Reactions of Pig Heart Lipoamide Dehydrogenase with Pyridine Nucleotides

EVIDENCE FOR AN EFFECTOR ROLE FOR BOUND OXIDIZED PYRIDINE NUCLEOTIDE

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The reactions of lipoamide dehydrogenase with NADH/NAD⁺ and two analogues, nicotinamide hypoxanthine dinucleotide (NHID/NIID⁺) and acetylpyridine adenine dinucleotide (APyADH/APyAD⁺) have been examined using steady state and stopped flow kinetic measurements. The pH profiles for vmax in the NADH-lipoamide, APyADH-lipoamide, dihydrolipoamide-APyAD⁺, and dihydrolipoamide-APyAD⁺ redox reactions are reported. The conversions of complexes of 2-electron reduced enzyme (EH₂) and oxidized pyridine nucleotide to complexes of oxidized enzyme and reduced pyridine nucleotide are accompanied by mandatory proton release, which is reflected in the pH dependence of vmax in the lipoamide reductase reactions.

Stopped flow kinetic measurements of the reduction of oxidized enzyme by saturating levels of NHIDH and APyADH have demonstrated an intermediate which appears to be a complex of oxidized enzyme and reduced pyridine nucleotide. The intermediate has absorbance properties which resemble those of intermediates seen on the reduction of other pyridine nucleotide-linked flavoproteins (Massey, V., Matthews, R. G., Foust, G. P., Howell, L. G., Williams, C. H., Jr., Zanetti, G., and Ronchi, S. (1970) in Pyridine Nucleotide-dependent Dehydrogenases (Sund, H., ed) pp. 393-411, Springer-Verlag, Berlin). This intermediate is formed within 3 ms whether [4S⁻H]APyADH or [4S⁻H]APyADH is used as the substrate, suggesting that formation of this intermediate precedes hydride transfer.

Oxidation of EH₂ by saturating levels of NH⁺ results in the rapid formation of an intermediate with long wavelength absorbance, maximal at 580 nm. This intermediate is shown to contain EH₂ bound to NH⁺, with the long wavelength absorbance attributable to thiolate to flavin charge transfer. Since thiolate to flavin charge transfer is maximal at 530 nm in EH₂, the shift in charge transfer absorbance associated with the binding of oxidized pyridine nucleotide may reflect an alteration in the oxidation-reduction potentials of flavin and disulfide/dithiol. Our results also indicate that the rate of formation of the 580 nm absorbance is unaffected by deuteration of NHDH in the 4S position, and that product release is the rate-limiting step in the reduction of oxidized enzyme by NHIDH at pH 7.6. We suggest that bound oxidized pyridine nucleotide may function not only as an acceptor of reducing equivalents from EH₂, but also as an effector which influences flavin-disulfide interaction in EH₂.

Lipoamide dehydrogenase is one of a group of enzymes in which both flavin and oxidation-reduction-active disulfide groups are constituents of the active site (1, 2). All of these enzymes catalyze pyridine nucleotide-linked oxidoreductions of substrates containing disulfide bonds (3). In 1960, Massey et al. (4) demonstrated that the enzyme mechanism is ping-pong, and that the individual half-reactions, which are completely reversible, may be studied conveniently in either direction. The two half-reactions may be summarized by Equations 1 and 2.

\[
\text{FAD} + S^- + HS \rightarrow \text{SH} + S^+ \quad (1)
\]

\[
\text{FAD} + S^- + NAD^+ \rightarrow \text{FAD} + NADH + H^+ \quad (2)
\]

The half-reaction in which dihydrolipoamide is oxidized, shown by Equation 1, is formally a base-catalyzed disulfide-dithiol interchange between enzyme and substrate. Thermodynamic measurements have shown that both protons initially present on dihydrolipoamide are transferred to the reduced enzyme in the physiological pH range (5, 6). We therefore refer to the 2-electron reduced enzyme as EH₂. The flavin, which shows no direct participation in the first half-reaction

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(Equation 1), is thought to function primarily in the second half-reaction, Equation 2, where it presumably mediates the transfer of electrons between the oxidation-reduction-active disulfide and pyridine nucleotide (3). This assumption is supported by x-ray data on the closely related enzyme, red cell glutathione reductase (7), which shows the flavin located between the oxidation-reduction-active disulfide and the bound oxidized pyridine nucleotide.

We began these studies in the hope of identifying intermediates in the pyridine nucleotide half-reaction. Since the reactions of lipoamide dehydrogenase with NADH or NAD\textsuperscript{+} are faster than 200,000/min at saturating substrate concentration, we have used analogues of NADH and NAD\textsuperscript{+} which react more slowly with lipoamide dehydrogenase, and our assumption has been that these analogues react by a basically similar mechanism. Fig. 1 shows a possible schematic of the events associated with the reduction of the enzyme at high pH (above 7.9) where the elements of a hydride ion are transferred to oxidized enzyme to form \( \text{EH}^- \). The events in this process are shown as the binding of reduced pyridine nucleotide (\( \text{R-PNH} \)), transfer of the elements of a hydride ion from reduced pyridine nucleotide to flavin (\( \text{FADH}^- \)), formation of a thiolate to flavin 4a-adduct (4a-adduct) as an intermediate in the transfer of electrons from reduced flavin to the oxidation-reduction-active disulfide, generation of a complex between enzyme with a reduced disulfide and oxidized pyridine nucleotide (\( \text{EH}^- \cdot \text{PN}^+ \)), and finally release of the oxidized pyridine nucleotide (\( \text{EH}\)). Thorpe and Williams (8) have shown that addition of NAD\textsuperscript{+} to a derivative of 2-electron reduced enzyme in which one of the active center thiol has been alkylated (\( \text{EHR} \)) results in the bleaching of the flavin, with appearance of absorption at 384 nm, spectral changes which are characteristic of the formation of flavin 4a-adducts. This observation suggested that NAD\textsuperscript{+} was influencing the interaction between the flavin and the active center thiol in the modified enzyme. The present work has led to the observation of both Michaelis complexes, \( \text{E-PNH} \) and \( \text{EH}^- \cdot \text{PN}^+ \), under a variety of conditions. However, we have not observed transient species with the absorbance properties to be expected of reduced flavins or complexes, \( \text{E-PNH} \) and \( \text{EH}^- \cdot \text{PN}^+ \), under a variety of conditions. These findings are in agreement with the earlier data of Massey et al. (4) which showed that the rate of reoxidation of the enzyme by lipoamide was rate-limiting for \( V_{\text{max}} \) at pH 6.3. Our previous work has shown that the pH dependence of \( V_{\text{max}} \) is governed by the ionization of \( \text{E} \) to \( \text{EH}^- \), and that the Michaelis complex of \( \text{EH}^- \cdot \text{LipS} \) is catalytically active while that of \( \text{E} \cdot \text{LipS} \) is catalytically incompetent. The apparent pK associated with both \( V_{\text{max}} \) and with the rate of reoxidation of the enzyme is 7.9, which is the actual pK associated with ionization of \( \text{K} \) (5, 6).

**Materials and Methods**

Lipoamide dehydrogenase purified from pig heart was purchased from Miles Laboratories (Seravon Division) and prepared as previously described (9). Dihydrolipoamide was generously donated by Richardson-Merrell, Inc. D-Lipoamide, NAD\textsuperscript{+}, NADH, NHD\textsuperscript{+}, NHDD, APyAD\textsuperscript{+}, and APyADH were purchased from Sigma, dithiothreitol, and lipoamide dehydrogenase, and then purified by chromatography on DEAE-52 according to the procedure described by Arnold et al. (10). Confirmation of the incorporation of deuterium in the S face of the methylene group on \([4S-\text{T}]\text{NIDH}\) was accomplished by oxidation of the deuterated NHDH by the \( R \)-specific enzyme, yeast alcohol dehydrogenase, and then examination of the resulting NHDH\textsuperscript{14} by proton magnetic resonance according to the method of Arakaki et al. (10).

Spectrophotometric measurements were performed with a Cary 118C recording spectrophotometer interfaced to a PDP/8R computer (11). Anaerobic titrations utilized ultrasonication equipment previously described (11). Steady state kinetic measurements of \( V_{\text{max}} \) were performed by fixed ratio extrapolation (12) or by conventional double extrapolation methods (4). Extinction coefficients for NADH and NHDD of 6.2 (\( \text{mm}^{-1} \cdot \text{cm}^{-1} \)) at 340 nm and for APyADH of 9.1 (\( \text{mm}^{-1} \cdot \text{cm}^{-1} \)) at 363 nm were used in the calculation of turnover numbers (13).

The rapid reaction experiments were carried out in a stopped flow apparatus designed for anaerobic work (14) and coupled to a Nova minicomputer system. This system permitted the recording of spectra of solutions in the stopped flow within about 10 s.

**Results and Discussion**

Agreement of \( V_{\text{max}} \) Obtained by Fixed Ratio and Double Extrapolation—When determinations of \( V_{\text{max}} \) for the dihydrolipoamide-NAD\textsuperscript{+} reductase by the fixed ratio extrapolation method (12) and by conventional double extrapolation are compared, the same values of \( V_{\text{max}} \) are obtained by these two methods. The fixed ratio extrapolation method has the advantages of simplicity and of the achievement of measured velocities which are closer to \( V_{\text{max}} \) than those generally obtained by conventional analysis, thus reducing the uncertainty associated with extrapolation. Accordingly, we have used both fixed ratio and conventional double extrapolation methods for determination of \( V_{\text{max}} \). The methods used are indicated in the figure legends.

The pH Dependence of \( V_{\text{max}} \), for the NADH-lipoamide Reductase Reaction—In the left-hand side of Fig. 2, the pH dependence of \( V_{\text{max}} \) for the NADH-lipoamide reductase reaction is compared with values obtained for the pseudo-first order rate constant characterizing the reoxidation of \( \text{EH}^- \) by saturating lipoamide (to be referred to as \( k_{\text{EH}^-} \)). The values for \( k_{\text{EH}^-} \) are taken from Ref. 6. It can be seen that the pH profiles are very similar, indicating that the rate of reoxidation of \( \text{EH}^- \) by lipoamide is almost completely rate-limiting at all pH values. These findings are in agreement with the earlier data of Massey et al. (4) which showed that reoxidation of the enzyme by lipoamide was rate-limiting for \( V_{\text{max}} \) at pH 6.3. Our previous work has shown that the pH dependence of \( k_{\text{EH}^-} \) is governed by the ionization of \( \text{E} \) to \( \text{EH}^- \), and that the Michaelis complex of \( \text{EH}^- \cdot \text{LipS} \) is catalytically active while that of \( \text{E} \cdot \text{LipS} \) is catalytically incompetent. The apparent pK associated with both \( V_{\text{max}} \) and with the rate of reoxidation of the enzyme is 7.9, which is the actual pK associated with ionization of \( \text{K} \) (5, 6).

The pH Dependence of \( V_{\text{max}} \) for the Dihydrolipoamide-NAD\textsuperscript{+} Reductase Reaction—The right-hand side of Fig. 2 compares the pH dependence of \( V_{\text{max}} \) in the dihydrolipoamide-NAD\textsuperscript{+} reductase reaction with values for the extrapolated first order rate constant, \( k_{\text{HiLipS}H} \), characterizing the rate of reduction of lipoamide dehydrogenase by saturating dihydrolipoamide. In agreement with Massey et al. (4), we find that the rate of reduction of the enzyme by dihydrolipoamide is equivalent to \( V_{\text{max}} \) at pH values of 7.6 or greater. As the pH is lowered, \( k_{\text{HiLipS}H} \) remains constant while \( V_{\text{max}} \) decreases. Similar observations on the pH dependence of \( V_{\text{max}} \) for rat liver enzyme dihydrolipoamide-NAD\textsuperscript{+} reductase activity at 37°C have been reported by Reed (15).

These results are not unexpected. As indicated in Equation 2, the oxidation of \( \text{EH}^- \) by NAD\textsuperscript{+} is accompanied by proton release, and becomes thermodynamically unfavorable at sufficiently low pH. From the fact that this pH dependence is expressed in \( V_{\text{max}} \), i.e., under conditions where saturation with substrates has been achieved, we may infer that NAD\textsuperscript{+} binds

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![Fig. 1. Scheme for pyridine nucleotide half-reactions of lipoamide dehydrogenase. The arrows indicate potential charge-transfer interactions and are drawn from donor to acceptor.](http://example.com/fig1.png)
The same vertical scale applies to both halves of Fig. 2.

V max obtained by double extrapolation; a, V max on going from EH2 to EH4, which is analogous to transfer of electrons from dithiol to flavin, is only about 3500 M-1 cm-1. Thus, if any NAD+ is formed in this experiment, it must be less than 20% of the enzyme concentration. Perhaps the most reasonable interpretation of these spectral changes, and one which is supported by the experiments to be described later, is that NAD+ binding at low pH perturbs the charge transfer absorption associated with EH2, without inducing gross dithiol to flavin electron transfer. As can be seen in Curve 1 of Fig. 3, EH2 itself shows long wavelength absorbance, although it retains considerable absorbance in the 450 nm region, indicating that the flavin remains largely oxidized and the electrons have primarily gone to reduce the active center disulfide. This long wavelength absorbance has been attributed to charge transfer between a thiolute anion formed on reduction of the active center disulfide and the oxidized flavin (2, 5).

Veger and Massey (16) had previously observed a species with 580 nm absorbance at pH 7.6, when enzyme was reduced with an excess of NAD+ in the presence of high levels of NAD+, but the enzyme species giving rise to the absorbance at 580 nm is not defined under these conditions.

Similarities between the Reactions of the Enzyme with APyADH and APyAD+ and Those with NADH and NAD+—Before proceeding with stopped flow studies of the reactions of lipoamide dehydrogenase with analogues of NADH and NAD+, it seemed important to establish that the reaction mechanism, as indicated by steady state kinetic studies, was not drastically altered. We planned to use one analogue pair in which the nicotinamide substituent had been altered, APyADH/APyAD+, and one analogue in which the nicotinamide moiety was unchanged, but the adenine moiety was altered, NIDII/NIDID+. NIDII and NIDID bind so poorly to oxidized and reduced enzyme, respectively, that steady state kinetic measurements are not possible.

On the other hand, APyADH and APyAD+ appear to bind quite well, and permit steady state kinetic measurements to be made. One might infer from this that the ADP portion of the pyridine nucleotide was the major determinant in the binding, as is also the case for the binding of pyridine nucleotides to many other dehydrogenases (17). In Fig. 4, the pH profile for V max in the dihydrolipoamide-APyAD+ reductase reaction is shown (Δ). The pH dependence of V max in both EH2 and its deprotonated form, EH−, but that proton release is mandatory for the completion of the reaction.

Titrations of EH2 with NAD+ at Low pH—These findings are corroborated by anaerobic titration of EH2 with NAD+ at pH 5.9, an experiment shown in Fig. 3. After reduction of the enzyme by addition of 0.8 eq of dithionite, the reduced enzyme was titrated with NAD+. The pronounced absorbance changes associated with NAD+ additions are indicative of the formation of a complex between EH2 and NAD+. This complex has a long wavelength absorbance which is maximal at about 580 nm and is green. The changes seen are not compatible with much formation of oxidized enzyme and NADH; in particular there is no increase in absorbance at 343 nm where EH2 and E are isosbestic and where the conversion of NAD+ to NADH should be associated with an extinction change of about 6200 M-1 cm-1. At 455 nm the absorbance is decreasing, while the extrapolated first order rate constant for oxidation of EHz by lipoamide, and are taken from Ref. 6. The solid line is a theoretical curve for reactivity governed by an apparent pK of 6.3. The data indicated by × represent values for the extrapolated first order rate constant for oxidation of EH2 by lipoamide, and are taken from Ref. 6. The solid line is a theoretical curve for reactivity governed by an apparent pK of 6.3. The same vertical scale applies to both halves of Fig. 2.

FIG. 2. Left, comparison of the pH dependence of V max in the NADH-lipoamide reductase reaction with the pH dependence of the rate constants for oxidation of 2-electron reduced enzyme by saturating lipoamide. O, V max obtained by double extrapolation; Δ, V max obtained by fixed ratio extrapolation. All assays were followed at 340 nm, at 22 °C, and the 3-ml assay solutions contained 0.05 M phosphate, citrate (pH 5.3), or pyrophosphate (pH 8.6) buffer, 1 mM EDTA, 2 mg of bovine serum albumin, lipoamide dehydrogenase, lipoamide, and NADH. NAD+ was also added to assay mixtures in amounts sufficient to prevent formation of 4-electron reduced enzyme: pH 8.5, no NAD+ added; pH 7.9, no NAD+ added; pH 7.0, 16.7 μM NAD+, pH 6.5, 96.7 μM NAD+; pH 5.9, 100 μM NAD+, pH 5.5, 100 μM NAD+. The data indicated by × represent values for the extrapolated first order rate constant for oxidation of EHz by lipoamide, and are taken from Ref. 6. The solid line is a theoretical curve for reactivity governed by an apparent pK of 6.3. The same vertical scale applies to both halves of Fig. 2.
Fig. 4. The pH dependence of the APyADH-lipoamide reductase and dihydrolipoamide-APyAD+ reductase reactions of lipoamide dehydrogenase. Cl profile of Vmax versus pH for the APyADH-lipoamide reductase activity of lipoamide dehydrogenase. Values were obtained from measurements of absorbance changes at 363 nm, 25°C, and Vmax at each pH was estimated by fixed ratio extrapolation. Assay mixtures were the same as those in Fig. 2, left, except that APyADH was substituted for NADH, and NAD+ was omitted. +, the extrapolated first order rate constant characterizing the rate of reduction of the enzyme by saturating APyADH, measured in the stopped flow at 530 nm, in 0.05 M phosphate, pH 5.9, 25°C; δ, profile of Vmax, as a function of pH for the dihydrolipoamide-APyAD+ reductase activity of lipoamide dehydrogenase. Values were obtained from measurements of absorbance changes at 25°C, 363 nm, and Vmax at each pH was estimated by fixed ratio extrapolation. Assay mixtures were the same as those in Fig. 2, right, except that APyADH+ was substituted for NAD+. x, values obtained for the extrapolated first order rate constants for the oxidation of EH2 by APyAD+, measured in the stopped flow at 530 and 363 nm at pH values of 6.3 and 7.5. The solid curve is a theoretical curve for reactivity associated with a pK of 7.6.

turnover with this substrate has a shape similar to that observed when NAD+ is used as a substrate, but the overall magnitude of Vmax is 5-fold lower at the pH optimum, indicating that the rate of reduction of the enzyme by dihydrolipoamide is no longer rate-limiting for Vmax with this substrate. Values for the rate of reoxidation of the enzyme by saturating APyAD+, kAPyAD (x——x), are also shown. They are in moderately good agreement with the rate of turnover at these pH values, indicating that reoxidation of the enzyme by APyAD+ is indeed largely rate-limiting for Vmax and pH-dependent.

Also shown in Fig. 4 are values obtained for Vmax in the APyADH-lipoamide reductase reaction (☐—☐). Above pH 7, such measurements are not possible because the equilibrium between products and substrates becomes increasingly unfavorable. (The oxidation-reduction potential of the APyADH/ APyAD+ couple is about 62 mV more positive than the corresponding NADH/NAD+ couple (18, 19).) Vmax has a value which is 25-fold lower than the rate of reoxidation of the enzyme by saturating lipoamide, and is pH-independent over the range where measurements are possible. Thus, reduction of the enzyme by APyADH must be rate-limiting in turnover, and this is substantiated by the agreement of Vmax with kAPyADH at pH 5.9. This is in marked contrast to our results with NADH as reductant, where Vmax depends entirely on the rate of oxidation of the enzyme by lipoamide.

In both the APyADH-lipoamide and the dihydrolipoamide-APyAD+ reductase reactions, fixed ratio extrapolations produce linear double reciprocal plots, indicating that a binary complex or ping-pong mechanism still prevails. (Ternary complex mechanisms which lead to converging lines when analyzed by the double extrapolation method, give rise to nonlinear fixed ratio extrapolations (12).

These results gave us some assurance that the basic mechanism for reaction of the enzyme with APyADH, or APyAD+, did not differ substantially from that with NADH or NAD+.

Intermediates in the Reduction-Oxidation of the Enzyme by APyADH/PyA+ — Parameters for the reduction of enzyme by APyADH and its oxidation by APyAD+ are summarized in Table I. The kinetically determined values for the dissociation constants indicate that both APyADH and APyAD+ bind lipoamide dehydrogenase reasonably tightly. When the reduction of enzyme by saturating levels of APyADH is examined in the stopped flow at either 25°C or 0°C, absorbance changes occur in the dead time of the instrument (about 2 ms). The spectrum of the intermediate formed in the dead time is shown in Fig. 5. The absorbance properties indicate that the flavin remains largely in the oxidized state. At lower APyADH concentrations, where the rate of oxidation of APyADH can be monitored directly, it was found that APyADH oxidation and the appearance of EH2 occurred at the same rate, with no indication of a dead time burst of APyADH oxidation. We are tentatively attributing the dead

![Color figure 5](http://www.jbc.org/)

**FIG. 5.** Species seen during the reduction of lipoamide dehydrogenase by APyADH at 1.5°C, pH 6.15. —, initial oxidized enzyme, 33 μM, in 0.1 M phosphate, after mixing with an equal volume of glass-distilled water; —, spectrum of the intermediate formed in the dead time of the instrument after mixing with 1000 μM APyADH in 0.002 M unneutralized Tris buffer; —, spectrum of the intermediate formed in the dead time of the stopped flow after mixing with 1000 μM [β-3H]APyADH in 0.002 M unneutralized Tris; —, spectrum of enzyme 3 s after mixing with 1000 μM APyADH.
time spectrum to a Michaelis complex between enzyme and APyADH. The absorbance properties of this intermediate resemble those of a series of intermediates which are formed very rapidly when other flavoprotein dehydrogenases are reduced by pyridine nucleotides (20), although the nature of these intermediates remains uncertain (20, 21). Massey and Ghisla (22) have attributed the long wavelength absorbance of these complexes to charge transfer between reduced pyridine nucleotide and oxidized flavin. In our case, the intermediate is then converted to EH₂, with the characteristic absorbance properties shown in Fig. 5, without the appearance of other detectable intermediates. If [4S-²H]APyADH is used as substrate, the intermediate still forms in the dead time at 25°C, but now the rate of appearance of EH₂ is decreased 2.6-fold. This indicates that a step involving hydrogen transfer is at least partially rate-limiting in the conversion of the E-APyADH complex to EH₂.

When EH₂ is oxidized by saturating levels of APyAD⁺, a spectrally distinct intermediate is formed in the dead time of the stopped flow (Fig. 6). From the absorbance properties of the enzyme solution at 375 nm, it is clear that little if any APyADH has been formed in the dead time, and the species presumably is an EH₂-APyAD⁺ complex. This species then is converted to oxidized enzyme and APyADH in a reaction which is biphasic at all wavelengths examined. However, as the spectrum at 12 ms indicates, there is no evidence for accumulation of either reduced flavin or a 4a-adduct during the conversion of EH₂-APyAD⁺ to E and APyADH. Both reduced flavin and 4a-adduct species would have substantial bleaching of the 450 nm absorbance band (5, 8).

Since kₐ[APyAD] is at least 300-fold slower than kₐ[NAD] (see Table I), it was of interest to determine whether the reoxidation of 4-electron reduced flavin, or EH₂, would be equally slow. In EH₂, both the flavin and the oxidation-reduction-active disulfide are reduced, and after APyAD⁺ binding, the flavin could presumably transfer hydride to the bound APyAD⁺ without any requirement for movement of electrons from disulfide to flavin. As is evident from Table I, this reaction is uncompetitively rapid, and has an extrapolated rate constant of 80,000/min at pH 6.3. This indicates that the geometry of the enzyme-substrate complex is not intrinsically unfavorable for hydride transfer. The much slower oxidation of EH₂ by APyAD⁺ (475/min under the same conditions) may reflect a rate-limiting step in the events prior to hydride transfer from reduced flavin to pyridine nucleotide as well as a less favorable free energy of reaction.

Reactions of the Enzyme with NHDH and NHD⁻—Fig. 7 shows the results of stopped flow measurements of the reduction of the enzyme at levels of NHDH which are about 50% saturating, and at pH 6.15. An intermediate is formed in the dead time of the stopped flow and this intermediate has spectral properties which are very similar to the intermediate observed on reduction by APyADH. This species then decays to form a spectrum characteristic of EH₂ again without the appearance of further intermediates. The inset to Fig. 7 shows that the rate of reaction of E with NHDH is saturable, and is characterized by an extrapolated first order rate constant for the formation of EH₂ of about 20,000/min.

A rather different picture is seen if the reduction of the enzyme by NHDH is examined at pH 7.6. These results are shown in Fig. 8. Here the final concentration of NHDH is about 5 μM, which is about 50% saturating at this pH. Before EH₂ is formed, we observe the formation of a spectrum (at 12.5 ms) which is clearly not simply a mixture of E and EH₂, and which does not resemble the dead time spectrum seen at pH 6.2. The absorbance has decreased relative to oxidized enzyme from 425 to 500 nm, and there is a pronounced long wavelength band with absorbance extending out beyond 700 nm, and a maximum at about 580 nm. The spectrum of this intermediate is very similar to that of the “green” intermediate formed when EH₂ is titrated with NAD⁺ at low pH (Fig. 3). On the left side of Fig. 8 are shown some of the absorbance traces from which the spectra shown on the right were calculated. The absorbance at 700 nm rises very rapidly after mixing, in a reaction which is complete in about 12.5 ms. This long wavelength absorbance then falls. In contrast, the absorbance at 530 nm continues to rise throughout the first 60 ms, reflecting first the production of the transient species and then the formation of EH₂. The kinetics of the formation and decay of the transient species is well delineated at 435 nm where E and EH₂ are almost isosbestic. Absorbance decreases are seen in the rapid phase at this wavelength, followed by a return of absorbance in the slow phase. Our final trace, at 395 nm, was intended to ascertain whether appreciable amounts of 4a-adduct, which would be expected to absorb at this wavelength, were formed in the rapid phase of this reaction.
It shows that the rapid phase of reduction is associated with only a slight rise in absorbance so that little if any 4a-adduct can accumulate.

Further experimentation was intended to clarify the nature of the green intermediate just described. There are several reasonable possibilities. The transient spectrum in Fig. 8 is unlikely to be a Michaelis complex between oxidized enzyme and NHDH (E-PNH in Fig. 1) because it looks so different from the dead time complex formed at pH 6.2, or the Michaelis complex between E and APyADH. We might be seeing partial formation of a species in which the flavin is reduced and there is charge transfer between the reduced flavin and NHD+ (FADH+ in Fig. 1). Alternatively, the spectrum might define a species in which electron transfer is complete, and the absorbance is due to charge transfer between a thiolate anion formed on reduction of the active center disulfide and the oxidized flavin-NHD+ complex (EH2-PN+ in Fig. 1).

Examination of the effect of [4S-2H]NHDH on the rate of formation of the transient intermediate indicated that there was no observable kinetic isotope effect on either the rate of formation of the green intermediate or on its rate of decay to form EH2. Since the transfer of electrons from pyridine nucleotide to flavin requires the cleavage of the carbon-deuterium bond on the S face of deuterated NHDH, one would expect a substantial isotope effect on the formation of a reduced flavin intermediate if this cleavage is the rate-limiting step. Deuteration of the [4S-2H]NHDH used was confirmed by proton magnetic resonance spectroscopy.

If the green intermediate we have observed on NHDH reduction of the enzyme is ascribable to a Michaelis complex between EH2 and NHD+, and the disappearance of this intermediate reflects the slow dissociation of NHD+ from this complex, then we should expect to see the EH2-NHD+ complex accumulate when EH2 is oxidized by saturating levels of NADH (EHR) and NHDH (EHR-PN+ in Fig. 1).

We have also examined the spectral changes seen when EH2 is titrated with NHD+ at low pH. At pH 5.9, a titration of EH2 with NHD+ leads to spectral changes which are very similar to those seen on titration of EH2 with NAD+. Thus, our failure to observe formation of the green intermediate on reduction of the enzyme by NHDH at pH 6.2 must be due to a change in rate-limiting step, rather than to an effect of pH on the absorbance of the EH2-NHD+ complex.

The interpretation of the green intermediate as a species in which the electrons are primarily localized on the reduced active center disulfide is strengthened by the observation of very similar absorbance changes when aminopyridine adenine dinucleotide (AAD+), an oxidized pyridine nucleotide which does not appear to be oxidation-reduction-active, is added to enzyme in which one of the active center thiol has been alkylated (23). In reduced alkylated enzyme (EHR) the electrons are "locked" in the disulfide, and cannot be transferred completely to the flavin to form dihydroflavin. Thus, the absorbance changes seen on addition of AAD+ to EHR cannot be attributed to any species in which the flavin is present as dihydroflavin or its anion, and is most reasonably attributed to charge transfer between the active center thiolate anion and the flavin.
and the oxidized flavin-AAD' complex. In agreement with
this assignment, the long wavelength absorbance of the EHR-
AAD' complex disappears at low pH values, in association
with an apparent pK of 5.8 and is abolished by the addition of
mercurials which react with one of the active center thiols.2
The apparent pK of 5.8 may thus reflect the protonation of
the thiolate anion, with resultant disappearance of charge
transfer absorbance.

The interconversion of the EH₂-NHD* complex with EH₂
and NHD* results in spectral perturbations although no elec-
tron transfer is occurring. In particular, the long wavelength
absorbance band shifts from a maximum at about 530 nm in
EH₂ to one at 580 nm in EH₂-NHD*. In uncomplexed EH₂
the oxidation-reduction potential of the active center dithiol/
disulfide is considerably more positive than that of the flavin
(5). Therefore, the electrons in EH₂ are located principally in
the dithiols and the flavin appears oxidized, and the long
wavelength absorbance arises from charge transfer between
thiolate anion and oxidized flavin. When NHD* binds, the
shift in the maximum of the charge transfer band reflects a
change in the relative ground state oxidation-reduction potent-
tials of the charge transfer donor (thiolate) and acceptor
(FAD). In this case the shift to longer wavelengths associated
with NHD* binding shows that the transition energy associ-
ated with thiolate to flavin charge transfer in the excited state
has decreased and implies that the energy necessary for
ground state electron transfer is also lowered. Correlations
between the wavelength of charge-transfer bands and the oxidation-reduction potential differences between donors and
acceptors are well established (19, 24, 25).

These results imply that the binding of oxidized pyridine
nucleotide to EH₂ should influence the equilibrium distribu-
tion of electrons between the flavin and the oxidation-reduc-
tion-active disulfide so as to increase the amount of reduced
flavin. However, to detect such a redistribution of electrons
three requirements must be met: (a) the flavin potential must
be raised sufficiently close to the disulfide oxidation-reduction potential that appreciable amounts of reduced flavin are pres-
ent at equilibrium, (b) the redistribution of electrons must not
be obscured by perturbations of the EH₂ absorbance which
are unrelated to electron transfer, and (c) the experiment
must be performed under conditions where addition of ox-
idized pyridine nucleotide to EH₂ does not result in electron
transfer to form oxidized enzyme and reduced pyridine nu-
cleotide. In practice, the latter requirement restricts us to
measurements with substrates at low pH, where oxidation of
EH₂ does not proceed, or to measurements in which analogues
like AAD*, which are not oxidation-reduction-active, are used.

Fig. 3 shows the result of a low pH experiment, and as
previously discussed there is no decisive evidence for the for-
mation of reduced flavin. However, the perturbation of the
EH₂ spectrum by NAD* is sufficiently marked that up to 40%
reduced flavin at equilibrium could go undetected (see above).
The results of an experiment in which the complex of oxidized
enzyme and AAD' was reduced by dithionite are shown in
Fig. 10. Comparison of the spectrum of EH₂ (Curve 1 in Fig.
3) with the EH₂-AAD' spectrum indicates that the charge
transfer band of the latter is markedly enhanced, and slightly
shifted to longer wavelengths. The absorbance changes at
wavelengths below 500 nm are generally consistent with en-
hanced charge transfer, and might indeed also be representa-
tive of some increase in the content of reduced flavin. How-
ever, the gross perturbation of the charge transfer by AAD'
precludes quantitative determination of reduced flavin con-
tent. The effect of AAD' on the spectrum of EH₂ is similar

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Fig. 10. Absorbance properties of the complex of EH₂ and AAD*. Oxidized enzyme, 26.9 µM, in 0.05 M phosphate buffer, pH 7.6, was
titrated with AAD'. At a final concentration of 500 µM AAD*, the
absorbance changes were complete and the spectrum of the E-
AAD' complex was measured against a cuvette which contained 500
µM AAD' in buffer. The E-AAD' complex was then titrated with
aliquots of a standardized dithionite solution and spectra measured
after each addition. Additions of dithionite were continued until the
enzyme appeared fully reduced. The linear portions of the titration
curve were then extrapolated to their intersection, as described in
Refs. 6 and 11 to yield values for the absorbance of the EH₂-AAD'
complex at 530 and 450 nm. The spectrum shown here for the EH₂-
AAD' complex is calculated as described in Ref. 11 and represents
the absorbance properties of fully formed EH₂-AAD*.

When Escherichia coli lipoamide dehydrogenase is examined
(28).

Within these limitations, failure to see appearance of signif-
ican quantities of reduced flavin on addition of AAD' to EH₂
may also be related to the chemical characteristics of AAD'.
As already mentioned, this analogue does not appear to be
oxidation-reduction-active, and shows 340 nm absorbance and
blue fluorescence (27). Although AAD' is formally an oxidized
pyridine nucleotide, these properties suggest that it shares
many of the characteristics of a reduced pyridine nucleotide,
pnbeacuse the β-amino substituent donates electron
density into the pyridine ring. We have previously established
(28) that addition of NADH to EH₂ leads to absorbance
changes which are qualitatively similar to those seen on
addition of AAD' to EH₂ (enhanced charge transfer absorb-
ance with very little shift to longer wavelength) and in contrast
to the absorbance properties of EH₂-NAD' or EH₂-NHD*
complexes. Thus, we are unable to examine directly the effect
of oxidized pyridine nucleotide on the electron distribution
between disulfide and flavin.

Regardless of its effect on electron distribution in EH₂, the
bound oxidized pyridine nucleotide may affect the ease of
flavin-disulfide interaction, and the rate of any conformational
changes which might govern product release. The rate-limit-
ing step for reduction of the enzyme by NHDH at pH 7.6
occurs after electron transfer is complete and while NHDH* is
still bound, (the conversion of EH .PN* to EH in Fig. 1)
and may reflect a conformational change in the apoprotein
which permits NHDH* to dissociate and which alters the energy
barrier for thiolate to flavin charge transfer. When NHDH is
the reductant, product release occurs at a rate which is about
10,000/min, while when NADH is the reductant the overall
process, and hence product release, must occur at a rate which
is greater than 200,000/min. In either case, the rate of steps
subsequent to hydride transfer from reduced pyridine nucleo-
tide to flavin are governed by the particular pyridine nucleo-
tide bound, indicating an effector role for oxidized pyridine
nucleotide. Furthermore, the conversion of E·NHDH to EH₂-
NHD' is not associated with a rate-limiting hydrogen transfer
from pyridine nucleotide to flavin, suggesting that steps in the
reaction other than hydride transfer are rate-limiting in the formation of \(E_{H_2}\)-NADH.

While these studies present the first evidence for such an effector role for oxidized pyridine nucleotide in native enzyme, the studies of Thorpe and Williams (8) had implicated NADH as an effector in modified enzyme (EHR). Addition of NADH to EHR results in spectral changes characteristic of the formation of a thiolate to flavin 4a-adduct. Thus, addition of NADH to modified reduced enzyme under conditions where formation of dihydrolipofavin or of NADH are precluded, still results in the movement of electrons toward the flavin. These results again suggest that NADH binding alters the relative oxidation-reduction potentials of the reduced disulfide and the flavin.

Our results also tell us something about the pH dependence of the pyridine nucleotide half-reaction. \(V_{\text{max}}\) is pH-dependent for both the dihydrolipoamide-APyADH reductase and dihydrolipoamide-NADH reductase reactions. This pH dependence is not due to the half-reaction in which dihydrolipoamide is oxidized, since the conversion of \(E-H_2\)-LipS\(_2\) to \(E-H_2\)-LipS\(_2\) is pH-independent and the release of Lip\(_2\) from \(E-H_2\)-LipS\(_2\) is much more rapid with \(V_{\text{max}}\) at low pH (6). Therefore, the pH dependence of \(V_{\text{max}}\) must reflect events in the pyridine nucleotide half-reaction, either in central complex formation or during release of reduced pyridine nucleotide. In the case of the reaction of \(E-H_2\) with APyADH, the conversion of \(E-H_2\)-APyAd to E-APyADH can be directly measured in the stopped flow. This rate of conversion has been shown to be pH-dependent, and to correspond to the \(V_{\text{max}}\) for dihydrolipoamide-APyADH turnover at the same pH. Furthermore, we have shown that little or no NADH is formed when \(E-H_2\) and NADH are mixed at pH 5.9, suggesting that the production of \(E\)-NADH requires prior proton release. Our results are consistent with the scheme shown below for the oxidation of \(E-H_2\) by pyridine nucleotide.

\[
\begin{align*}
E_{H_2} & \rightleftharpoons E_{H_2} \cdot PN^+ \\
K_1 & \quad \quad K_2 \\
EH & \rightleftharpoons EH^+ \cdot PN^- \quad k_f \quad E \cdot PNH \rightleftharpoons E
\end{align*}
\]

According to this scheme, \(E_{H_2}\)-PN\(^+\) will accumulate in the steady state under conditions where the pH is below pH\(_K\) and both PN\(^-\) and dihydrolipoamide are saturating. The result will be a decrease in \(V_{\text{max}}\) at low pH and also a decrease in the observed rate of conversion of \(E_{H_2}\)-PN\(^-\) to E-PNH if the half-reaction is measured in the stopped flow, occurrences which have been documented in the present work.

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Reactions of pig heart lipoamide dehydrogenase with pyridine nucleotides.

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