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Redox Properties of Microsomal Reduced Nicotinamide Adenine Dinucleotide-Cytochrome b_5 Reductase and Cytochrome b_5†

Takashi Iyanagi

ABSTRACT: Hepatic NADH-cytochrome b_5 reductase was reduced by 1 mol of dithionite or NADH per mol of enzyme-bound FAD, without forming a stable semiquinone or intermediate during the titrations. However, the addition of NAD^+ to the partially reduced enzyme or illumination in the presence of both NAD^+ and EDTA yielded a new intermediate. The intermediate had an absorption band at 375 nm and the optical spectrum resembled anionic semiquinones seen on reduction of other flavin enzymes. Electron paramagnetic resonance measurements confirmed the free-radical nature of the species. To explain the results, a disproportionation reaction between the oxidized and reduced NAD^+ complexes (E-FAD-NAD^+ + E-$FADH_2$-NAD^+ $\rightleftharpoons$ 2E-FADH·-NAD^+) is assumed. Potentiometric titration of NADH-cytochrome b_5 reductase at pH 7.0 with dithionite gave a midpoint potential of −258 mV; titration with NADH gave −160 mV. This difference may be due to a difference in the relative affinity of NAD^+ for the reduced and oxidized forms of the enzyme. The effects of pH on the midpoint potential of the NAD^+-free enzyme were very similar to those which have been measured with free FAD. At pH 7.0, midpoint potentials of trypsin-solubilized and detergent-solubilized cytochrome b_5 were 13 and 0 mV, respectively.

Reduced nicotinamide adenine dinucleotide-cytochrome b_5 reductase (EC 1.6.2.2) of hepatic microsomes is a flavoprotein containing 1 mol of FAD per mol of enzyme (Strittmatter and Velick, 1957). It is an electron transfer component involved in the biosynthesis of monoenoic fatty acids (Oshino et al., 1971; Enoch et al., 1976) and ethanolamine plasmalogen (Fritz et al., 1974).

The solubilized enzyme from membrane catalyzes one-electron transfer from NADH[1] to various electron acceptors such as cytochrome b_5, ferricyanide (Strittmatter and Velick, 1957), and quinones (Iyanagi and Yamazaki, 1969). The reaction mechanism has been studied by Strittmatter (1965), who concluded from spectrophotometric and kinetic data that it involves successive one-electron oxidations of reduced enzyme to which NAD^+ is bound, and that the rate of the second phase of reoxidation of cytochrome b_5 reductase is dependent on the pyridine nucleotide substituent. This suggests that a flavin semiquinone is involved as a catalytic intermediate. The intermediate observed during reoxidation of NAD^+-free reduced enzyme by ferricyanide showed the spectral characteristics of a blue (neutral) semiquinone (Strittmatter, 1965), but no EPR studies have yet been reported. Strittmatter (1965) also observed that interaction between a semiquinone intermediate and the pyridine nucleotide affects the reactivity of the flavin with electron acceptors. The redox potential of NADH-cytochrome b_5 reductase has been reported by Iyanagi et al. (1974), but has not been analyzed in terms of the interaction between oxidized or reduced enzyme and the pyridine nucleotide. In order to understand the electron-transfer mechanisms from a two-electron donor, NADH, to a one-electron acceptor, cytochrome b_5, it is important to study the redox properties of NAD^+-bound or -free enzyme. In the present paper the oxidation-reduction properties of purified NADH-cytochrome b_5 reductase and cytochrome b_5 are reported.

Materials and Methods

Enzyme Preparation. NADH-cytochrome b_5 reductase was prepared from pig livers according to the method of Takesue and Omura (1970) with some modification (Iyanagi and Yamazaki, 1969). The concentration of enzyme was determined spectrophotometrically by the use of the extinction coefficient of 10.2 mM^{-1} cm^{-1} at 460 nm (Strittmatter and Velick, 1957). Trypsin-solubilized cytochrome b_5 was prepared from rabbit microsomes by the method of Omura and Takesue (1970). Detergent-solubilized cytochrome b_5 was prepared from rabbit microsomes by a method described elsewhere

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[1] Abbreviations used are: EPR, electron paramagnetic resonance; N, the number of electrochemical equivalents involved in any given oxidation-reduction process (Clark, 1960); I, ionic strength; NAD^+ and NADH, oxidized and reduced nicotinamide adenine dinucleotide; E-FAD, enzyme-bound flavin adenine dinucleotide (NADH-cytochrome b_5 reductase), oxidized form; E-FADH·, semiquinone form; E-$FADH_2$, reduced form; EDTA, ethylenediaminetetraacetic acid.

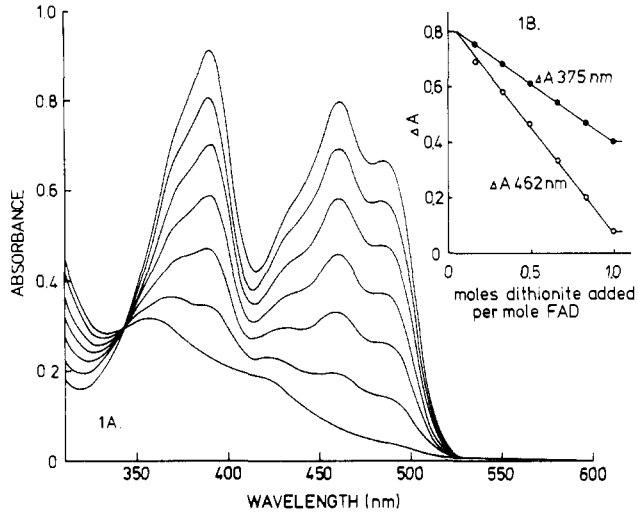


FIGURE 1: Anaerobic titration of NADH-cytochrome b_5 reductase with sodium dithionite. (A) NADH-cytochrome b_5 reductase, 78 μM in I = 0.1 potassium phosphate buffer (pH 7.0), was titrated with 5.2 mM sodium dithionite dissolved in 0.01 M potassium phosphate buffer (pH 8.35). The experimental curves were not corrected for dilution. The volume change during titration was less than 4% of the original volume. (B) The decrease of absorbance at 375 and 462 nm obtained from A was plotted vs. mol of dithionite added per mol of FAD.

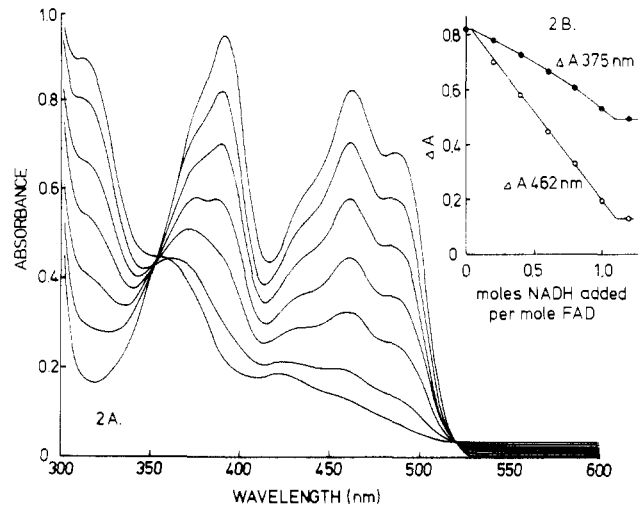


FIGURE 2: Anaerobic titration of NADH-cytochrome b_5 reductase with NADH. (A) NADH-cytochrome b_5 reductase, 80.4 μM in I = 0.1 potassium phosphate buffer (pH 7.0), was titrated with 4.5 mM NADH dissolved in I = 0.1 potassium phosphate buffer (pH 7.0). The experimental curves were not corrected for dilution. (B) The decrease of absorbance at 375 and 462 nm obtained from A was plotted vs. mol of NADH added per mol of FAD.

(Iyanagi and Mason, 1973). The cytochrome b_5 content was determined from the difference spectrum reduced minus oxidized, taking $\Delta\epsilon(424\text{-}409) = 185\ \text{mM}^{-1}\ \text{cm}^{-1}$ according to Omura and Sato (1964).

Materials. NADH and NAD^+ were purchased from the Oriental Yeast Co.; FAD, from Sigma Chemical Co.; toluidine blue, from Tokyo Kasei Co.; indigodisulfonate, from the National Aniline Co.; safranine T, from the Aldrich Chemical Co., Inc.; neutral red, from Wako Chemical Co. Sodium dithionite solutions were prepared and spectrophotometrically standardized with FAD, for which an extinction coefficient of $11.3\ \text{mM}^{-1}\ \text{cm}^{-1}$ was determined at 455 nm (pH 7.0). Nitrogen gas (Nippon Sanso Co., minimum purity, 99.999%) was used in the anaerobic titration systems. All other reagents were the best grade commercially available.

Methods. Optical spectra were measured with a Shimazu Model UV-200 spectrophotometer, in a sample compartment thermostated at 25 ± 1 °C. NADH-cytochrome b_5 reductase activity was measured by the method of Takesue and Omura (1970). EPR derivative absorption spectra were observed with a Varian Model E-line spectrometer with 100-kHz field modulation, and other conditions as noted. The EPR spectra at liquid nitrogen temperature and room temperature were recorded in collaboration with Drs. M. Tamura and K. Hayashi at Osaka University. A capillary tube of 0.8 mm i.d. was used at room temperature.

Titrations were performed anaerobically under nitrogen at 25 ± 1 °C according to the method and using the apparatus of Iyanagi et al. (1974). The potentiometric titration techniques were essentially the same as those employed by Dutton (1971). The anaerobic titration cuvette was continuously stirred and maintained under an atmosphere of highly purified nitrogen, thermostated at 25 ± 1 °C. The anaerobic titration cuvette contained NADH-cytochrome b_5 reductase or cytochrome b_5, an appropriate mediator or mixture of mediators, and aliquots of reductant added anaerobically. The resultant potentials were measured with a system comprising a Type P101 platinum electrode (Radiometer, Copenhagen, Denmark), a saturated calomel electrode (Type K404, Radiometer), and Horiba pH/mV meter (Hitachi-Horiba Co., Tokyo, Japan).

Results

Anaerobic Reduction of NADH-Cytochrome b_5 Reductase. Figure 1 shows the results obtained when oxidized enzyme was titrated anaerobically with dithionite. The oxidized flavoprotein showed absorption peaks at 390 and 462 nm, and a shoulder at 484 nm. The addition of 1 mol of dithionite per mol of enzyme-bound FAD yielded the typical spectrum of the fully reduced enzyme. The enzyme was reduced without forming a stable semiquinone or the intermediate. The fully reduced enzyme was then back titrated with potassium ferricyanide. Complete oxidation of the reduced enzyme was obtained by the addition of 2 mol of potassium ferricyanide per mol of enzyme-bound FAD without detectable intermediate (data not shown). When the physiological substrate, NADH, was used as reductant, it was found that the addition of 1.1 mol of NADH per mol of enzyme-bound FAD yielded the fully reduced enzyme (Figure 2), as has been reported by Strittmatter (1963). A new, quite flat, absorption band in the long-wavelength region appeared, which has been shown to be due to the formation of a charge-transfer complex between NAD^+ and reduced flavin. A 317-nm band, which results from interaction with a sulfhydryl group of the enzyme with NAD^+, also formed (Strittmatter, 1963). Since both NADH and dithionite are two-electron donors, it may be concluded that the enzyme accepts two electrons per molecule of enzyme-bound FAD and, therefore, that FAD is the only oxidation-reduction active group in the enzyme.

Photoreduction of NADH-Cytochrome b_5 Reductase. NADH-cytochrome b_5 reductase can be reduced by illumination, under anaerobic conditions, in the presence of a high concentration of EDTA (Massey and Palmer, 1966). As shown in Figure 3, when NADH-cytochrome b_5 reductase was reduced by light and EDTA in the absence of NAD^+, the partially reduced enzyme (Figure 3, curve E) was almost identical with that of half-reduced enzyme obtained by reduction of oxidized enzyme with dithionite (Figure 1). In this case, prolonged illumination was required to obtain the fully reduced

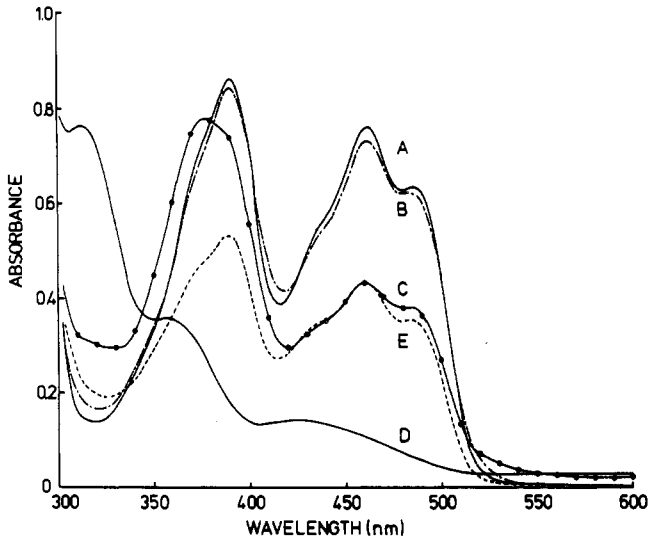


FIGURE 3: Effect of light on NADH-cytochrome b_5 reductase under anaerobic conditions in the presence of EDTA. NADH-cytochrome b_5 reductase, 74.5 μM in 0.1 M potassium phosphate buffer (pH 7.0), was reduced by light in the presence of 20 mM EDTA at 15 °C: curve A, oxidized enzyme; curve B, curve A plus 74.5 μM NAD^+ addition; curves C and D, the mixture of curve B was irradiated by light and recorded at 100 min and 20 h, respectively; curve E, curve A was illuminated for 72 h. The illumination was a tungsten 100-W spotlight lamp.

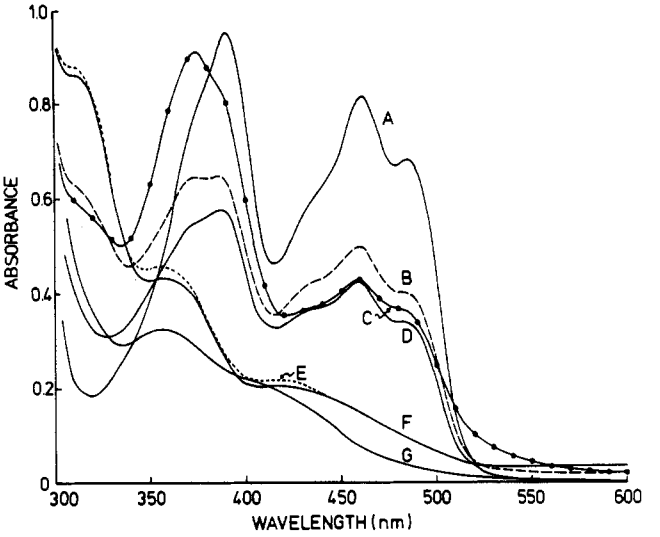


FIGURE 4: Effect of NAD^+ on the spectra of the half-reduced or fully reduced enzyme. NADH-cytochrome b_5 reductase, 80 μM in I = 0.1 potassium phosphate buffer (pH 7.0), was reduced by sodium dithionite and then titrated by NAD^+: curve A, oxidized enzyme; curve B (- -), curve D plus 0.5 equiv of NAD^+ per mol of FAD; curve C (-●-), curve B plus an additional 1.5 equiv of NAD^+ per mol of FAD; curve D, half-reduced enzyme by dithionite; curve E, curve F plus an additional 1 equiv of NAD^+ per mol of FAD; curve F, curve G plus 1 equiv of NAD^+ per mol of FAD; curve G, fully reduced enzyme by dithionite.

enzyme. The reduction rate was much slower than that in the presence of NAD^+. During light illumination in the presence of both EDTA and NAD^+, a new spectrum with the peak at 375 nm and a flat absorption band in the long-wavelength region was generated (Figure 3, curve C). The fully reduced enzyme in the presence of NAD^+ was obtained at about 20 h (Figure 3, curve D). When reduced enzyme was mixed thoroughly with air, the NAD^+-free enzyme was oxidized much faster than NAD^+-bound enzyme. This result suggests that the autoxidation of the flavin is retarded by NAD^+ binding.

Effect of NAD^+ on the Spectra of Half-Reduced and Fully Reduced Enzyme. The fully and half-reduced enzymes formed by reduction with dithionite were titrated with NAD^+. When fully reduced enzyme (Figure 4, curve G) was titrated with 1 mol of NAD^+ per mol of FAD, the resultant spectrum (Figure 4, curve F) was almost identical with that obtained by reduction of oxidized enzyme with NADH (Figure 2). A further addition of 1 equiv of NAD^+ per mol of FAD caused only a small change in the spectrum (Figure 4, curve E). The spectrum (Figure 4, curve B) observed on addition of 0.5 equiv of NAD^+ per mol of FAD to half-reduced enzyme (Figure 4, curve D) was very similar to that of the half-reduced enzyme formed during the course of NADH titration (Figure 2). Surprisingly, a further addition of 1.5 equiv of NAD^+ per mol of FAD caused a large increase in the 375-nm absorbance (Figure 4, curve C), but it appears to contain a substantial proportion of oxidized and reduced enzyme, as judged fom the spectrum. This result indicated that a new spectrum could be expected to develop from equilibration of oxidized and reduced enzyme-NAD^+ complexes. The spectrum was very similar to that of illumination obtained in the presence of EDTA and NAD^+ (Figure 3, curve C). The new absorbing species resembled anionic semiquinones seen on reduction of other flavin enzymes (Massey and Palmer, 1966).

EPR Spectra of NADH-Cytochrome b_5 Reductase. The free-radical nature of the new species described above was shown by its EPR spectrum. The addition of 0.5 equiv of NAD^+ per mol of FAD to half-reduced enzyme does indeed

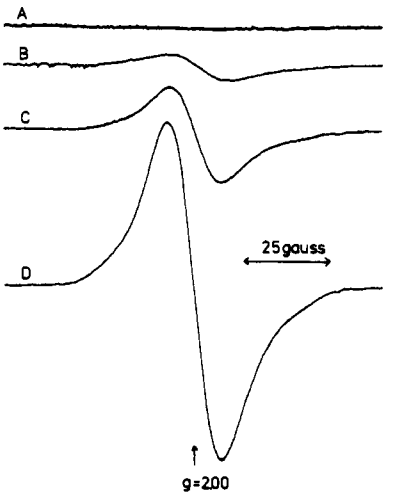


FIGURE 5: Electron paramagnetic resonance spectra of NADH-cytochrome b_5 reductase: curve A, NADH-cytochrome b_5 reductase, 250 μM in I = 0.1 potassium phosphate buffer (pH 7.0), oxidized enzyme; curve B, NADH-cytochrome b_5 reductase, 250 μM in I = 0.1 potassium phosphate buffer (pH 7.0) was reduced by NADH (1.25 mM, final concentration) under anaerobic conditions; curve C, NADH-cytochrome b_5 reductase, 250 μM in I = 0.1 potassium phosphate buffer (pH 7.0), was reduced by NADH (125 μM, final concentration) under anaerobic conditions; curve D, NADH-cytochrome b_5 reductase, 250 μM in I = 0.1 potassium phosphate buffer (pH 7.0), was reduced by NADH (125 μM, final concentration) in the presence of NAD^+ (125 μM, final concentration) under anaerobic conditions and the system was allowed to stand for 5 min at room temperature. All EPR spectra were observed at microwave power 5 mW, modulation amplitude 5 G, and T = −173 °C.

cause the appearance of a free-radical signal at g = 2.00 with a line width of 16 G, as shown in Figure 5, curve D. However, during the course of the titrations of oxidized or reduced enzyme by dithionite or ferricyanide, such a spectrum was not observed (Figure 1). It is therefore clear that the semiquinone form is stabilized by the binding of NAD^+. A small EPR signal was also observed with half-reduced enzyme formed by addi-

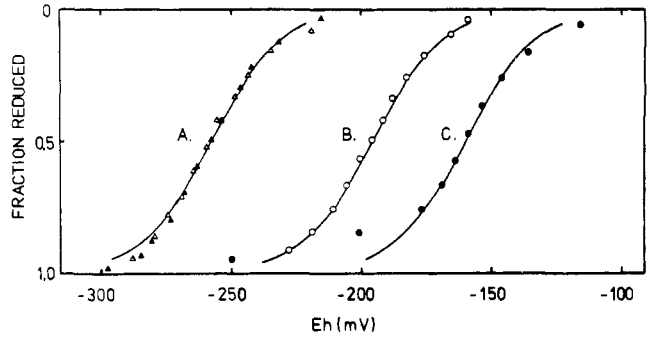


FIGURE 6: Potentiometric titration of NADH-cytochrome b_5 reductase: NADH-cytochrome b_5 reductase, 66 μM; mediator, indigodisulfonate, 2 μM, plus safranine T, 2 μM, and in 3.5 mL of I = 0.1 potassium phosphate buffer (pH 7.0). This mixture was titrated with dithionite (curve A, △) or NADH (curve C, ●) under anaerobic conditions. In the case of curve A, the system was back-titrated with ferricyanide (▲). Oxidation-reduction potential of free FAD, 75 μM in I = 0.1 potassium phosphate buffer (pH 7.0), was measured in the absence of mediators (curve B, ○). The solid lines drawn through the data points present theoretical curves calculated for an N = 2 titration.

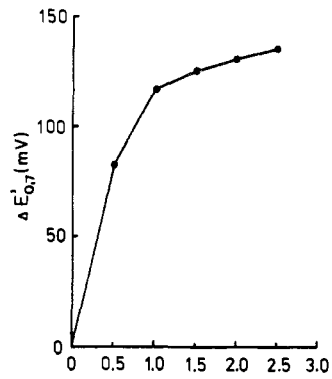


FIGURE 7: Effect of NAD^+ on the oxidation-reduction potentials of NADH-cytochrome b_5 reductase. Half-reduced enzyme (by 0.5 equiv addition of dithionite per mol of FAD), 68.6 μM, and mediators, indigodisulfonate, 2 μM, plus safranine T, 2 μM, in 3.5 mL of I = 0.1 phosphate buffer, was titrated with NAD^+. The numbers on the abscissa indicate the ratio of moles of NAD^+/enzyme, bound FAD.

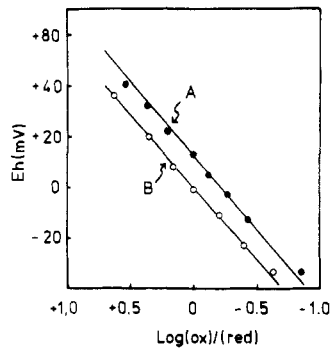


FIGURE 8: Potentiometric titration of cytochrome b_5: cytochrome b_5, 50 μM; mediator, toluidine blue, 2 μM, plus indigodisulfonate, 2 μM, and in 3 mL of I = 0.1 potassium phosphate buffer (pH 7.0), titrated with dithionite under anaerobic conditions; curve A, trypsin-solubilized cytochrome b_5; curve B, detergent-solubilized cytochrome b_5.

tion of 0.5 equiv of NADH per mol of FAD (Figure 5, curve C). If the free-radical signal arose from the species with the 375-nm peak, that species is a semiquinone. The half-reduced enzyme formed by reduction with dithionite (Figure 1) is not a semiquinone. That is, the intensity at 375 nm of the half-reduced enzyme formed by reduction with NADH (Figure 2) was slightly higher than that of the half-reduced enzyme obtained by reduction with dithionite (Figure 1). The EPR signal disappeared almost by addition of excess NADH, as shown in Figure 5, curve B. A quantitative estimate of the spin concentration in the intermediate (Figure 4, curve C) was made by a comparison of the double integral of its EPR signal observed at room temperature, with the corresponding double integral of the EPR signal from *p*-benzosemiquinone (Narni et al., 1966; Iyanagi and Mason, 1973). The spin concentration was 31% of the total flavin. These results suggest that the new peak at 375 nm is due to the formation of a free-radical species from enzyme-bound flavin.

Oxidation-Reduction Potentials of NADH-Cytochrome b_5 Reductase and Cytochrome b_5. The oxidation-reduction potentials of NADH-cytochrome b_5 reductase were measured by direct potentiometry, titrating with dithionite as a reductant and potassium ferricyanide as an oxidant, in the presence of small amounts of indigodisulfonate and safranine T as mediators. The midpoint potential, $E_{0,7}'$, was calculated from the titration data shown in Figure 6 (curve A) to be −258 mV, N = 2, at pH 7.0 and 25 °C. When the enzyme was titrated with NADH as reductant, the midpoint potential, $E_{0,7}'$, was calculated from the titration data shown in Figure 6 (curve C) to be −160 mV, at pH 7.0 and 25 °C. The observed data points were in good agreement with theoretical curves for N = 2 between 10 and 75% reduction, but deviated at over 75% reduction. The difference of approximately 98 mV in the midpoint potentials observed from dithionite and NADH titration is due to the interaction between NAD^+ and reduced enzyme. In fact, a difference, $\Delta E_{0,7}'$, of 83 mV was observed on the addition of 0.5 equiv of NAD^+ per mol of FAD to the half-reduced enzyme formed by reduction with dithionite (Figure 7). This increase of redox potential was accompanied by an increase of absorption at 375 nm. The midpoint potentials of trypsin-solubilized cytochrome b_5 and detergent-solubilized cytochrome b_5 were compared by direct potentiometry, with dithionite as a reductant, in the presence of small amounts of toluidine blue as mediator. In Figure 8 is shown a plot of log oxidized/reduced (ox/red) vs. E_h. The intercept at log ox/red = 0 yielded values of $E_{0,7}'$ of 0 mV for detergent-solubilized cytochrome b_5 and 13 mV for trypsin-solubilized cytochrome b_5, respectively. The slopes correspond to one-electron processes (N = 1). These values are in reasonable agreement with a previously reported midpoint potential of 2 mV at pH 7.0, obtained by a different method (Velick and Strittmatter, 1956).

The E_0' values obtained for the NADH-cytochrome b_5 reductase and the E_0'-pH relationship for NADH-cytochrome b_5 reductase are given in Figure 9. Indigodisulfonate and safranine T in the pH range 6.0–8.0 and safranine T and Neutral red at pH 9.0 were used as mediators. The experimental points of the E_0' vs. pH curve were very similar to those obtained with free FAD (Lowe and Clark, 1956). The value of the pK_r' = 6.5 (Figure 9) is very similar to that reported for free FAD (Lowe and Clark, 1956).

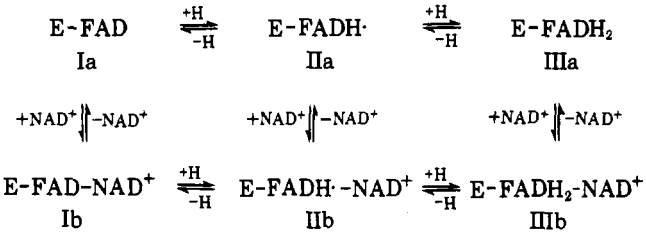
Discussion

NADH-cytochrome b_5 reductase and cytochrome b_5 are the electron-transfer components involved in mixed function oxidase systems (Oshino et al., 1971; Enoch et al., 1976). Similar flavoproteins have been reported to function in the transfer of electrons to nonheme iron proteins such as adrenodoxin, putidaredoxin, and rubredoxin. The flavoproteins, adrenodoxin reductase, putidaredoxin reductase from *Pseu-*

domonas putida, and rubredoxin reductase from *Pseudomonas oleovorans,* contain 1 FAD per mol of enzyme in a single polypeptide chain (Chu and Kimura, 1973; Gunsalus, 1968; Ueda and Coon, 1972). An exception seems to be hepatic NADPH-cytochrome P-450 reductase, which contains one molecule each of FAD and FMN per protein molecule, and each flavin may have an individual function. That is, one flavin accepts two reducing equivalents from NADPH, and the other acts as a one-electron carrier in the electron transfer from NADPH to cytochrome P-450 (Iyanagi and Mason, 1973; Iyanagi et al., 1974). Intramolecular transfer of reducing equivalent by NADPH-sulfite reductase appears similar to that in hepatic NADPH-cytochrome P-450 reductase with respect to flavin components (Siegel et al., 1971). The sequence of electron transfer for all those systems may be summarized as follows: electron donor → flavoprotein → one-electron carrier → oxygenase → O_2.

An interesting problem in these systems is the mechanism of stepdown from two-electron donors to a succession of equipotential one-electron transfer steps. Strittmatter (1965) has reported that reduced NADH-cytochrome b_5 reductase is oxidized in two steps by one-electron acceptors such as cytochrome b_5 or ferricyanide. This mechanism implies a semioxidized flavin intermediate during the reaction. The new spectral species observed with NADH-cytochrome b_5 reductase might be such an intermediate (Strittmatter, 1965). Such an intermediate can also be expected in adrenodoxin reductase, putidaredoxin reductase, and rubredoxin reductase, because their nonheme iron substrates are one-electron carrier acceptors. However, a so-called O_2-stable semiquinone as observed with NADPH-cytochrome P-450 reductase (Iyanagi and Mason, 1973) has not been formed.

In principle, NADH-cytochrome b_5 reductase, which contains 1 mol of FAD per mol of enzyme, can exist in six redox states:



Whether or not these states are all observably distinct from one another depends upon several factors, including the midpoint potential of each one-electron redox couple, and the degree of overlap of the potentials of the E-$FADH_2$/E-FADH·/E-FAD and E-$FADH_2$-NAD^+/E-FADH·-NAD^+/E-FAD-NAD^+ systems. It is possible, however, to observe several steps during the titration by NADH or dithionite. In the present study we have observed several states.

When NADH-cytochrome b_5 reductase was titrated with dithionite, the reductase accepted 2 electrons per molecule of FAD, without the formation of a semiquinone species. However, when the reductase was titrated with NADH, the extent of the semiquinone formation was markedly dependent on the concentration of NAD^+, as judged from the optical spectra (Figure 4, curve C) and the EPR signal (Figure 5, curve D). These observations can be explained in terms of the following set of equations (1-3).

$$\text{E-FAD} + \text{E-FADH}_2 \rightleftharpoons 2\text{E-FADH}\cdot \quad (1)$$

$$\text{E-FAD} + \text{NAD}^+ \rightleftharpoons \text{E-FAD-NAD}^+ \quad (2)$$

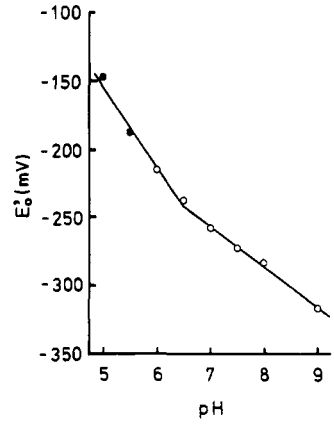


FIGURE 9: Effect of pH on the oxidation-reduction potential of NADH-cytochrome b_5 reductase: NADH-cytochrome b_5 reductase, 63 μM; a mixture of mediator, indigodisulfonate, 2 μM, plus safranine T, 2 μM (in the pH range 6.0-8.0), or safranine T, 2 μM, plus neutral red, 2 μM (pH 9.0), and in 3.5 mL of I = 0.1 acetate buffer (●) or phosphate buffer (○), titrated with sodium dithionite under anaerobic conditions.

$$\text{E-FAD-NAD}^+ + \text{E-FADH}_2\text{-NAD}^+ \rightleftharpoons 2\text{E-FADH}\cdot\text{-NAD}^+ \quad (3)$$

The reductase is reduced to the fully reduced form (state IIIa) by dithionite without forming a semiquinone. Therefore, the semiquinone formation in a mixture of fully reduced (state IIIa) and oxidized (state Ia) enzyme is very small (eq 1), even in the presence of mediators (such as methyl viologen, safranine T, and indigodisulfonate) required for electron transfer between flavoprotein molecules. The half-reduced state formed by reduction with NADH seems to contain appreciable amounts of semiquinone species (Figure 2 and Figure 5, curve C). The addition of NAD^+ to the half-reduced enzyme caused an increase in semiquinone concentration (Figure 4, curve C). NAD^+ forms a complex with oxidized enzyme as well as reduced enzyme, as judged by the difference spectrum (Figure 3, curve B). The dissociation constant, K_o, for NAD^+ binding to oxidized enzyme was estimated to be about 2×10^{-4} M^{-1}, from a difference spectrum (data not shown). Therefore, formation of a semiquinone species dependent upon the concentration of NAD^+ may be explained by eq 2 and 3. The results suggest that NAD^+-bound semiquinone (state IIb) is more stable than NAD^+-free semiquinone (state IIa). The new spectrum with a peak at 375 nm is very different from that of a blue type semiquinone with a broad absorption band from 500 to 650 nm (Strittmatter, 1965). This difference can be attributed to the interaction between NAD^+ and the semiquinone form (state IIa). NAD^+-free semiquinone (state IIa) is the neutral species, but NAD^+-bound semiquinone (state IIb) is the anionic form, corresponding to the structure E-FAD^-·-NAD^+, and NAD^+ may act as a stabilized group. The EPR signal line width, 16 G (Figure 5, curve D), is similar to that of anionic semiquinones (Palmer et al., 1971). Yagi (1975) reported that the binding of benzoate to D-amino acid oxidase shifts the semiquinone form from the anionic form to the neutral state. The present results might conversely represent a shift from the neutral to the anionic form on binding of pyridine nucleotide, although an anionic semiquinone is rather unusual in a dehydrogenase (Massey et al., 1969).

The midpoint potential of free FAD was −196 mV at pH 7.0, measured with the apparatus used in the present studies (Figure 6, curve B). In the case of enzyme-bound FAD, the redox potential, $E_{0,7}'$, was −258 mV, by titrating with dithionite (Figure 6, curve A). This change in $E_{0,7}'$ between free

and enzyme-bound FAD is related to the association constants for the interaction of the apoenzyme with the oxidized and reduced FAD (Vesting, 1955). The shift in potential, $\Delta E_{0,7}' = -62$ mV, implies that oxidized FAD binds about 100 times more tightly to enzyme than does reduced FAD. The positive shift in potential, $\Delta E_{0,7}' = 98$ mV, observed between NADH and dithionite titration is due to NAD^+ binding about 2×10^3 times more tightly to reduced enzyme than to oxidized enzyme. The dissociation constant, K_r, for binding of NAD^+ to reduced enzyme can be calculated from $K_o = 2 \times 10^{-4}$ and $E_{0,7}' = 98$ mV (Vesting, 1955). The value of K_r was estimated to be about 4×10^{-7} M^{-1}. The further shift of $E_{0,7}'$ observed on addition of NAD^+ might be due to the increased concentration of the semiquinone species (Figure 7). However, if this change is due to semiquinone formation alone, the observed midpoint potential should be unchanged, as pointed out by Michelis (Clark, 1960). This also implies that new spectral species (Figure 4, curve C) contain charge-transfer complexes. In order to understand the exact electron distribution in the proposed structure, E-FAD^{-}·-NAD^+, additional structural and kinetic studies must be made. Such studies are under investigation.

Redox potential values similar to those of NADH-cytochrome b_5 reductase have been reported for adrenodoxin reductase (−291 mV without $NADP^+$, −198 mV with $NADP^+$) (Lambeth and Kamin, 1976) and putidaredoxin reductase (−283 mV) (Gunsalus and Lipscomb, 1972). In the case of NADPH-cytochrome P-450 reductase, very similar redox potentials were observed for both systems by titration of the enzyme with NADPH and dithionite (Iyanagi et al., 1974). Thus, in this enzyme strong binding of $NADP^+$ to reduced enzyme is not expected.

Finally, it is of interest to compare the oxidation–reduction potentials of NAD^+ bound and free enzyme. The present studies have shown that NAD^+ forms a complex with reduced enzyme with a low dissociation constant, but binds relatively weakly to oxidized enzyme. This preferential binding shifts the redox potential of the enzyme by about 100 mV. The new intermediate observed by equilibration of the oxidized and reduced enzyme in the presence of NAD^+ showed properties of free radicals. A more detailed kinetic analysis of the intermediates in a bivalent flavin molecule will yield useful information about the electron-transfer mechanism from NADH to cytochrome b_5.

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