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One-Electron Oxidation–Reduction Properties of Hepatic NADH–Cytochrome b_5 Reductase†

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ABSTRACT: The one-electron oxidation–reduction properties of flavin in hepatic NADH–cytochrome b_5 reductase were investigated by optical absorption spectroscopy, electron paramagnetic resonance (EPR), and potentiometric titration. An intermediate with a peak at 375 nm previously described by Iyanagi (1977) [Iyanagi, T. (1977) *Biochemistry 16*, 2725–2730] was confirmed to be a red anionic semiquinone. The NAD^+-bound reduced enzyme was oxidized by cytochrome b_5 via the semiquinone intermediate. This indicates that electron transfer from flavin to cytochrome b_5 proceeds in two successive one-electron steps. Autoxidation of the NAD^+-bound reduced enzyme was slower than that of the NAD^+-free reduced enzyme and was accompanied by the appearance of an EPR signal. Midpoint redox potentials of the consecutive one-electron-transfer steps in the presence of excess NAD^+ were $E_{m,1} = -88$ mV and $E_{m,2} = -147$ mV at pH 7.0. This corresponds to a semiquinone formation constant of 8. The values of $E_{m,1}$ and $E_{m,2}$ were also studied as a function of pH. A mechanism for electron transfer from NADH to cytochrome b_5 is discussed on the basis of the one-electron redox potentials of the enzyme and is compared with the electron-transfer mechanism of NADPH–cytochrome P-450 reductase.

NADH–cytochrome b_5 reductase (EC 1.6.2.2), a single-subunit, mono-FAD-containing enzyme, and cytochrome b_5 function as an electron-transport chain from NADH to a terminal oxidase desaturase in the endoplasmic reticulum (Strittmatter et al., 1974; Ohnishi et al., 1975; Okayasu et al., 1981). The flavoprotein accepts two electrons from NADH with concomitant production of a long-wavelength-absorbing NAD^+-reduced flavoprotein charge-transfer complex and can transfer reducing equivalents from NADH to the one-electron acceptor cytochrome b_5 (Strittmatter, 1965).

Strittmatter (1965) has proposed that by using both the fully reduced flavin and the semiquinone as reductants, NADH–cytochrome b_5 reductase can reduce 2 equiv of cytochrome b_5 in successive one-electron steps. In a previous study (Iyanagi, 1977), we demonstrated that addition of NAD^+ to the partially reduced enzyme yields a new intermediate with an absorption band at 375 nm, showing the characteristic features of a red semiquinone. Hemmerich & Massey (1979) have suggested that in a flavoprotein involved in one-electron transfers, the blue neutral semiquinone is an obligatory intermediate. Indeed, a blue semiquinone is observed in NADPH–cytochrome P-450 reductase (Masters et al., 1965; Iyanagi & Mason, 1973; Yasukochi et al., 1979; Oprian & Coon, 1982) and NADPH–adrenodoxin reductase (Lambeth & Kamin, 1977; Kitagawa et al., 1982). On the other hand, enzymes of the dehydrogenase–oxidase group generate a red-colored radical species. Thus, the observed anionic red semiquinone in the dehydrogenase–electron transferase NADH–cytochrome b_5 reductase is rather unusual. A more

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detailed kinetic analysis of the intermediate of the bivalent flavin molecule will yield useful information about the electron-transfer mechanism from NADH to cytochrome b_5.

In the present study, we have further characterized the red semiquinone intermediate. We have focused on the following points by using stopped-flow, electron paramagnetic resonance (EPR),[1] and potentiometric titrations: (1) the formation mechanism of the intermediate; (2) the nature of the intermediate as a free radical; (3) one-electron oxidation–reduction potentials of the enzyme-bound flavin. A mechanism for electron transfer from NADH to cytochrome b_5 is discussed with special attention to one- and two-electron-transfer reactions.

Materials and Methods

Materials

NADH and NAD^+ were purchased from Oriental Yeast Co., Triton N-101 [alkylphenoxypoly(ethoxyethanol)] was from Sigma, sodium deoxycholate was from Nakarai Co., DE-52 was from Whatman, and 5′-ADP-agarose [agarose–hexane–adenosine 5′-diphosphate (type 2)] was from P-L Biochemicals. 5-Deazaflavin was a generous gift from Dr. Vincent Massey of the University of Michigan. Cytochrome b_5 was purified from pig liver by the method of Omura & Takesue (1970). Hydroxylapatite was prepared by the method of Levin (1962).

Methods

Optical spectra were measured with a Hitachi Model 200-10 spectrophotometer in a sample compartment thermostated at 25 °C. The EPR derivative absorption was measured with a JEOL JES-FX spectrometer. The EPR spectra at room temperature were recorded in collaboration with Dr. Masahiro Kohno at JEOL Ltd., using a flat cell. The spin concentration was measured by using the "air-stable semiquinone" of NADPH–cytochrome P-450 reductase as described by Iyanagi et al. (1978). Stopped-flow experiments were performed with a Union Giken RA 401 stopped-flow spectrometer (Iyanagi et al., 1981).

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 described in Iyanagi (1977). The anaerobic titration cuvette was continuously stirred and maintained under an atmosphere of highly purified nitrogen. All titration solutions contained 5 mM glucose and 1 μM glucose oxidase of ensure continued anaerobiosis during the titration. The anaerobic titration cuvette contained NADH–cytochrome b_5 reductase, 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 a Beckman pH/mV meter (Beckman Model 3500 digital pH meter).

NADH–cytochrome b_5 reductase activity was monitored at 556 nm by using ϵ = 19 mM^{-1} cm^{-1} (Omura & Takesue, 1970). The concentration of the enzyme was determined spectrophotometrically on the basis of a molar extinction coefficient of ϵ = 10.2 mM^{-1} cm^{-1} at 460 nm (Strittmatter & Velick, 1957).

SDS gel electrophoresis was carried out according to the method of Weber & Osborn (1969) using cytochrome *c*, chymotrypsinogen, carbonic anhydrase, ovalbumin, and bovine serum albumin.

Solubilization and Purification of NADH–Cytochrome b_5 Reductase. (*A*) *Lysosome-Solubilized Enzyme.* Lysosome-solubilized NADH–cytochrome b_5 reductase was prepared by the method of Iyanagi et al. (1969) with some modification (Takesue & Omura, 1970; Schafer & Hultquist, 1980). Pig liver (about 1.5 kg) was homogenized for 6 min with 4 volumes of ice-cold 0.15 M KCl by using a Matsushita Model MX-140S homogenizer. The homogenates were centrifuged at 10000*g* for 20 min; the supernatant solution was adjusted to pH 5.5 with acetic acid and after 2 h centrifuged at 10000*g* for 30 min at 4 °C. The precipitate, "acid-precipitated microsomes", was dissolved in 0.1 M potassium phosphate buffer, pH 7.7, containing 1 mM EDTA and stored at −20 °C. The microsomes (4 L) from pig liver (about 1.5 kg) were adjusted to pH 5.6, incubated at 37 °C for 3 h, and centrifuged at 10000*g* for 20 min, and the supernatant solution was adjusted to pH 7.5 with sodium hydroxide. The solubilized reductase was recovered from the supernatant fluid by fractionation with ammonium sulfate (45–75%) and applied to a Sephadex G-100 column (8 × 100 cm) previously equilibrated with 50 mM potassium phosphate buffer, pH 7.7, containing 1 mM EDTA. The active fraction was diluted 5-fold with distilled water and applied to a DEAE (DE-52) column (3.5 × 35 cm) previously equilibrated with 10 mM potassium phosphate buffer, pH 7.7, containing 0.1 mM EDTA; the column was washed with 500 mL of the same buffer, and the reductase was eluted with 50 mM potassium phosphate buffer, pH 7.7, containing 0.5 mM EDTA. The active yellow fraction was applied to a 5′-ADP–agarose column (1.5 × 20 cm) previously equilibrated with 10 mM potassium phosphate buffer, pH 7.7, containing 0.1 mM EDTA. Approximately 10–20% of the enzyme did not bind to the 5′-ADP–agarose column. The column was washed with 300 mL of the same buffer, and the reductase was eluted with 2 mM ADP in 10 mM potassium phosphate buffer, pH 7.7, containing 0.1 mM EDTA. The active fraction was applied to a hydroxylapatite column (3.5 × 20 cm) equilibrated with 10 mM potassium phosphate buffer, pH 7.7, containing 0.1 mM EDTA. The column was washed with 500 mL of 10 mM buffer, and the reductase was then eluted with a linear gradient of 10–100 mM potassium phosphate buffer, pH 7.7, containing 0.1 mM EDTA.

(*B*) *Detergent-Solubilized Enzyme.* Microsomes were prepared from pig liver by the method of Iyanagi et al. (1978). About 20 g of microsomal protein was suspended (to 10 mg of protein/mL) in 0.1 M Tris–acetate buffer, pH 7.6, containing 2% Triton N-101, 0.4% sodium deoxycholate, 10% (v/v) glycerol, and 1 mM EDTA. The mixture was stirred for 30 min at 4 °C and centrifuged at 78000*g* for 120 min. The supernatant solution was applied to a DEAE-cellulose (DE-52) column (5 × 40 cm) previously equilibrated with 0.1 M Tris–acetate buffer, pH 7.6, 10% glycerol, 0.2% Triton N-101, 0.2% deoxycholate, and 1 mM EDTA. Unadsorbed fractions, containing reductase activity, were dialyzed against 10 mM potassium phosphate buffer (pH 7.7) containing 0.5% Triton N-101, 0.2% deoxycholate, 0.1 mM EDTA, and 10% glycerol and applied to a DE-52 column (5 × 30 cm) equil-

[1] Abbreviations: EPR, electron paramagnetic resonance; *N*, number of electrochemical equivalents involved in any given oxidation–reduction process; E_h, oxidation–reduction potential referred to the standard hydrogen electrode; $E_{m,x}$, midpoint potential at pH_x (Clark, 1960); E–FAD_{ox}, oxidized NADH–cytochrome b_5 reductase; E–FADH·, neutral blue radical; E–FAD^{-}·, anionic red radical; E–$FADH_2$, fully reduced enzyme, protonated; E–$FADH^-$, fully reduced enzyme, unprotonated; E–FAD_{red}, fully reduced enzyme; E–FAD_{sq}, semiquinone form of the enzyme; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane.

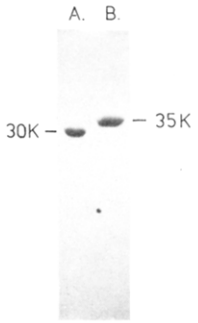


FIGURE 1: SDS gel electrophoresis of NADH–cytochrome b_5 reductase. Gels A and B show the lysosome-solubilized enzyme (10 μg) and detergent-solubilized enzyme (10 μg), respectively. The molecular weight of purified enzyme was determined by reference to the mobility of standard proteins.

ibrated with the same buffer. The reductase was eluted with a linear gradient of 0–0.5 M KCl in 10 mM potassium phosphate buffer, pH 7.7, containing 0.5% Triton N-101, 0.2% deoxycholate, 10% glycerol, and 0.1 mM EDTA. The active yellow fraction was dialyzed against 10 mM potassium phosphate buffer, pH 7.7, containing 0.1% Triton N-101, 10% glycerol, and 0.1 mM EDTA and applied to a 5′-ADP–agarose column (1.5 × 20 cm) previously equilibrated with the same buffer. The column was washed with 300 mL of the same buffer, and the reductase was eluted with 2 mM ADP in 10 mM potassium phosphate buffer, pH 7.7, containing 0.1% Triton N-101, 10% glycerol, and 0.1 mM EDTA. The active fraction was applied to a hydroxyapatite column (3.5 × 20 cm) equilibrated with 10 mM potassium phosphate buffer, pH 7.7, containing 0.1% Triton N-101, 10% glycerol, and 0.1 mM EDTA. The column was washed with 500 mL of the same buffer, and the reductase then was eluted with a linear gradient of 10–100 mM potassium phosphate buffer, pH 7.7, containing 0.1% Triton N-101, 10% glycerol, and 0.1 mM EDTA.

Results

Effect of pH on the Maximal Rate of Cytochrome b_5 Reduction by NADH–Cytochrome b_5 Reductase. Lysosome- and detergent-solubilized NADH–cytochrome b_5 reductases were purified from pig liver microsomes by using 5′-ADP–agarose affinity chromatography (see Materials and Methods). The apparent molecular weights for lysosome- and detergent-solubilized enzymes were 30K and 35K, respectively (Figure 1). The K_m (0.4–0.5 μM) for NADH was similar in both enzymes. Similarly, the value of K_m for cytochrome b_5 (30–40 μM) was not significantly different for the lysosome- or the detergent-solubilized enzyme. The rate of reduction of cytochrome b_5 was dependent on ionic strength. Therefore, V_{max} for cytochrome b_5 was measured at constant ionic strength and at saturation levels of NADH (100 μM). Figure 2A shows the maximal turnover of NADH–cytochrome b_5 reductase. The values decreased with increasing pH in the range from pH 6 to pH 9.0. A similar result was obtained for the detergent-solubilized enzyme (data not shown). The reduction of enzyme-bound flavin by NADH was directly measured by stopped flow. A pH dependence similar to that for V_{max} in steady-state reduction of cytochrome b_5 was obtained (Figure 2B). These results confirm that the reduction of flavin in the reductase by NADH is the rate-limiting step (Strittmatter, 1965).

Binding of NAD$^+$ to Oxidized NADH–Cytochrome b_5 Reductase. A titration of oxidized enzyme (E–FAD$_{ox}$) with NAD$^+$ resulted in perturbation of the flavin spectrum and inhibition of activity. The difference spectrum generated by

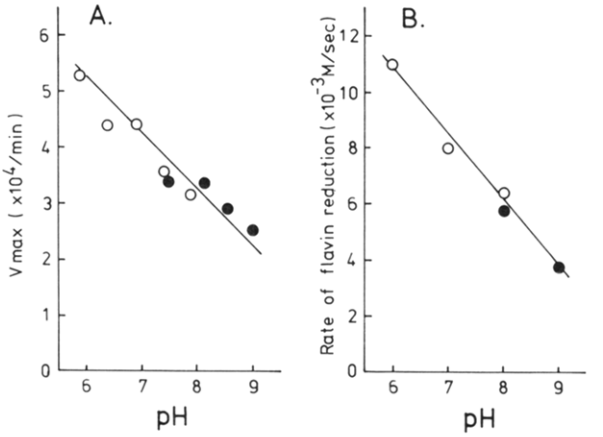


FIGURE 2: Effect of pH on the V_{max} of NADH–cytochrome b_5 reduction and flavin reduction by NADH. (A) Cytochrome b_5 reduction was measured in 100 μM NADH and 1.1 × 10^{-9} M lysosome-solubilized enzyme and at various concentrations of cytochrome b_5. The V_{max} for cytochrome b_5 was determined from double-reciprocal plots of (cytochrome b_5 reduction)$^{-1}$ vs. [cytochrome b_5]$^{-1}$. (B) Initial velocity of flavin reduction by NADH measured as described by Strittmatter (1965). The reactants were 290 μM NADH and a solution containing 16 μM NADH–cytochrome b_5 reductase and 25 μM potassium ferricyanide. The stopped-flow mixing was followed at 462 nm (25 °C). In (A) and (B), open circles were obtained in 5 mM potassium phosphate buffer and closed circles in 5 mM Tris–acetate buffer, and solutions were adjusted to constant ionic strength (I = 0.1) with KCl.

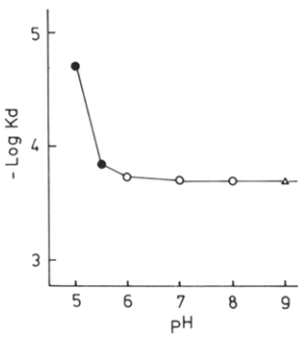


FIGURE 3: Effect of pH on the K_d. NADH–cytochrome b_5 reductase, 80 μM in 2.0 mL of 0.1 M buffer, was titrated with NAD$^+$ (8.0 mM); the reference cuvette contained the same concentration and volume of NADH–cytochrome b_5 reductase. The apparent K_d value was determined from the plot of $1/\Delta A$ (406–458 nm) vs. $1/[NAD^+]$. Buffers used were 0.1 M acetate buffer (●), 0.1 M potassium phosphate buffer (○), and 0.1 M Tris–acetate buffer (△).

the addition of excess NAD$^+$ showed positive peaks at 406, 477, and 513 nm and negative peaks at 458 and 490 nm. Double-reciprocal plots of absorbance changes vs. NAD$^+$ added were linear, yielding an apparent dissociation constant (K_d) of 2 × 10^{-4} M. This value was very close to the K_i for NAD$^+$ of 3 × 10^{-4} M which was determined in kinetic experiments at 25 °C (data not shown). Inhibition by NAD$^+$ was competitive with respect to NADH. A titration of oxidized enzyme with NADP$^+$ did not result in perturbation of the flavin spectrum (data not shown). These results suggest that NAD$^+$ binds to a specific site of the oxidized enzyme with a low dissociation constant. The K_d was determined over the pH range from 5.0 and 9.0, and the results are shown in Figure 3. The apparent K_d for NAD$^+$ was almost constant over the pH range 5.5–9.0 but significantly decreased at pH 5.0.

Dithionite Titration of NADH–Cytochrome b_5 Reductase in the Presence of NAD$^+$. We previously reported that the oxidized enzyme (E–FAD$_{ox}$) when reduced by dithionite does not form any stable semiquinone or intermediate (Iyanagi, 1977). However, the addition of NAD$^+$ to the half-reduced

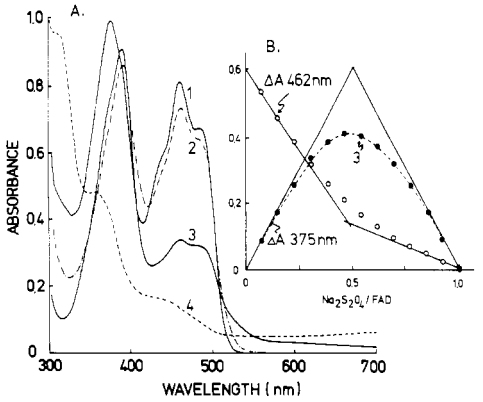


FIGURE 4: Anaerobic titration of NADH–cytochrome b_5 reductase with dithionite in the presence of NAD^+. (A) NADH–cytochrome b_5 reductase, 86 μM in 0.1 M potassium phosphate buffer, pH 7.0, was titrated with dithionite (10 mM) in the presence of 2 mM NAD^+ and 2 μM indigodisulfonate. The experimental curves were not corrected for dilution. Curve 1, fully oxidized enzyme; curve 2, curve 1 plus 2 mM NAD^+; curve 3, recorded at the stage representing approximately 50% reduction; curve 4, fully reduced enzyme. Intermediate curves have been omitted for clarity. (B) Titration curves showing the changes in absorbance at 375 and 462 nm after successive additions of dithionite. The number 3 designates the experimental point at which the spectrum shown in curve 3 of (A) was recorded. The solid lines are extrapolated linear phases in the absence of disproportionation, and the dashed line is calculated from the extrapolated end points and a formation constant for the intermediate (I) of 8, K_I = [intermediate]^2/([E-FAD_{ox}][E-FAD_{red}]).

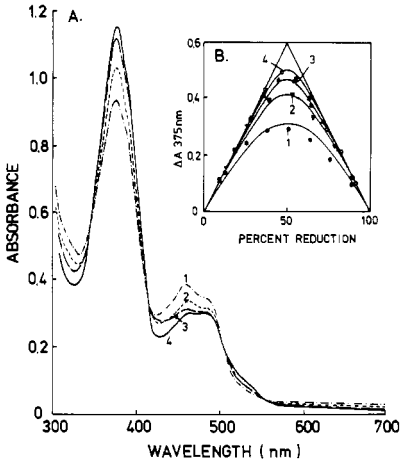


FIGURE 5: Anaerobic titration of NADH–cytochrome b_5 reductase with dithionite in the presence of NAD^+ at four different pH values. (A) NADH–cytochrome b_5 reductase, 86 μM in 0.1 M buffer [curve 1, pH 6.0 (acetate): curve 2, pH 7.0 (potassium phosphate); curve 3, pH 8.0; curve 4, pH 9.0 (Tris–acetate)], was titrated with dithionite (10.5 mM) in the presence of 2 mM NAD^+ and 2 μM indigodisulfonate, respectively. Then each spectrum was recorded at half-reduced enzyme concentration. (B) The titration curves show the change of absorbance at 375 nm after successive additions of dithionite. The formation of an intermediate was plotted as the percent reduction. The solid lines drawn through the data points represent theoretical curves calculated for a semiquinone formation constant, K_s = [E-FAD_{sq}]^2/([E-FAD_{ox}][E-FAD_{red}]), of 2.5 [pH 6.0 (●)], 8 [pH 7.0 (▼)], 18 [pH 8.0 (△)], and 35 [pH 9.0 (○)], respectively. The numbers 1–4 designate the experimental points at which the spectra shown in (A) were recorded.

enzyme caused an increase in a new spectral species with an absorption peak at 375 nm, showing its free-radical nature. Figure 4 shows the results observed when the oxidized enzyme was titrated anaerobically with dithionite in the presence of excess NAD^+. Formation of intermediate was observed during the dithionite titration (Figure 4A), and the maximum concentration was obtained at a half-reduction of the enzyme (Figure 4B). Formation curves for the intermediate (Figure 4B) conformed to

$$E\text{-}FAD_{ox} + E\text{-}FAD_{red} \rightleftharpoons 2(\text{intermediate})$$

The formation of an intermediate was increased in the alkaline pH range, as shown in Figure 5, and the spectrum at pH 9.0 is very similar to that of the anionic red semiquinone of pyruvate oxidase (Mather et al., 1982). The above results suggest that a disproportionation equilibrium between individual enzyme-bound flavin molecules is allowed in the presence of NAD^+.

Semiquinone Form of NADH–Cytochrome b_5 Reductase. We have previously observed an EPR spectrum resembling that of the "red form" of flavin semiquinone in NADH–cytochrome b_5 reductase and proposed the structure E-$FAD^{\cdot-}$–NAD^+ as a semiquinone form for the spectral species with an absorption peak at 375 nm (Iyanagi, 1977). In order to understand the exact electron distribution in this structure, we studied the kinetics of semiquinone formation and performed a quantitative estimate of the spin concentration. The oxidized enzyme (E-FAD_{ox}) was mixed with 0.5 equiv of NADH in the presence of a high concentration of NAD^+, in the stopped-flow apparatus (Figure 6). Since NADH donates 2 reducing equiv to the flavoprotein, the reaction should result in a mixture of the oxidized and reduced enzymes. After being mixed in the presence of the mediator indigodisulfonate which is required for electron transfer between enzyme molecules, an increase in absorption at 375 nm was accompanied by an increase in the EPR signal, while in the absence of mediator these two parameters increased very slowly. These results indicate that the formation of the semiquinone form is due to

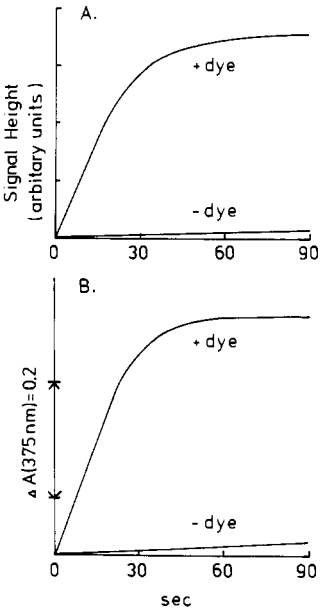


FIGURE 6: Time course of flavin free-radical formation produced from a mixture of the oxidized and reduced NADH–cytochrome b_5 reductases. The enzyme and NADH were rapidly mixed (dead time about 20 ms) anaerobically in an EPR flat cell with a two-jet mixer so as to produce a final concentration of 77.5 μM NADH–cytochrome b_5 reductase, 38.8 μM NADH, and 2 mM NAD^+ in the presence or absence of 2 μM indigodisulfonate (dye), in 0.1 M potassium phosphate buffer (pH 7.0) at 25 °C. The magnetic field was set at the signal maximum. The increase of free-radical formation (A) and the absorbance change at 375 nm (B) were measured in different cells under the same conditions.

a disproportionation reaction between the oxidized and reduced enzyme molecules.

A quantitative estimate of the spin concentration of the intermediate was made by a comparison of the double integral of its EPR signal observed at room temperature with the

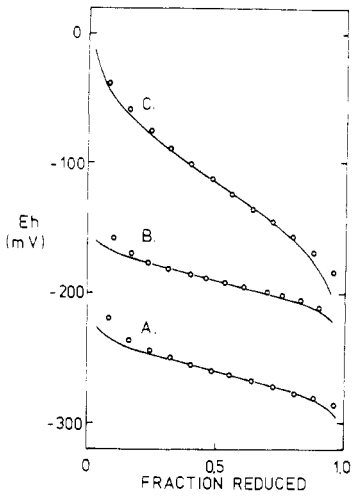


FIGURE 7: Potentiometric titration of NADH–cytochrome b_5 reductase. NADH–cytochrome b_5 reductase (86 μM), mediators, and indigodisulfonate (2 μM) plus safranine T (2 μM) in 3 mL of 0.1 M potassium phosphate buffer (pH 7.0) were titrated with dithionite (A), NADH (B), or dithionite in the presence of 2 mM NAD^+ (C). The solid lines drawn through the data points represent theoretical curves calculated for an $N = 2$ titration (curves A and B). For curve C, the theoretical curve was calculated for $K_s = 8$ (Clark, 1960).

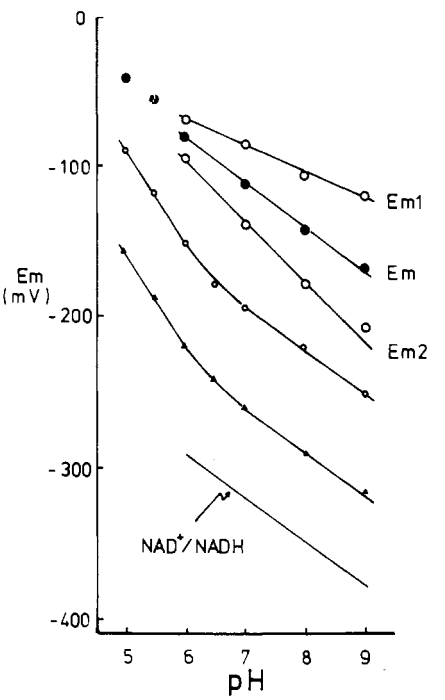


FIGURE 8: pH dependence of $E_{m,1}$ and $E_{m,2}$, midpoint potentials of two one-electron-transfer steps of flavin in NADH–cytochrome b_5 reductase. Individual $E_{m,1}$ and $E_{m,2}$ values at different pHs were calcualted from the K_s values presented in Figure 5B. The midpoint potentials of two electron-transfer steps, the redox couples E–FAD_{ox}/E–FAD_{red} [dithionite titration (△)] and E–FAD_{ox}/E–FAD_{red}–NAD^+ [NADH titration (○)], were measured as described in Iyanagi (1977).

corresponding double integral of the EPR signal of the air-stable semiquinone (FAD–FMNH) of NADPH–cytochrome P-450 reductase. The spin concentration was approximately 70% of the total flavin at 50% reduction. The value is in good agreement with the semiquinone formation constant (K_s), $K_s = [E\text{–}FAD_{sq}]^2/([E\text{–}FAD_{ox}][E\text{–}FAD_{red}]) = 8$, calculated from the titration curve shown in Figure 5B. These results indicate that the new peak at 375 nm is due to the formation of a free-radical species from enzyme-bound flavine. The microwave power saturation curve, the signal shape, and the line width of 16 G (data not shown) were very similar to those observed with the semiquinone of D-amino acid oxidase, which shows red-colored semiquinone anions (Palmer et al., 1971).

Semiquinone Formation during Oxidation by Oxidized Cytochrome b_5 of Reduced NADH–Cytochrome b_5 Reductase. 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. In fact, such an intermediate was observed when oxidized enzyme (77.5 μM) was mixed with equimolar NADH plus cytochrome b_5 in the stopped-flow apparatus (data now shown). The spin concentration of the intermediate obtained after mixing was about 60% of the total flavin. Therefore, at completion of the reaction, the one-electron-containing form predominated. In the presence of excess cytochrome b_5 (195 μM), the reduced enzyme was completely oxidized within the dead time of the instrument (about 20 ms) (data not shown). These results directly demonstrate that the reduced enzyme is oxidized by two successive one-electron transfers.

One-Electron Redox Potentials of NADH–Cytochrome b_5 Reductase. The oxidation–reduction potentials of NADH–cytochrome b_5 reductase were measured by direct potentiometry, titrating with dithionite or NADH (Iyanagi, 1977). The midpoint potential, $E_{m,7}$, was −258 mV ($N = 2$) by reduction with dithionite, and −160 mV ($N = 2$) by reduction with NADH. The observed data for NADH titration deviated at over 75% reduction for $N = 2$, but the observed data points for the enzyme used in the present study gave good curves between 0 and 100% reduction for $N = 2$, and the midpoint potential, $E_{m,7}$, was calculated from the titration data to be −194 mV (Figure 7B). The reason for this difference is not clear, but the enzyme used in the previous study may have contained some denatured protein which was removed in the preparation used in these experiments by 5′-ADP–agarose affinity chromatography (see Materials and Methods).

In the present study, the oxidation–reduction potentials of NADH–cytochrome b_5 reductase were measured by dithionite titration in the presence of excess NAD^+. The observed data points deviated from theoretical curves for $N = 2$, as shown in Figure 7, curve C. This is due to the formation of the semiquinone species, as predicted from Figures 4–6. The midpoint potentials for a one-electron redox couple of NAD^+-bound enzyme, E–FAD_{ox}/E–FAD_{sq} (with potential $E_{m,1}$) and E–FAD_{sq}/E–FAD_{red} (with potential $E_{m,2}$), can be calculated by the semiquinone formation constant and potentiometric methods (Clark, 1960). From the data obtained in Figure 5B, a value of 8 for the semiquinone formation constant (K_s) is estimated at pH 7.0, and the values of $E_{m,1}$ and $E_{m,2}$) are calculated from these two parameters, namely, K_s and E_m, on the basis of the following two equations: (i) $E_{m,1} - E_{m,2} = 60 \log K_s$ and (ii) $E_{m,1} + E_{m,2} = 2E_m$. The $E_{m,1}$ and $E_{m,2}$ values obtained at pH 7.0 were −88 and −147 mV, respectively. The potentiometric titration curve gave good fits for a semiquinone formation constant of 8 (Figure 7, curve C). In order to analyze one-electron redox potentials of the enzyme in the physiological pH range, experiments similar to those shown in curve C of Figure 7 were performed over a pH range from 5.0 to 9.0. Plots of $E_{m,1}$ and $E_{m,2}$ as a function of pH are shown in Figure 8. At pH 5.5 and 5.0, the enzyme solution was turbid during the titrations. Therefore, the values of $E_{m,1}$ and $E_{m,2}$ at pH 5.0 and 5.5 were not calculated. A 30 mV/pH unit slope for the oxidation–reduction potential of E_m was observed in the pH range 6.0–9.0. $E_{m,1}$ and $E_{m,2}$ can be assigned to the redox couples E–FAD–NAD^+/E–FAD^-·–NAD^+ and E–FAD^-·–NAD^+/E–$FADH^-$–NAD^+, re-

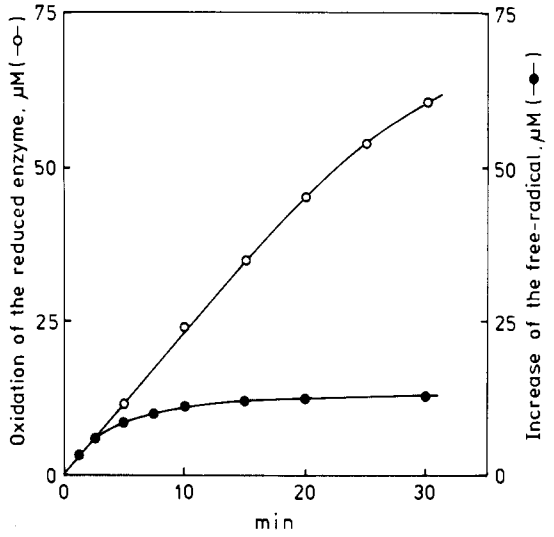


FIGURE 9: Reoxidation of NAD^+-bound reduced NADH–cytochrome b_5 reductase by air. NADH–cytochrome b_5 reductase, 86 μM in 0.1 M potassium phosphate buffer, pH 7.0, was reduced by a stoichiometric amount of NADH in the presence of air. The increase in absorbance at 462 nm (○) and the EPR signal (●) were measured in different cells under the same conditions.

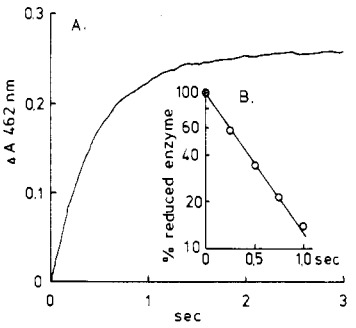


FIGURE 10: Reoxidation of NAD^+-free reduced NADH–cytochrome b_5 reductase by O_2. NADH–cytochrome b_5 reductase, 58 μM in 0.1 M potassium phosphate buffer, pH 7.0, was reduced by light irradiation in the presence of 5 mM EDTA and 1 μM 5-deazaflavin and was mixed with O_2 solution in a stopped-flow apparatus. Final concentrations were 29 μM enzyme, 0.6 mM O_2, and 0.1 M potassium phosphate buffer, pH 7.0. (A) Time course of enzyme oxidation; (B) first-order plot of the absorbance change at 462 nm.

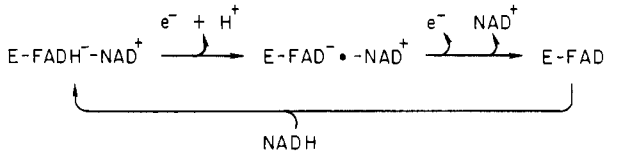
spectively, in the presence of high concentrations of NAD^+.

Reactivity with Oxygen of Reduced NADH–Cytochrome b_5 Reductase. When NADH-reduced enzyme was exposed to air, an increase in absorbance at 462 nm was observed and was accompanied by the appearance of a free radical as shown in Figure 9. A red-type semiquinone during the oxidation was observed, as judged from optical spectra (data not shown). The initial velocity of the oxidation of NADH-reduced enzyme was similar to that of the formation of the free radical, and a lag phase was not observed (Figure 9). The results indicate that a red-type semiquinone is indeed produced by direct oxygen oxidation. On the other hand, NAD^+-free reduced enzyme, which is reduced by light irradiation in the presence of 5-deazaflavin and EDTA (Massey & Hemmerich, 1978), was rapidly oxidized by oxygen, with a first-order rate constant of $k = 2.1\ s^{-1}$ (Figure 10). Monophasic reoxidation curves were observed at wavelengths of 462 and 375 nm, respectively.

Discussion

Cytochrome b_5 reductase used in the present studies was isolated in two forms different in molecular weight by 5K (Figure 1). The enzyme in vivo is bound to the endoplasmic reticulum, and when prepared in the lysosome-solubilized form, the hydrophobic anchoring portion is cleaved, making it water soluble (Crabb et al., 1980). The fact that no functional differences were detected between the detergent-solubilized enzyme, which contains the hydrophobic portion, and the lysosome-solubilized enzyme, which does not, suggests that the membrane-intercalated segment has a purely structural role.

Scheme I: Mechanism of NADH-Cytochrome b_5 Reduction Catalyzed by NADH-Cytochrome b_5 Reductase[a]



[a] Cytochrome b_5 is reduced by the electron (e^-).

NADH–cytochrome b_5 reductase is an enzyme containing one FAD molecule which catalyzes the transfer of electrons from NADH to cytochrome b_5:

$$NADH + 2cyt\ b_5(Fe^{3+}) \rightarrow NAD^+ + 2cyt\ b_5(Fe^{2+}) + H^+$$

The initial product of NADH reduction of the reductase is a charge-transfer complex in which NAD^+ is tightly bound (4×10^{-7} M) to the reduced flavoprotein (Iyanagi, 1977). This form of the enzyme reduces 2 equiv of the one-electron acceptor cytochrome b_5 in two successive steps (Strittmatter, 1965). In fact, the present study directly demonstrates that the two-electron-reduced enzyme ($E–FADH^-–NAD^+$) is oxidized via the flavosemiquinone species by cytochrome b_5. This indicates that NADH–cytochrome b_5 reductase flavin functions as a good converter from the two-electron donor NADH to the one-electron acceptor cytochrome b_5. The proposed mechanism for cytochrome b_5 reduction of NADH–cytochrome b_5 reductase is summarized in Scheme I.

Strittmatter (1965) reported that the intermediate observed during reoxidation of NAD^+-free reduced enzyme by ferricyanide showed the spectral characteristic of a blue (neutral semiquinone). On the other hand, this and previous studies (Iyanagi, 1977) demonstrate a red-type semiquinone. The optical spectrum of this species with a peak at 375 nm (Figure 5, curve 4) is very similar to that of the anionic red semiquinone of pyruvate oxidase (Mather et al., 1982), and a 16-G line width in the EPR signal shows the properties of anionic spectra (Palmer et al., 1971). The reductase is reduced to the fully reduced form by dithionite without forming a stable semiquinone in the absence of NAD^+ (Iyanagi, 1977). The semiquinone form of the enzyme is therefore stabilized in the presence of NAD^+. The discrepancy in semiquinone type between our studies and those of Strittmatter can be attributed to the interaction between NAD^+ and the semiquinone form (Iyanagi, 1977). However, preliminary studies using pulse radiolysis showed that a red semiquinone anion is formed by a one-electron reduction of the oxidized enzyme and similar spectra are observed at pH 7.0 in both the presence and absence of NAD^+ (K. Kobayashi, T. Iyanagi, and K. Hayashi, unpublished results). These data indicate that the semiquinone species observed in Figure 5 is not due to the rapid conversion from the blue to the red form but is directly formed on the one-electron oxidation of the fully reduced enzyme. Recently, a red anionic semiquinone was reported in yeast flavocytochrome b_2 (Capeillère-Blandin et al., 1975), succinate dehydrogenase (Edmondson et al., 1981; Ohnishi et al., 1981), and pyruvate oxidase (Mather et al., 1982), which are dehydrogenase electron-transfer flavoproteins. Furthermore, a quantitative estimate of spin concentration indicates that the

spectral species with a peak at 375 nm is a semiquinone itself (Figure 4). Thus, these data suggest that the two one-electron reoxidations of fully reduced enzyme (E–FADH$^-$–NAD$^+$) occur each with the loss of an electron and a proton as shown in Scheme I. Finally, reoxidation of the flavoprotein of a fully oxidized form (E–FAD) allows the dissociation of NAD$^+$ from the flavoprotein, as suggested by the much higher dissociation constant (2×10^{-4} M) for the interaction of NAD$^+$ and the oxidized enzyme (Figure 3).

The one-electron oxidation–reduction potentials, $E_{m,1}$ and $E_{m,2}$ of NADH–cytochrome b_5 reductase, were measured in the presence of excess NAD$^+$. Deviation of the curves from those predicted for a simple two-electron oxidation–reduction, $N = 2$, reflects the degree of stabilization of the semiquinone by NAD$^+$. The separation in redox potential ($E_{m,1} - E_{m,2}$) depends on the K_s value (Clark, 1960). At alkaline pH, the semiquinone form was more stable than at acid pH, and a large separation was observed as shown in Figure 8. Furthermore, the value of $E_{m,7}$, the couple of E–FAD/E–FADH$^-$–NAD$^+$, shifts from −195 to −115 mV in the presence of excess NAD$^+$ (Figure 7). If this change is due to semiquinone formation alone, the observed midpoint potentials should be unchanged, as pointed out by Michaelis (Clark, 1960). The shift is then due to the stabilization of the semiquinone state by the binding of NAD$^+$.

It is interesting to discuss the physiological relevance of a shift of the redox potential induced by NAD$^+$ binding to the reduced enzyme species and the separation of $E_{m,1}$ and $E_{m,2}$. Reduction with NADH produces the reduced flavoprotein–NAD$^+$ complex, in which the strong binding of NAD$^+$ to the reduced enzyme produces a more favorable free-energy change than that for reduction of flavoprotein in the absence of NAD$^+$ (Figure 7). Therefore, these changes are a driving force for the reduction of the enzyme by NADH, as discussed for adrenodoxin reductase by Lambeth & Kamin (1977). Transfer of an electron from E–FADH$^-$–NAD$^+$ to cytochrome b_5 produces the semiquinone species E–FAD$^{\cdot}$–NAD$^+$, and a further electron to a second cytochrome b_5 is supplied from E–FAD$^{\cdot}$–NAD$^+$ (Scheme I). The reduced enzyme species E–FADH$^-$–NAD$^+$ and E–FAD$^{\cdot}$–NAD$^+$ are stabilized by the binding of NAD$^+$. The free energy for the electron transfer from NADH to cytochrome b_5 is extremely favorable, as shown in Figure 11. However, a large oxidation–reduction potential gap between NADH/NAD$^+$ ($E_{m,7} = -320$ mV) and cytochrome $b_5(Fe^{3+})$/cytochrome $b_5(Fe^{2+})$ ($E_{m,7} = 0$ mV) is divided into smaller steps by several redox states of the enzyme-bound flavin, as shown in Figure 11, and the large activation energy would be decreased by each oxidation–reduction reaction of the enzyme-bound flavin. Each electron of NADH is transferred from the relatively constant oxidation–reduction potentials of enzyme-bound flavin to cytochrome b_5.

The reduction of flavin in NADH–cytochrome b_5 reductase is the rate-limiting step (Strittmatter, 1965), and the V_{max} of cytochrome b_5 reduction is relatively constant in the range of pH 6–9 (Figure 2). The differences in oxidation–reduction potentials between the NAD$^+$/NADH couple and the E–FAD$_{ox}$/E–FAD$_{red}$–NAD$^+$ couple are almost constant in the range of pH 6–9 (Figure 8). These observations suggest that there is a clear relationship between the difference in oxidation–reduction potential and the rate of electron transfer between these groups.

NADH–cytochrome b_5 reductase can catalyze one-electron transfer from NADH to various electron acceptors, such as ferricyanide (Strittmatter & Velick, 1957), quinones (Iyanagi & Yamazaki, 1969; Powis & Appel, 1980), and semidehydroascorbate (Iyanagi & Yamazaki, 1969). The reduced enzyme is also oxidized by oxygen. The NAD$^+$-free reduced enzyme is rapidly oxidized by oxygen with a first-order rate constant of $k = 2.1$ s^{-1} at pH 7.0 (Figure 10), while NAD$^+$-bound reduced enzyme is reoxidized very slowly by oxygen ($t_{1/2} \cong 20$ min at pH 7.0) as shown in Figure 9. A different reactivity for oxygen may be explained by the redox potential of each state. That is, the redox couple E–FAD/E–FADH$^-$, $E_{m,7} = -260$ mV, is capable of one-electron reduction of oxygen [$E_{m,7} = -270$ to -330 mV (Sawada et al., 1975)] whereas the redox couple E–FAD/E–FADH$^-$–NAD$^+$, $E_{m,7} = -194$ mV, is not (Figure 11). Powis & Appel (1980) have discussed a similar mechanism for quinone reduction by NADH–cytochrome b_5 reductase and showed that the one-electron oxidation–reduction potential is an important factor in the reduction of quinones by flavoproteins catalyzing one-electron transfer.

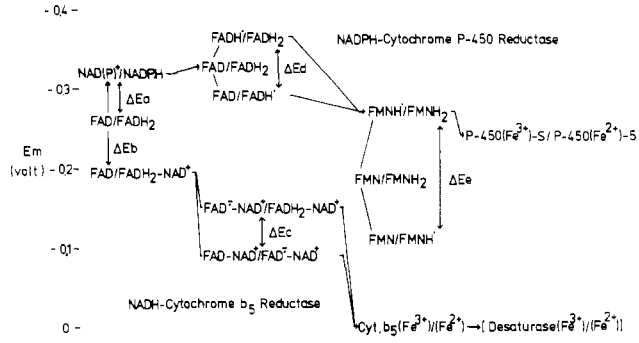


FIGURE 11: One-electron oxidation–reduction potentials (pH 7.0) of the components of hepatic microsomal electron-transport systems. Electrons are transferred from NAD(P)H to oxygenase, cytochrome P-450, and desaturase via flavoproteins. The values of midpoint potentials for half-reactions of FAD and FMN of NADPH–cytochrome P-450 reductase were determined by Iyanagi et al. (1974). Those values for NADH–cytochrome b_5 reductase are obtained from the data of the present study. The redox potential of substrate (hexobarbital)-bound cytochrome P-450 is determined to be $E_{m,7} = -237$ mV (Sligar et al., 1979), but that of desaturase is not determined. FAD and FMN represent the enzyme-bound flavins of NADH–cytochrome b_5 reductase and NADPH–cytochrome P-450 reductase, respectively.

Electron transfer from NADPH to cytochrome P-450 in liver microsomes has been shown to proceed via an FAD–FMN flavoprotein (Iyanagi et al., 1978; Vermilion et al., 1981) and does not require an iron–sulfur protein (Lu & Coon, 1968). From analysis of the spectrophotometric and potentiometric titration experiments (Iyanagi et al., 1974; Oprian & Coon, 1982), we have proposed that the two flavins have separate roles in catalysis; FAD serves as an electron acceptor from NADPH, and FMN participates as an electron carrier in the process of electron transfer from NADPH to cytochrome P-450. The difference in potential, $\Delta E_d = 75$ mV, between the couples FAD/FADH and FADH/FADH$_2$ with $E_{m,7}$ (Figure 11) is comparatively narrow, and FAD can participate in both one-electron and two-electron transfer (Massey & Hemmerich, 1978). On the other hand, the difference in redox potential, $\Delta E_e = 160$ mV, between the couples FMN/FMNH and FMNH/FMNH$_2$ (Figure 11) is enormously widened, and the couple FMNH/FMNH$_2$ can participate as a one-electron carrier (Mayhew & Massey, 1969; Iyanagi et al., 1981; Vermilion et al., 1981). In NADH–cytochrome b_5 reductase, the difference in redox potential between the couples FAD–NAD$^+$/FAD$^{\cdot}$–NAD$^+$ and FAD$^{\cdot}$–NAD$^+$/FADH$^-$–NAD$^+$ is $\Delta E_c = 60$ mV (Figure 11). Therefore, FAD in this enzyme can participate in both one-electron and two-electron transfer

similar to FAD in NADPH–cytochrome P-450 reductase, and cytochrome b_5 functions as a one-electron carrier to desaturase. The sequence of electron transfer for both systems is summarized as follows: two-electron donor → dehydrogenase → one-electron carrier → oxygenase → O_2.

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Registry No. NADH–cytochrome b_5 reductase, 9032-25-1.

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