Measurement of the Oxidation-Reduction Potentials for Two-Electron and Four-Electron Reduction of Lipoamide Dehydrogenase from Pig Heart*

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The oxidation-reduction potential, $E_m$ for the couple oxidized lipoamide dehydrogenase/2-electron reduced lipoamide dehydrogenase has been determined by measurement of equilibria of these enzyme species with lipoamide and dihydrolipoamide or with oxidized and reduced azine dyes. $E_m$ is $-0.280 \text{ V}$ at pH 7, and $\Delta E_m/\Delta \text{pH}$ is $-0.06 \text{ V}$ in the pH range 5.5 to 7.6. Values for $E_m$, the oxidation-reduction potential for the couple 2-electron reduced enzyme/4-electron reduced enzyme, were obtained from measurements of the extent of dismutation of 2-electron reduced enzyme to form mixtures containing oxidized and 4-electron reduced enzyme. $E_m$ is $-0.346 \text{ V}$ at pH 7, and $\Delta E_m/\Delta \text{pH}$ is $-0.06 \text{ V}$ in the pH range 5.7 to 7.6. Spectra of oxidized enzyme and 4-electron reduced enzyme do not show variations with pH over this range, but the spectrum of the 2-electron reduced enzyme is pH-dependent, with the molar extinction at 530 nm changing from 3250 $\text{ M}^{-1} \text{ cm}^{-1}$ at pH 8 to 2050 $\text{ M}^{-1} \text{ cm}^{-1}$ at pH 5.2. The pH-dependent changes which are observed in the absorption properties of the 2-electron reduced enzyme are consistent with the disappearance of a charge transfer complex between an amino acid side chain and the oxidized flavin at the lower pH values, with the apparent pK of the side chain at pH 5. It has been suggested that the 530 nm absorbance of 2-electron reduced enzyme is due to a charge transfer complex between thiolate anion and oxidized flavin, and we propose that the thiolate anion is stabilized by interaction with a protonated base.

The thermodynamic data predict that the amount of 4-electron reduced enzyme formed when the enzyme is reduced by excess NADH will be pH-dependent, with the greatest amounts seen at low pH values. These data support earlier evidence (Matthews, R. G., Wilkinson, K. D., Ballou, D. P., and Williams, C. H., Jr. (1976) in Flavins and Flavoproteins (Singer, T. P., ed) pp. 464-472; Elsevier Scientific Publishing Co., Amsterdam) that the role of NAD$^+$ in the NADH-lipoamide reductase reaction catalyzed by lipoamide dehydrogenase is to prevent accumulation of inactive 4-electron reduced enzyme by simple reversal of the reduction of 2-electron reduced enzyme by NADH.

Lipoamide dehydrogenase (NADH:lipoamide oxidoreductase, EC 1.6.4.3) catalyzes the reversible reaction

$$\text{NAD}^+ + \text{dihydrolipoamide} \rightarrow \text{lipoamide} + \text{NADH} + \text{H}^+ \tag{1}$$

The enzyme accepts and donates 2 electrons in its catalytic cycle (1). Since the enzyme contains both FAD and an oxidation-reduction-active disulfide, it can accept 4 electrons under various forcing conditions (2). We were interested in studying the oxidation-reduction properties of lipoamide dehydrogenase for two reasons. The first was to clarify the conditions under which 4-electron reduced enzyme, which is thought to be inactive in catalysis, is formed. Since the oxidation-reduction potentials of both substrate couples, NADH/NAD$^+$ and dihydrolipoamide/lipoamide, have been measured as a function of pH, determination of the $E/E_{\text{H}^+}$ and $E_{\text{H}^+_2}/E_{\text{H}_2}$ couples as a function of pH should help us to predict the degree to which $E_{\text{H}_2}$ will be produced under defined conditions. Our second goal was to determine the protonic stoichiometry of the reduction of $E$ to $E_{\text{H}_2}$ and of $E_{\text{H}_2}$ to $E_{\text{H}_3}$. Kosower (4) has proposed that $E_{\text{H}_3}$, with its broad 530 nm absorption band, is a charge transfer complex between a thiolate anion (formed on

* The abbreviations used are: $E$, oxidized lipoamide dehydrogenase; $E_{\text{H}_2}$, 2-electron reduced lipoamide dehydrogenase; $E_{\text{H}_3}$, 4-electron reduced lipoamide dehydrogenase. The nomenclature for oxidation-reduction potentials follows the recommendations of Clark (3); $E_m$ the midpoint potential for the oxidized enzyme/2-electron reduced enzyme; $E_m$ the midpoint potential for the couple 2-electron reduced enzyme/4-electron reduced enzyme; $E_{\text{H}_3}$ the midpoint potential at pH 7; $E_n$, the potential with reference to the standard hydrogen electrode.

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reduction of the active center disulfide) and the oxidized flavin. If this were so, then one would expect the protonation of the thiolate anion to be associated with changes in the absorbance of $\text{EH}_2$ and with a break in the slope of plots of $E_\lambda$, the midpoint potential of the $E$/$E\text{H}_2$ couple, versus pH. Sene
now-Garwood and Garwood (5) have studied the attack of thiols on disulfide bonds in model systems, and their results have provided support for the possibility of concomitant electrophilic and nucleophilic catalysis of disulfide bond cleavage. It has been suggested (6) that in lipoamide dehydrogenase an adjacent base might enhance the reactivity of the attacking thiol. The pH optimum for dihydrolipoamide-NAD$^+$ reductase activity is at 7.9 (7), although dihydrolipoamide is not appreciably dissociated in solution at that pH. It was felt that a knowledge of the protonic stoichiometry of reduction of the enzyme as a function of pH would be important in defining possible reaction mechanisms for this enzyme.

Oxidation-reduction potentials for the NADH/NAD$^+$ couple have been determined by Burton (8) who gives a value for $E_{\text{m}}$, of $-0.320$ V (25$^\circ$), in agreement with earlier data of Burton and Wilson (9). Rodkey (10) has shown that the slope of the midpoint potential of the NADH/NAD$^+$ couple is $-0.03$ V over the pH range from 6.6 to 10.5. Oxidation-reduction potentials for the dihydrolipoamide/lipoamide couple have been determined by Massey (7) and by Sanadi et al. (11). $E_{\text{m}}$, for the lipoamide couple is $-0.287$ V, and the slope of the midpoint potential versus pH is $-0.06$ V from pH 6 to pH 8.5.

Lipoamide dehydrogenase accepts 4 electrons from dithi
nite (12). Addition of 1 mol of dithionite/mol of flavin produces an intermediate with a pronounced long wavelength absorption. The spectrum of this intermediate is indistinguishable from that produced by reduction of the enzyme with dihydrolipoamide. This 2-electron reduced enzyme (EH$^-$) is the catalytically active species, and its reoxidation by lipoamide and other lipoic acid derivatives has been shown to be rate-limiting at pH 6.3 in the NADH-lipoamide reductase reaction (1, 13). Conditions leading to the production of 4-electron reduced enzyme (EH) lead to decreases in the turnover number of the enzyme (2).

Massey and Veeger (2) first demonstrated the absolute requirement for NAD$^+$ during catalysis of the NADH-lipo
amide reductase reaction at pH 6.3, and showed that NAD$^+$ was required for catalytic activity in order to prevent the accumulation of $\text{EH}_2$ during turnover. The requirement for NAD$^+$ as an activator has been studied as a function of pH by Voetberg and Veeger (14) using lipoic acid as the electron acceptor, and by Matthews et al. (13), using lipoamide as the electron acceptor, and in both cases the degree of activation exerted by NAD$^+$ decreases with increasing pH. Previous work (13) has established that NAD$^+$ exerts this effect by influencing the equilibrium of reaction (2).

$$\text{EH}_2 + \text{NADH} + \text{H}^+ \rightarrow \text{EH}_2^- + \text{NAD}^+$$

and that the rate at which $\text{EH}_2$ is reoxidized by NAD$^+$ is very fast. The observed rate constant is $693$ s$^{-1}$ with 65 $\mu$M NAD$^+$ and 17 $\mu$M enzyme.

Sears and Sanadi (15) attempted to estimate the midpoint potential of enzyme-bound flavin by measurements of changes in absorbance at 460 nm on addition of NADH to oxidized enzyme. These measurements were complicated by the fact that the 460 nm absorbance of E and $\text{EH}_2$ is not the same, so that changes at 460 nm were due to production of both $\text{EH}_2$ and $\text{EH}_2^-$. Also NADH and NAD$^+$ both bind E, $\text{EH}_2$, and $\text{EH}_2^-$ tightly so that they might distort equilibrium measurements of the reaction given by Equation 2. Using these measurements they obtained a value for the midpoint potential of the $\text{EH}_2$/$\text{EH}_2^-$ couple of $-0.332$ V at pH $7$. Because of the ambiguities in these measurements, we felt it necessary to rederive the oxidation-reduction potential of lipoamide dehydrogenase.

**MATERIALS AND METHODS**

Lipoamide dehydrogenase purified from pig heart was purchased from either Boehringer or Sigma and prepared as described previously (16). Di-Dihydrolipoamide was generously donated by Richardson-Merrell, Inc. Di-Lipoamide was purchased from Sigma, dithionite was obtained from Matheson, Coleman and Bell, safranine T from ICN Pharmaceuticals, and phenoasfranine from British Drug House.

Anaerobic titrations were performed with an all glass titration apparatus originally described by Burleigh et al. (17) and modified by elimination of the septum as described by Lambeth and Palmer (18). The enzyme solution was made anaerobic with seven alternating cycles of nitrogen (purified as described previously (16)) and vacuum. Dithionite solutions were prepared and then standardized by anaerobic spectrophotometric titration according to the method described by Burleigh et al. (17). Dihydrolipoamide solutions were standardized by titration into 5,5'-dithiobis(2-nitrobenzoic acid) using the conditions previously described (16) for measurement of protein thiol groups, except that guanidine was omitted. Lipoamide solutions were standardized by measurement of the absorbance at 340 nm, using 0.77 $\text{m}^{-1}$ cm$^{-1}$ for the molar extinction coefficient of lipoamide in dilute aqueous solution. Anaerobic aqueous solutions of lipoamide or dihydrolipoa
mide were prepared by dilution of a concentrated stock solution in methanol, and were degassed in the same ways as dithionite solutions prior to introduction into the titrating syringe. Fresh solutions were prepared and standardized immediately before each experiment. Restandardization of dihydrolipoamide solutions at the conclusion of experiments showed no significant decline in thiol titer.

**Determination of Extinction Coefficient of EH$_2$—**The extinction coefficient of $\text{EH}_2$ at 530 nm as a function of pH was determined by a series of titrations of the enzyme with standardized dithionite. The molar extinction coefficient of $\text{EH}_2$ could be obtained by extrapolation of the linear portion of the titration curve. In all cases the molar extinction coefficient for addition of 1 eq of dithionite was corrected for the oxidation of small amounts of dithionite by residual oxygen at the beginning of the titration, as evidenced by the small lag in appearance of 530 nm absorption at the beginning of the titration.

**Determination of Equilibrium between Enzyme and Dihydrolipoamide—**Equilibrium determinations were performed in a Cary 118 C recording spectrophotometer equipped with a thermostatically controlled sample compartment. Temperature was maintained at 25$^\circ$.

Prior to each experiment the pH of the enzyme solution was measured in a Beckman expanded scale pH meter. The enzyme solution was then passed through a Millipore filter and 3.00 ml of solution were introduced into the titration apparatus. The enzyme spectrum was recorded before and after anaerobiosis and any change in concentration due to evaporation during anaerobiosis was noted. Aliquots of dihydrolipoamide were introduced into the cuvette from the attached 100 $\mu$l Hamilton gas-tight syringe, which was equipped with a hand-operated dispenser which released 2 $\mu$l of titrant each time the button was depressed. After each addition the absorbance was monitored until changes were complete and then the absorbance at 455 and 530 nm was measured. Complete spectra were recorded at intervals on the concentration mode of the Cary 118 C so that spectra could be automatically corrected for any dilution. Since $\text{EH}_2$ is the only enzyme species present which absorbs light at 530 nm, the concentration of this species was determined from changes in absorbance at 530 nm, after correction for any dilution. If less than 80% of the enzyme is present as $\text{EH}_2$, the concentration of $\text{EH}_2$ formed during dihydrolipoamide titrations due to

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*All enzyme concentrations and stoichiometries given in this paper will be per mol of enzyme-bound FAD. There are 2 mol of FAD bound/mol of enzyme.

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2 K. Wilkinson and C. H. Williams, Jr., unpublished results.
Oxidation-Reduction Potentials of Lipoamide Dehydrogenase

the disproportionation can be neglected, and the concentration of oxidized enzyme, E, can be determined by difference. Knowing the total enzyme concentration, the concentration of EH2 formed will then be equivalent to the concentration of lipoamide formed, and the concentration of dihydrolipoamide can be determined by subtraction. The oxidation reduction potential (E0) of the system at equilibrium after each addition of dihydrolipoamide was calculated from the Nernst equation:

$$E_0 = E_{m, pH} + \frac{RT}{N_F} \ln \left[\frac{[oxidant]}{[reductant]}\right]$$

where $E_{m, pH}$ is the midpoint potential for the couple oxidant/reductant at the pH value specified; R is the gas constant; T, the absolute temperature; F, the Faraday; and N_F, the number of electrochemical equivalents. N_T is 2 for lipoamide, and the midpoint potential of the lipoamide couple at any given pH was determined by extrapolation of the data of Massey (7). The midpoint potential (E0) for the reduction of E to EH2 was determined for each point in the titration curve by equating the potential equations for the lipoamide-dihydrolipoamide and E/EH2 couples. The resulting values for $E_{m, pH}$ (2/E/2E2) were averaged. The use of the Nernst equation to calculate midpoint potential values relies on the ability to measure ratios of concentrations of unliganded E and EH2. If lipoamide, or dihydrolipoamide, or both, bind preferentially to one oxidation-reduction species of the enzyme, this would prejudice the calculations of $E_{m, pH}$, because the ratio of concentrations of E and EH2 determined from 530 nm absorbance will not correspond to the ratio of concentrations of unliganded E and EH2. Experiments addressing this point are described below.

Data from titrations of oxidized enzyme with dihydrolipoamide can also be used to estimate the extinction coefficient of EH2 at 530 nm. The quadratic Equation 4

$$K - [EH2] [lipoamide] = \left[\frac{[E] - [EH2]}{[Lip]} - \frac{1}{2} \frac{[EH2]}{[lipoamide]} \right]$$

describes the relationship between E and EH2 after addition of a given concentration of dihydrolipoamide (Lip) to an initial concentration of enzyme, E0, if the initial concentration of oxidized lipoamide is zero, and if complexation between enzyme and lipoamide species can be neglected. A computer program (written by Mr. Keith Wilkinson) calculated values for K and for the extinction coefficient of EH2 at 550 nm which best fitted the data points.

For titrations of EH2 with lipoamide, the enzyme was first reduced with 1 eq of dithionite. The concentration of EH2 at equilibrium was determined from the absorbance at 530 nm, and the concentration of EH2 formed was determined from the decrease in absorbance at 499 nm, where E and EH2 are isoelectric. The extinction coefficients of E and of EH2 at 499 nm are 4000 M^-1 cm^-1 at pH 6.12, and the extinction coefficient of EH2 is 3300 M^-1 cm^-1 at 499 nm). Initial additions of lipoamide produced the quantitative conversion of EH2 to EH4 as well as a partial production of E, and the amount of dihydrolipoamide produced by conversion of EH2 to EH4 could be calculated from the initial concentration of EH2. Equilibria following subsequent additions of lipoamide to the enzyme involved only lipoamide and dihydrolipoamide and EH2 and E. The concentrations of each species could be determined from measurements of absorbances at 530 and 455 nm. Concentrations of dihydrolipoamide and E were adjusted to correct for concentrations of EH2 and E present before the addition of lipoamide. These data could then be used to calculate $E_{m, pH}$, and the midpoint potential for the E/EH2 couple.

**Determination of Equilibria between Enzyme and Azine Dyes—**

Oxidation-reduction potential determinations were also made spectrophotometrically by titrating mixtures of lipoamide dehydrogenase and phenasafarain or safranine T with dithionite. A solution containing enzyme (approximately 50 μM) and dye (15 μM) was made anaerobic. Small increments of dithionite were added and the percentage reductions of enzyme and dye were determined after equilibration was reached following each addition. Preliminary experiments showed isosbestic points at 404 nm between oxidized and reduced forms of phenasafarain, and between oxidized and reduced forms of safranine T. The concentration of EH2 could be determined by measurement of changes in absorbance at 404 nm. The molar extinction coefficient at 404 nm for EH2 minus E was determined for each pH value from the ratio of absorbance changes at 404 nm to those at 530 nm in a companion titration of the enzyme with dithionite or dihydrolipoamide. Dye reduction was determined from decreases in absorbance at 498 nm, where E and EH2 are isobestic and the reduced forms of the dyes have no absorbance. The oxidation-reduction potential (E0) for the system at equilibrium was calculated from the Nernst Equation 3. The midpoint potential of our sample of phenasafarain at pH 7, 20°C, was determined potentiometrically by Dr. Fred Guengerich (19) who obtained a value of -0.044 V, in good agreement with the midpoint potential of -0.248 V calculated for those conditions from the data given by Clark (3, p. 415). Midpoint potentials for the phenasafarain couple at 20°C and the pH values measured in our titrations were calculated according to the equation cited by Clark (3, p. 415). The midpoint potentials of several samples of safranine T were determined potentiometrically by Dr. Guengerich and found to be more negative than published values. Dr. Marion Stankovich determined the midpoint potential of safranine T obtained from ICN Pharmaceuticals by potentiometric measurement of the reduction of a 20 μM dye solution in 0.05 M phosphate buffer, pH 7.6 at 20°C. The data obtained showed good agreement with the theoretical curve for $E_{m, pH}$ versus per cent reduction corresponding to a midpoint potential of -0.298 V. The calculated midpoint potential for safranine T under these conditions, based on the data given by Clark (3), is -0.295 V. This sample of safranine T was used for all enzyme-safranine T equilibrium determinations. E0 used for reference potentials for the dye couples in each experiment were corrected for concentrations of dye used in the particular experiment according to the formulas cited by Clark (3, p. 415).

**Stopped Flow Measurements of Absorbance of EH2—**

Borohydride reductions of lipoamide dehydrogenase were accomplished by adding 0.2 to 5 mg of solid borohydride to anaerobic enzyme solutions. In 10 mM phosphate buffer, at pH 8.0, dismutation of the EH2 produced on borohydride reduction occurs very slowly (over many hours) and almost quantitative concentrations of EH2 can be produced. Stopped flow experiments were performed in Buffer B at the pH of a solution of anaerobic borohydride reduced enzyme was lowered by mixing with 100 mM buffer solutions at various pH values. These experiments were performed using a modified Gibs son stopped flow spectrophotometer. The initial enzyme solution was prepared in 10 mM phosphate buffer, pH 7.6, and the pH was raised to 7.95 following addition of borohydride. The pH values of the solutions after mixing in the stopped flow were measured by collecting the effluent from the stopping syringe and measuring the pH in an expanded scale Radiometer pH meter.

**RESULTS**

Figs. 1 and 2 illustrate the course of anaerobic reduction of lipoamide dehydrogenase by dithionite at pH 6.3. The reduction occurs in two phases. During the first phase, 530 nm absorbance increases, while the absorbance at 455 nm, where E absorbs maximally, decreases to 77% of its initial value. Further additions of dithionite lead to the disappearance of 530 nm absorbance, and to much larger decreases in the absorbance at 455 nm, which drops to 8% of its initial value. Addition of more dithionite (not shown) caused no further change in the visible spectrum; the presence of unreacted dithionite was indicated by increases in absorbance at about 320 nm. The correspondence between dithionite added and maximum 530 nm absorption indicates that 1 mol of dithionite/mol of FAD reduces the enzyme to the red intermediate, EH4, and a 2nd mol of dithionite reduces the enzyme to the fully reduced species, EH2, in agreement with previously reported titrations at pH 7.6 (12).

Intermediate spectra during the first half of the titration revealed isosbestic points at 498, 439, 385, and 342 nm. During the second half of the titration, an isosbestic point is observed at 313 nm until slight excursions of dithionite interfere. The spectrum of EH4 indicates that the flavin is now fully reduced. The spectrum shows a peak centered at 352 nm, and a shoulder, at about 410 nm. Such spectra are characteristic of neutral dihydrolflavins in aprotic, nonpolar solvents (20).

At the beginning of a titration with dithionite, spectral changes were complete before measurements could be made. At pH values below 7, additions of dithionite approaching 1

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300 350 400 450 500 550 600 650
Wavelength (nm)

FIG. 1. Reduction of lipoamide dehydrogenase with sodium dithionite. Enzyme, 244 nmol in 3 ml of 0.05 M phosphate buffer, pH 6.30, was titrated at 25° with 0.62 mM sodium dithionite dissolved in 0.05 M sodium pyrophosphate buffer, pH 8.3. ---, oxidized, anaerobic enzyme; ---, after addition of 0.32 mol of dithionite/mol of flavin; ---, 0.64 mol of dithionite/mol of flavin; ---, 1.04 mol of dithionite/mol of flavin; ---, 1.68 mol of dithionite/mol of flavin; ---, 2.08 mol of dithionite/mol of flavin. These spectra were not corrected for dilution.

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mol/mol of flavin caused initial decreases of absorbance at 530 nm, followed by slower increases which were monitored until they were completed. The rate of reduction of EH₃ to EH₄ at these pH values was quite rapid. These observations suggested that in this region of the titration additions of dithionite caused rapid reduction of some EH₂ to EH₄ followed by comproportionation of E and EH₄ to form the equilibrium concentration of EH₃. A preliminary experiment in which equal amounts of E and EH₄ at pH 6.3 were mixed anaerobically, and the rise in 530 nm absorbance on comproportionation monitored, indicated that the rate of comproportionation to form EH₂ was too rapid to be accurately measured in a Cary spectrophotometer. At higher pH values, the rate at which dithionite reduced EH₂ to EH₄ was slower, and comproportionation was not observed during the course of dithionite titrations. After each addition the absorbance was monitored until changes were complete.

Extinction coefficients for fully formed EH₄ at 455 and 530 nm can be obtained by extrapolation of the linear portions of a titration curve. Points near 50% reduction lie off the linear portions of the curve, indicating dismutation of EH₂ to E and EH₄ according to the equation

\[ 2 \text{EH}_2 \xrightarrow{k_1} \text{EH}_4 + K \]

Values for the formation constant, \( K \), where \( K = \frac{[\text{EH}_4]^2}{[\text{EH}_2][E]} \), were obtained by measurement of the ratio of 530 nm absorbance actually observed on addition of 1 eq of dithionite to the 530 nm absorbance obtained by extrapolation of the linear regions of the titration curve. At pH 6.3, data from two separate dithionite titrations indicated the formation of 86.4 and 90.9% EH₄ with values for \( K \) of 160 and 400, respectively. The difference in oxidation-reduction potential (\( E_g - E_i \)) between the couples E/EH₂ and EH₂/EH₄ was calculated using the following equation

\[ E_g - E_i = 0.0296 \log K \]

The two dithionite titrations yielded values for \( E_g - E_i \) of 0.065 and 0.077 V, respectively.

Before examining the effect of pH on the spectrum of EH₄, a control experiment was performed in which aliquots of a concentrated solution of enzyme in 10 mM phosphate buffer, pH 7.6, were diluted into 50 mM phosphate buffer solutions of varying pH. The spectrum of oxidized enzyme showed the same absorbance at 455 nm at pH 5.8 as at pH 8.0, and the absorption spectra agreed within 1% at all wavelengths between 300 and 700 nm.

A series of dithionite titrations were performed at pH values from 5.2 to 8.2. The variation of the extrapolated extinction of EH₄ at 530 nm as a function of pH is shown by the circles in Fig. 3. Between pH 5.2 and pH 8.14, the extinction at 530 nm of EH₄ increases from 2050 to 3250 M⁻¹ cm⁻¹. While the magnitude of the difference spectrum seen on reduction of E to EH₂ varies considerably with pH, the form of the difference spectrum changes little. During dithionite titration at pH 5.75, isosbestic points in the conversion of E to EH₂ are seen at 499, 440, 387, and 343 nm, while at pH 8.14, isosbestic points are observed at 498, 438, 384, and 345 nm.

The extent of dismutation of EH₂ after addition of 1 eq of dithionite appears the same within experimental error over the pH range from 5.2 to 8.2, and values for \( E_g - E_i \) lie between 0.057 and 0.077 in all titrations.

When the enzyme is titrated anaerobically with reduced lipoamide (Fig. 4) the spectral changes seen on reduction of E to EH₂ are the same as those seen in the first phase of dithionite titration. In Fig. 5, the extinction at 530 nm is
FIG. 2. Titration of lipoamide dehydrogenase with sodium dithionite. Plot of absorbance at 455 nm and 530 nm versus dithionite added. Enzyme, 110 nmol in 3 ml of 0.05 m phosphate buffer, pH 6.30, was titrated at 25° with 8.76 mm sodium dithionite. The arrows indicate points in the titration curve corresponding to 0, 1, and 2 eq of dithionite, respectively, after correction for the initial oxidation of dithionite by traces of residual oxygen.

plotted against equivalents of dihydrolipoamide added. It can be seen that the magnitude of the extinction changes observed at 530 nm increases with increasing pH. Values for the 530 nm molar absorbance of EH₂ obtained from a computer analysis of the data (see "Materials and Methods") were used to estimate the end points shown in the figure. These values for the extinction coefficients of EH₂ at various pH values (shown as circles in Fig. 3) agree very well with the data obtained by dithionite titration. The smooth curve drawn through the data points presented in Fig. 3 was used to provide values for the molar extinction of EH₂ at 530 nm as a function of pH, so that 530 nm absorbance could be related to EH₂ concentration at a given pH value.

Once the extinction of EH₂ at 530 nm is known, difference spectra calculated from data obtained on partial reduction of the enzyme can be used to construct the spectra of fully formed EH₂ at different pH values. Fig. 6 shows spectra of EH₂ calculated from data obtained during dihydrolipoamide titrations at pH 5.5 and pH 8.2.

Fig. 3 also presents data obtained immediately after borohydride reduction of lipoamide dehydrogenase at pH 5.55 (solid square) and data obtained when a solution of EH₂ produced by borohydride reduction at pH 8 was mixed in the stopped flow apparatus with 0.1 m phosphate or citrate/phosphate buffers (open triangles). These stopped flow experiments were performed in collaboration with Professor Vincent Massey. The values for the extinction coefficient of EH₂ at 530 nm indicated by these data points were reached within a few seconds at all pH values, although subsequent slow changes were seen at pH 4.5 and pH 4.1 which presumably were due to the instability of the enzyme at these pH values. Preliminary experiments in which EH₂ was formed at pH 6.3 and the pH was then raised by tipping in aliquots of 1 M unneutralized Tris indicate that the forms of EH₂, seen at high and low pH are in reversible equilibrium, and that raising the pH produces the increase at 530 nm which would be expected from the curve shown in Fig. 3.

Data obtained from lipoamide titrations at various pH values can now be used to calculate values for the E/EH₂ couple at these pH values. The validity of data obtained in this manner rests on two conditions: (a) that under the conditions of the titrations, complex formation between enzyme and...
Fig. 4. Titration of lipoamide dehydrogenase with dihydrolipoamide. Enzyme, 126 nmol in 3 ml of 50 mM phosphate buffer, pH 6.23, was titrated with 2.65 mM dihydrolipoamide dissolved in glass-distilled water containing 1.7% methanol. 1, oxidized enzyme; 2, after addition of 31.8 nmol of dihydrolipoamide; 3, 63.6 nmol of dihydrolipoamide; 4, 95.4 nmol of dihydrolipoamide; 5, 133 nmol of dihydrolipoamide; 6, 196 nmol of dihydrolipoamide; 7, 254 nmol of dihydrolipoamide.

Fig. 5. Effect of pH on the course of titrations of lipoamide dehydrogenase with dihydrolipoamide. Extinctions at 530 nm are plotted versus moles of dihydrolipoamide added per mol of enzyme flavin. The values at infinite lipoamide concentration were obtained as described in the text. Conditions: O—O, 75.6 nmol of enzyme in 50 mM citrate phosphate buffer, pH 5.50; △—△, 73.8 nmol of enzyme in 50 mM phosphate buffer, pH 6.79; 0—0, 73.8 nmol of enzyme in 50 mM phosphate buffer, pH 7.94.

Lipoamide is negligible, or equal in strength for both oxidation-reduction states of the enzyme; (b) that thermodynamic equilibrium has been achieved.

The similarity of the spectra of EH₂ produced by reduction with dithionite and with lipoamide suggested that lipoamide might not form a complex appreciably with the enzyme if the enzyme concentration was kept sufficiently low. To test for complexation, titrations of the enzyme with lipoamide were performed using different enzyme concentrations. Since the equilibrium constant is given by $K = [\text{lipoamide}][\text{EH}_2]/[\text{dihydrolipoamide}][\text{E}]$, if the enzyme concentration is doubled, the amount of lipoamide which must be added to achieve 50% reduction of the enzyme will also be approximately doubled and the extent of complexation should increase approximately 4-fold. If complexation occurred preferentially between one oxidation-reduction form of the enzyme and one oxidation-reduction form of lipoamide, so as to shift the measured midpoint potential, the calculated midpoint potential should shift as the enzyme concentration is varied.

Fig. 7 shows plots of $E_m$ versus per cent reduction calculated from titrations of 11.7, 24.8, and 42 μM enzyme at pH 6.23, and from titrations of 8.9 and 29.4 μM enzyme at pH 7.41. No variations in the averaged midpoint potentials calculated from the data obtained at the different enzyme concentrations and the same pH could be found. The solid lines drawn through the data points shown in Fig. 7 represent theoretical curves of $E_m$ versus per cent reduction calculated for a 2-electron reduction assuming the $E_m$ calculated from the data points. The observed data points are in good agreement with theoretical curves between 30 and 80% reduction. Accordingly, only data obtained in this range of reduction of E to EH₂ were used for the calculation of midpoint potential values.

Absorbance changes following anaerobic addition of dihydrolipoamide to enzyme solutions were rapid, and appeared complete within 1 or 2 min after addition. Following each addition, absorbance changes at 530 nm were monitored for at least 5 min to ensure that equilibrium was achieved.

Fig. 8 shows the calculated midpoint potential values for the E/EH₂ couple obtained at each pH (open circles). The shaded circle indicates the midpoint potential calculated from experiments in which EH₂ was prepared by titration with dithionite, and then was oxidized to E by titration with lipoamide. The details of the calculations are described under “Materials and Methods.” Data from such reverse titrations give calculated midpoint potential which are 14 ± 5 mV more...
positive than the lipoamide/dihydrolipoamide couple. These values agree within experimental error with midpoint potential values calculated from data obtained on titration of E with dihydrolipoamide. Between pH 5.5 and pH 7.6, these values are 7 ± 3 mV more positive than the corresponding lipoamide/dihydrolipoamide couples.

The data indicate that ΔE_m/ΔpH for the E/EH_2 couple is -0.06 V between pH 5.5 and pH 7.6. The data points lie somewhat above the line corresponding to a -0.06 V slope above pH 7.6, suggesting the possibility of an ionization of EH_2. However, the instability of EH_2 at pH values above 8.2 precludes determination of the E/EH_2 couple at higher pH values.

Fig. 8 also shows values for the midpoint potential of the E/EH_2 couple calculated from dithionite titrations of mixtures of E and oxidized phenosafranine (open diamonds), from reverse titrations of EH_2 and reduced phenosafranine with ferricyanide (shaded diamond) and from titrations of mixtures of E and oxidized safranine T with dithionite (open triangles). These experiments yield values for the midpoint potential of the E/EH_2 couple which are in excellent agreement with the values calculated from dihydrolipoamide titration. The close agreement supports our contention that complex formation between lipoamide and the enzyme is not affecting the determinations of the midpoint potential of the E/EH_2 couple. Also shown in Fig. 8 are values for the midpoint potential of the EH_2/EH_2 couple (open squares). These values were calculated using the solid line fitting E_m versus pH for the E/EH_2 couple, and then subtracting the value of E_m - E, calculated from measurement of the EH_2 formation constant. Such indirect determinations of the midpoint potential of the EH_2/EH_4 couple must be regarded as approximate, but they suggest that the EH_2/EH_4 couple is about 0.066 V more negative than the E/EH_2 couple, and has a slope of about -0.06 V between pH 5.5 and 7.6. The spectrum of EH_2 produced on addition of 2 mol of dithionite/mol of flavin is identical with that shown in Fig. 1 in the pH range between 5.2 and 7.6. Above pH 7.6, the observed rate of reduction of EH_2 to EH_1 becomes very slow, and EH_1 becomes increasingly unstable, so that turbidity forms before reduction of EH_1 to EH_2 is completed.

DISCUSSION

The data presented in Fig. 8 establish, for the pH range between 5.5 and 7.6, that the net stoichiometry of reduction of pig heart lipoamide dehydrogenase can be represented by

\[ E + 2e^- + 2H^+ \leftrightarrow EH_2 \]  
\[ EH_2 + 2e^- + 2H^+ \leftrightarrow EH_4 \]  

Sears and Sanadi (21) first suggested that the spectrum of EH_2 with its characteristic 530 nm absorption might be due to a charge transfer complex between oxidized FAD and one of the nascent thiols produced on reduction of the active center disulfide. Kosower (4) suggested that the charge transfer complex might rather be between a thiolate anion and oxidized FAD. Massey and Ghisla (22) have noted the resemblance between the spectrum of EH_2 in lipoamide dehydrogenase and the spectra of other flavoproteins which exhibit charge transfer complexes between low molecular weight ligands and the oxidized enzyme-bound flavins. Examples of such flavoproteins, in which oxidized flavin serves as the electron acceptor, include old yellow enzyme (23, 24), butyryl-CoA dehydrogenase (25), and D- and L-amino acid oxidase (26-31). In the case of lipoamide dehydrogenase, where E has a spectrum characteristic of a simple oxidized flavoprotein, and only EH_2 shows long wavelength absorbance, the donor in the charge transfer complex must either be formed on 2-electron reduction, or moved into juxtaposition with the flavin by conformational changes in the protein accompanying its reduc-
Massey (12) has pointed out that 530 nm absorption on production of EH₂ is still observed at pH 5.6, a pH well below pK values of simple thiols. The present data establish that the 330 nm absorbance of 2-electron reduced enzyme does decrease with decreasing pH, and the spectrum of EH₂ appears to approach that of perturbed oxidized enzyme as the pH is lowered (Fig. 6). Such absorbance changes would be compatible with protonation of an anionic electron donor with consequent abolition of the charge transfer absorption band. The data indicate that the pK of the donor is approximately 5.

Furthermore, the slope of Eₘ versus pH for the E/EH₂ couple demonstrates that 2 protons and 2 electrons must be added to E to form EH₂. If we are to represent the red (530 nm absorbing) form of EH₂ as being due to charge transfer between a thiolate anion and oxidized flavin, this requires that a base which is unprotonated on E must accept a proton when EH₂ is formed. Thus, we could represent the formation of EH₂ at the pH values above 5.5 as follows:

\[
\text{FAD} + 2e^- + 2H^+ + S_\text{-SH} \rightarrow E \text{-SH} + S_\text{-SH}^+ + 2H^+
\]

The pK (of approximately 5) associated with the disappearance of 530 nm absorbance is very low for a thiolate anion, but is quite reasonable for a thiol base pair of the type \(S^- \cdots BH^+\). Husain and Lowe (32) have demonstrated such a thiol base pair in papain by modification of the enzyme with dibromopropanone to form a covalent linkage between histidine and the active site thiol.

Polgar (33) has measured the two pK values associated with the ion base pair in papain

\[
\text{SH} \cdots \text{BH}^+ \quad \text{pK}_1 \quad \text{S}^- \cdots \text{BH}^+ \quad \text{pK}_2 \quad \text{S}^- \cdots \text{B}
\]

by measuring the rate of alkylation of papain by haloacetamides and found that pK₁ is 4.00 and pK₂ is 8.40. pK values of approximately 5 and 8.2 for the putative ion base pair in lipoamide dehydrogenase would be quite sufficient to explain the -0.06 V slope observed in Fig. 8 between pH 5.5 and pH 7.6. Since the pH dependence of the EH₂ spectrum indicates that pK₁ may be at a pH value near 5, it would be of great interest to determine the slope of the E/EH₂ couple in the
pH range from 4 to 5.5. Unfortunately, the enzyme is only transiently stable below pH 5, so that equilibrium measurements in this pH range are not possible.

The thermodynamic data in Fig. 8 support earlier evidence (13) that the role of NAD$^+$ in the NADH-lipoamide reductase reaction catalyzed by lipoamide dehydrogenase is to prevent accumulation of EH$_2$ by simple reversal of the reduction of EH$_2$ by NADH. Below pH 6.1, the midpoint potential of the NADH/NAD$^+$ couple lies below that of the EH$_2$/EH$^+$ couple, while above pH 6.1 their relative positions are reversed. Thus, the disappearance of the requirement of NAD$^+$ for the catalysis of the NADH-lipoamide reductase reaction is readily explained. Williams (6) has calculated that the midpoint potential of the EH$_2$/EH$^+$ couple should be $-0.306 \text{ V}$ at pH 6.3, on the basis of the extent of flavin reduction seen when lipoamide dehydrogenase is reduced with 3 eq of NADH at pH 6.3 (1). The results shown in Fig. 8 confirm this value for the EH$_2$/EH$^+$ couple exactly.

It is of interest to note that both the spectrum of EH$_2$ at pH values between 5.2 and 7.6 and the $-0.06 \text{ V}$ slope of the midpoint potential of the EH$_2$/EH$^+$ couple in this pH range suggest that the dihydroflavin does not ionize in this pH range. This is in marked contrast to flavodoxin where the midpoint potential of the semiquinone/dihydroflavin couple shows a break in slope at pH 5.8 (34).

We are hesitant to ascribe the E/EH$^+$ and EH$_2$/EH$^+$ couples to the reduction of specific moieties on the flavoprotein because we do not know the internal distribution of the 2 electrons on EH$_2$. While the spectrum of EH$_2$ is similar to spectra of charge transfer complexes between simple oxidized flavoproteins and low molecular weight ligands, we cannot exclude the possibility that 25% or so of the EH$_2$ molecules have their electrons localized on the flavin, and that the E/EH$^+$ couple and the EH$_2$/EH$^+$ couple therefore reflect complex reductions rather than simple serial reduction of the disulfide and then of the flavin.

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