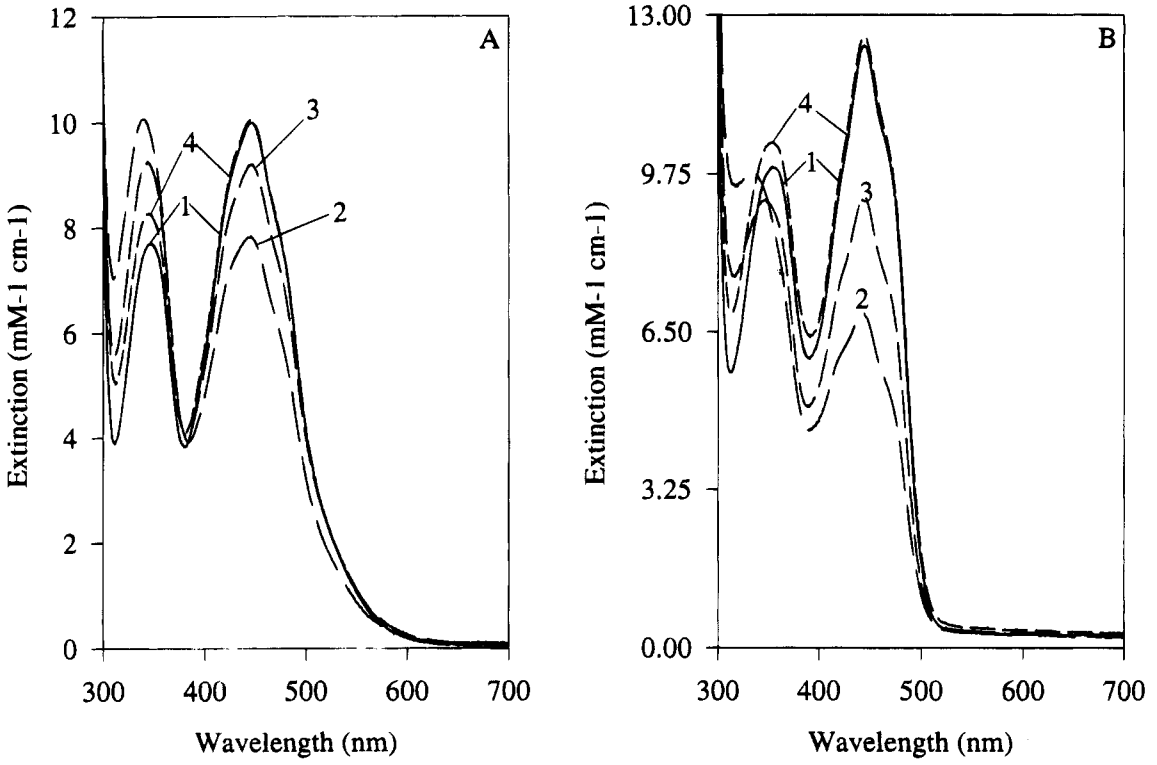


FIGURE 3: Reduction of C44S and C49S with NADH and reoxidation with $K_3Fe(CN)_6$ at pH 7.6. (A) C44S (27.6 nmol) was in 100 mM sodium/potassium phosphate buffer. (1) Oxidized anaerobic enzyme, (2) after addition of 1 equiv (two electrons) of NADH, (3) addition of 1 equiv (one electron) of $K_3Fe(CN)_6$, and (4) addition of another 1 equiv (one electron) of $K_3Fe(CN)_6$. (B) C49S (30.7 nmol) was in 100 mM sodium/potassium phosphate buffer. (1) Oxidized anaerobic enzyme, (2) after addition of 1 equiv (two electrons) of NADH, (3) addition of 1 equiv (one electron) of $K_3Fe(CN)_6$, and (4) addition of another 1 equiv (one electron) of $K_3Fe(CN)_6$.

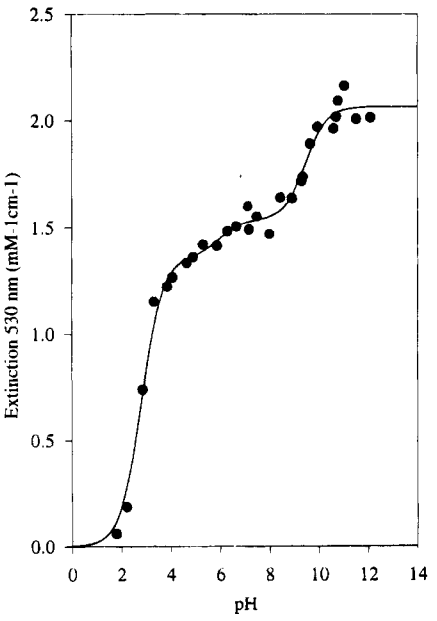


FIGURE 4: The extinction coefficient at 530 nm of C44S as a function of pH. Enzyme (47–55 μM) was mixed aerobically in the stopped-flow spectrophotometer with buffers having a constant ionic strength of 0.5 M at 4 °C. The following buffers were used: glycine hydrochloride, pH 1.8–2.22; citrate–phosphate, pH 2.87–7.17; Tris–phosphate, pH 7.11–9.36; glycine–NaOH, pH 9.31–10.7; and arginine–NaOH, pH 10.78–12.1. The solid line is a theoretical curve fit to three pK_a values.

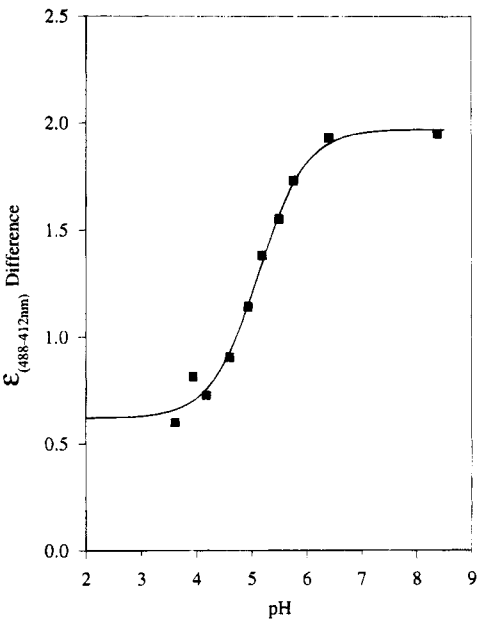


FIGURE 5: Extinction changes upon reacting C49S with MMTS, as a function of pH. Enzyme (30–33 μM) was mixed aerobically in the stopped-flow spectrophotometer with 100 mM buffers containing 10–20-fold excess MMTS + 5% acetonitrile at 25 °C. The pH range was 3.6–8.4 with the following buffers: citrate–phosphate, pH 3.6; acetate, 3.9–5.8; phosphate, 6.2–7.6; and Tris–phosphate, 8.4. The solid line is a theoretical curve fit to one pK_a of 5.1 for the interchange thiol.

transfer complex, species IId, 13% (Wilkinson & Williams, 1979). The covalent intermediate, species IIc, has been observed only in the pig heart enzyme having the interchange thiol alkylated (Thorpe & Williams, 1976b).

The absorbance spectrum of C44S was like that of wild type EH_2. The extinction at 530 nm for C44S was 1970 M^{-1} cm^{-1}, which is comparable to the extrapolated value of 2100 M^{-1} cm^{-1} for wild type EH_2 (Wilkinson & Williams,

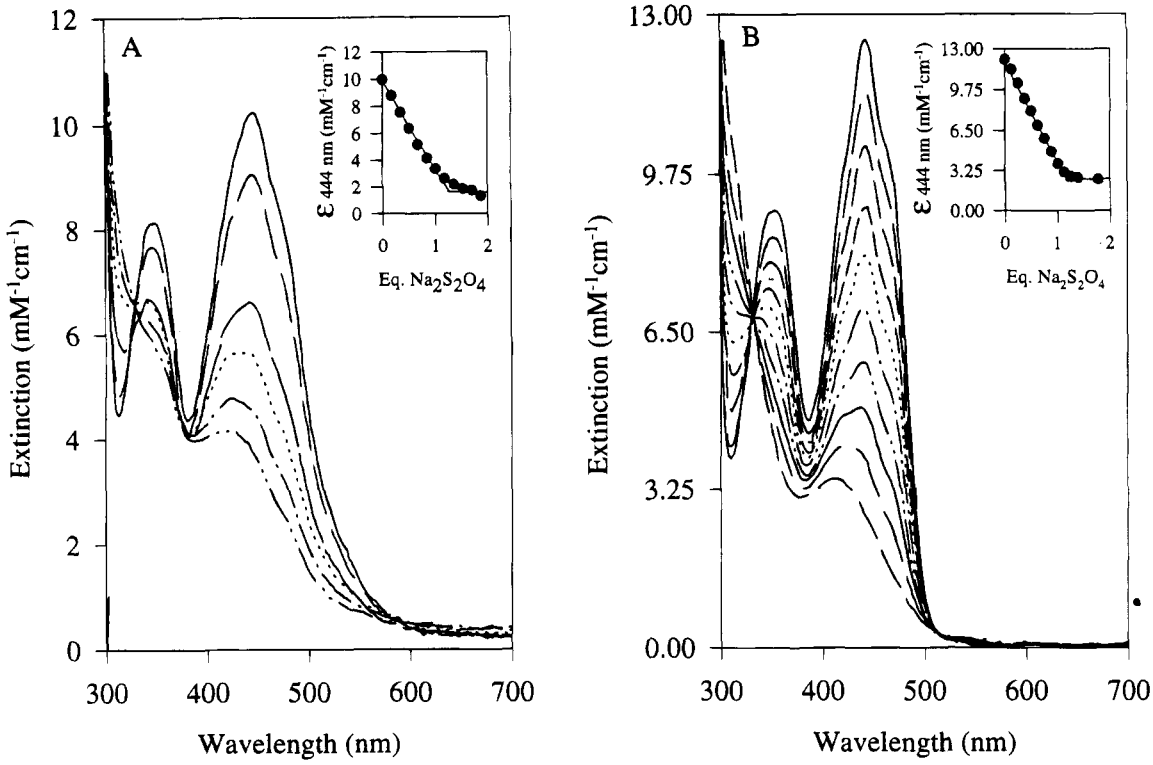
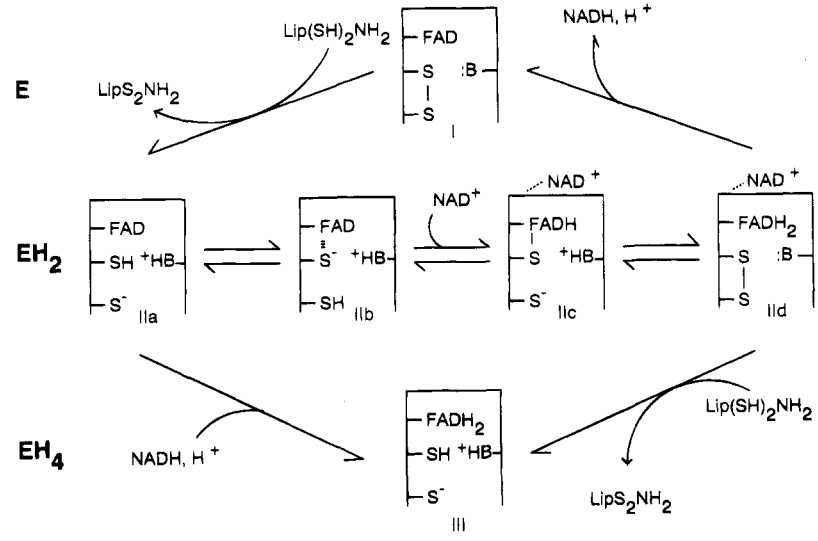


FIGURE 6: Anaerobic reduction of C44S and C49S by dithionite at pH 7.6. (A) C44S enzyme (57.6 μM) was prepared in 100 mM sodium/potassium phosphate, pH 7.6, containing 0.3 mM EDTA with 5% methyl viologen. Inset: The point of intersection is equal to 1.1 equiv of dithionite. (B) C49S enzyme (37.1 μM) was prepared in 100 mM sodium/potassium phosphate, pH 7.6, containing 0.3 mM EDTA with 5% methyl viologen. Inset: The point of intersection is equal to 1.1 equiv of dithionite.

Scheme 1: The Catalytic Cycle for Lipoamide Dehydrogenase[a]



[a] Taken from Maeda-Yorita et al., 1991.

1979). The fluorescence excitation spectrum of C44S indicated that the fluorophore was enzyme-bound FAD, as is the case with wild type EH_2. The fluorescence emission of C44S at 25 °C was approximately 5% that of oxidized wild type enzyme and is comparable to the fluorescence of wild type EH_2. Thus, the two C44S species detectable by absorbance and fluorescence appear to be analogous to species IIb and species IIa, respectively, of wild type enzyme.

The absorbance spectrum of C49S was similar to that of the oxidized wild type enzyme, although the spectrum was less well resolved, with an 11 nm blue shifted flavin peak. The fluorescence excitation spectrum of C49S had the same characteristics as did its absorbance spectrum. It should also be noted that the fluorescent wild type EH_2 species (species IIa) had an extrapolated absorbance spectrum similar to that of C49S, with a less well resolved flavin peak that was blue shifted (Wilkinson & Williams, 1979). C49S showed significantly more fluorescence than that of the C44S enzyme, amounting to 33% of the wild type E_{ox} fluorescence. However, the lack of dependence of the fluorescence of C44S on temperature is in contrast to the pronounced temperature dependence of the fluorescence of oxidized wild type enzyme. It is possible that the temperature dependence is related to an intact disulfide bond, since the wild type EH_2 fluorescence (species IIa) is also independent of the temperature.

The catalytic activity of C44S and C49S in assays using dihydrolipoamide was so low as to be almost unmeasurable (Table 2). This was entirely expected since the altered enzymes lack a disulfide with which to interchange with dihydrolipoamide. However, C44S and C49S have the ability to catalyze several other reactions that only involve the FAD in transhydrogenation between NADH and alternate electron acceptors such as oxygen, ferricyanide, DCIP, or pyridine nucleotide analogs (Straub, 1939; Massey, 1958). Structural studies have shown that the enzyme has separate binding sites for the pyridine nucleotide on the *re* face of the isoalloxazine ring and for dihydrolipoamide on the *si* face of the isoalloxazine ring. The enzyme disulfide is interposed between the binding site for dihydrolipoamide and the *si* side of the isoalloxazine (Schierbeek et al., 1989). This suggested that any alterations on the *si* side of the flavin (the disulfide) would not have major effects on FAD reduction by NADH or on the subsequent interaction of alternate substrates with the reduced flavin, and the data in Table 2 bear this out. The electron transferase, diaphorase, and oxidase activities were of the same order of magnitude as those of wild type enzyme, with the exception of the NADH/ferricyanide activity of C44S that was 20-fold lower.

Ferricyanide was the only acceptor having a reasonably high turnover number with wild type lipoamide dehydrogenase, C44S and C49S, and it was the only one electron acceptor used in the assays of these enzymes. The rate for the wild type enzyme is hyperbolically dependent on the square of the ferricyanide concentration, indicating the concerted reoxidation of reduced wild type enzyme by two molecules of ferricyanide.

The spectra of C44S in Figure 3A and of C49S in Figure 3B show no indication of flavin semiquinone accumulation during reoxidation of enzyme in equilibrium with 1 equiv of NADH. It can be seen from spectrum 2 that C49S is more reduced in equilibrium with 1 equiv (two electrons) of NADH (approximately 50%) than is C44S under the same conditions (approximately 30%). C49S is about half reoxidized by the first equivalent (1 electron) of ferricyanide while C44S is about two-thirds reoxidized. Given the high redox potential of ferricyanide (+425 mV), it can be assumed that it reacts quantitatively. After reduction by NADH, oxidized and reduced enzymes are in equilibrium with NAD^+ and NADH. The first equivalent of ferricyanide will reoxidize C44S with its lower redox potential more fully than C49S. The redox properties of C44S and C49S will be discussed in detail in the following paper (Hopkins & Williams, 1995).

The thiolate−FAD charge-transfer absorbance has been used to monitor the protonation state of the thiolate donor in the pig heart enzyme. Two pK_a values were observed, one at 4.3 attributed to the flavin-interacting thiol and one at 8.7 attributed to the base (His^{452}) (Sahlman & Williams, 1989a). The ion pair interaction, in which the thiol pK_a is lowered and the imidazole pK_a is raised, is analogous to those in papain and glyceraldehyde-3-phosphate dehydrogenase (Polgar & Halasz, 1982). Similar analysis of lipoamide dehydrogenase from *E. coli* was not possible due to the several species at the EH_2 level and the consequent decrease in the amount of thiolate−FAD charge-transfer complex (Sahlman & Williams, 1989b). With the interchange thiol removed in C44S, the thiolate−FAD charge-transfer absorbance can be used to monitor titration of the Cys^{49} thiol. The major change in the charge-transfer absorbance, with a pK_a value of 2.7, can be attributed to the thiol of Cys^{49}, and the lesser change, with a pK_a value of 9.5, to the imidazole of $His^{444'}$ (Figure 4). The pK_a of the interchange thiol, Cys^{44}, in C49S has been estimated as 5.1.

It is of interest that the pK_a values for the charge-transfer thiol in enzymes having the interchange thiol modified, mercuric reductase (Cys to Ser, $pK_a = 5.2$; Cys to Ala, $pK_a = 6.3$) (Schultz et al., 1985; Distefano et al., 1989) and glutathione reductase (Cys to Ala, $pK_a = 5.7$) (Deonarain et al., 1990), are considerably higher than the pK_a of 2.7 determined for C44S. The pK_a of 2.7 for the charge-transfer thiol is comparable with the pK_a of 3.7 attributed to this thiol in glutathione reductase having the interchange thiol alkylated (Sahlman & Williams, 1989a). Clearly, the differences in the pK_a values for the charge-transfer thiol (2.7) and the interchange thiol (5.1) are evidence of the particular milieu of each thiol reflecting their functions—chiefly the proximity of the electron-deficient FAD to Cys^{49}. Other factors, including the proximity of the base, lead to the lowering of these pK_a values, especially that of Cys^{49}, namely, hydrogen bonding to the 2′-hydroxyl of the ribityl side chain and the dipole of a long helix (Karplus & Schulz, 1989; Schierbeek et al., 1989).

In conclusion, the properties of the C44S and C49S altered enzymes have demonstrated the roles of the nascent thiols of the two-electron-reduced wild type enzyme from *E. coli*, and each having unique properties has allowed characterization of certain features of lipoamide dehydrogenase at the two-electron-reduced level. C44S with its thiolate−FAD charge-transfer absorbance is analogous to species IIb (Scheme 1), and C49S with its flavin fluorescence is analogous to species IIa, the spectrally distinct species hypothesized for the wild type enzyme at the EH_2 level. The removal of one titratable thiol has allowed the assignment of a macroscopic pK_a to the remaining thiol with reasonable certainty. Further studies utilizing these properties will focus on the effects of pyridine nucleotide ligands and the redox properties of the FAD in the absence of the disulfide, as documented in the following paper (Hopkins & Williams, 1995).

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