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Nicotinamide-Dependent One-Electron and Two-Electron (Flavin) Oxidoreduction: Thermodynamics, Kinetics, and Mechanism

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1. Biological nicotinamide-dependent oxidoreduction consists of reversible $2e^-$ oxidoreduction of substrates. A mechanism involving subsequent $1e^-$ steps is shown to be very unfavourable due to the high energy of the nicotinamide radical.

2. Free energy relationships provide a convenient tool, allowing one to differentiate between hydride transfer and hydrogen atom transfer. It is concluded that biological nicotinamide-dependent, as well as flavin-nicotinamide oxidoreduction, proceed *via* hydride transfer but not *via* hydrogen atom transfer.

3. In flavin-nicotinamide oxidoreduction, flavin-nicotinamide charge transfer complexes are very likely the catalytic intermediates, preceding transfer of hydride ion. The energy of the long-wavelength charge transfer transition of zwitterionic oxidized-nicotinamide/reduced-flavin complexes is strongly dependent on polarity. It is maximal in a highly polar environment.

4. 5-Deazaflavins show the high thermodynamic radical instability of nicotinamides. They have to be considered as nicotinamide analog $2e^-$ oxidoreductants rather than flavin analogs, therefore, lacking the ability to catalyze reversible $1e^-$ oxidoreduction, essential for many flavoenzymes.

Studies on the mechanism of nicotinamide-dependent oxidoreduction so far have suffered from the fact that no experimental tools were available to distinguish between two major possible mechanisms: (a) rate-determining hydrogen atom transfer, followed or preceded by a fast one-electron transfer step [Eqns (1a, b)];

$$HNic_{red} + Acc \overset{fast}{\rightleftharpoons} Nic^+H^\cdot + Acc^{\cdot-} \overset{slow}{\rightleftharpoons} Nic^+_{ox} + AccH^- \quad (1a)$$

$$HNic_{red} + Acc \overset{slow}{\rightleftharpoons} Nic^\cdot + AccH^\cdot \overset{fast}{\rightleftharpoons} Nic^+_{ox} + AccH^- \quad (1b)$$

and (b) transfer of a hydride ion equivalent, by means of an ionic mechanism [Eqns (2a,b)] [1–4].

$$HNic_{red} + Acc \overset{slow}{\rightleftharpoons} Nic^+_{ox} + AccH^- \quad (2a)$$

$$HNic_{red} + Acc \overset{slow}{\rightleftharpoons} Nic_{red}\text{-}AccH \overset{fast}{\rightleftharpoons} Nic^+_{ox} + AccH^- \quad (2b)$$

Eqn (2a) describes direct transfer of a hydride ion from dihydronicotinamide to acceptor whereas Nic_{red}-AccH in Eqn (2b) symbolyzes a covalent (σ-type) intermediate between dihydronicotinamide and acceptor. Its fast heterolytic breakdown, involving simultaneous transfer of electrons *via* the covalent bond and transfer of a proton [4] would kinetically be indistinguishable from hydride ion transfer (simultaneous proton plus $2e^-$ transfer).

Model studies [5–11] have not so far provided conclusive answers as to which of the possible mechanisms is operating in reversible nicotinamide-dependent oxidoreduction, mainly because a detailed mechanistic study relating defined $1e^-$ and $2e^-$ dihydronicotinamide oxidation reactions has been lacking. Furthermore, knowledge of the $1e^-$ and $2e^-$ oxidation reduction equilibria involved has been scarce. Nicotinamide-dependent oxidoreduction of flavins has received special attention [12–20] because of its importance in many biological processes. Flavin-nicotinamide

Abbreviations. Fl_{ox} = flavoquinone; HFl$^\cdot$ = flavosemiquinone; H_2Fl_{red} = flavohydroquinone; Nic^+_{ox} = oxidized nicotinamide; Nic$^\cdot$ = nicotinamide radical; $HNic_{red}$ = 1,4-dihydronicotinamide; $MeNic^+_{ox}$/$HMeNic_{red}$ = *N*-methyl-1,4-dihydronicotinamide; $PrNic^+_{ox}$/$HPrNic_{red}$ = *N*-propyl-1,4-dihydronicotinamide; Acc = acceptor; NMR = nuclear magnetic resonance.

Enzymes. Lipoamide dehydrogenase (EC 1.6.4.3); 'Old Yellow Enzyme' or yeast NADPH dehydrogenase (EC 1.6.99.1); melilotate hydroxylase or melilotate, NADH : oxygen oxidoreductase (EC 1.14.13.4); alcohol dehydrogenase (EC 1.1.1.1); glyceraldehyde-3-phosphate dehydrogenase or D-glyceraldehyde-3-phosphate : NAD oxidoreductase (EC 1.2.1.12); glucose oxidase or β-D-glucose : oxygen 1-oxidoreductase (EC 1.1.3.4); catalase or hydrogen peroxide : hydrogen peroxide oxidoreductase (EC 1.11.1.6); xanthine oxidase or xanthine : oxygen oxidoreductase (EC 1.2.3.2); phosphodiesterase or oligonucleate 5′-nucleotidohydrolase (EC 3.1.4.1); D-amino acid oxidase or D-amino-acid : oxygen oxidoreductase (deaminating) (EC 1.4.3.3).

charge transfer complexes have been shown to arise during the reaction, but evidence has not been sufficient to determine whether they are precursors of oxidoreduction or whether they represent a side path.

In this paper the modes of $1e^-$ and $2e^-$ nicotinamide-dependent oxidoreduction are classified. The thermodynamics of the reaction have been investigated by means of potentiometry, polarography and spectrophotometry. Kinetics and free energy relationships of dihydronicotinamide oxidation by spirocyclohexylporphyrexide, as $1e^-$ acceptor, and by lumiflavin, as $2e^-$ acceptor, are reported. Furthermore, supporting evidence is presented that flavin-nicotinamide charge transfer complexes are precursors of oxidoreduction. The function and properties of these complexes are discussed.

EXPERIMENTAL PROCEDURE

Materials

Solvents and reagents were commercial products of the best available purity. NAD^+, NADH, phosphodiesterase, glucose oxidase and catalase were purchased from Boehringer, Mannheim; nicotinamide, 3-acetylpyridine and 3-ethylnicotinate from EGA, Steinheim. Lumiflavin was a gift from Hoffmann La Roche, Basel; 3-carboxymethyllumiflavin was synthesized according to Hemmerich [27] and 3-ethylcarboxymethyl-5,5-dichlorobarbituric acid was a gift from Dr W. R. Knappe, University of Konstanz, FB Biologie.

Syntheses

5-Deaza-10-methyl-3-sulfopropyl-isoalloxazine (Ia). 5 g 5-deaza-10-methyl-isoalloxazine, synthesized by the multistep synthesis of Weinstock *et al.* [21] were suspended in 300 ml of dry dimethylformamide. 30 g of K_2CO_3 and 27 g propanesultone [27] were added and the mixture was stirred at room temperature for one week. After this time nearly all starting material was converted to product (thin-layer chromatography in 1-butanol/CH_3COOH/H_2O, 5/2/3, by vol.). After addition of 50 ml of CH_3COOH, about 150 ml water were added cautiously to the suspension under vigorous stirring until solution took place. After standing at 4 °C over night, a precipitate formed. It was filtered and washed successively with 5-ml portions of water (three times) and several portions of ethanol to yield 7.2 g (93.7%) of compound Ia, as the potassium salt, containing traces of starting material. M.p. 274 °C. λ_{max} (ε) at pH 8, 0.1 M Tris (neutral species) 386 (9.8), 320 (9.5) nm ($mM^{-1}\,cm^{-1}$). NMR (^2H_2O) δ = 8.38 (5-H), 7.90–7.43 (6-H, 7-H, 8-H, 9-H), 3.75 (10-CH_3), 3.20–2.76, 2.35–1.70 [3-$(CH_2)_3$]ppm; *cf.* also [28] for ^{1}H-NMR of 3,10-dimethyl-5-deaza-isoalloxazine.

5-Deaza-1,5-dihydro-10-methyl-3-sulfopropyl-isoalloxazine (Ib). To a stirred solution of 387 mg compound Ia in 10 ml 0.25 M aqueous $NaHCO_3$, 870 mg of $Na_2S_2O_4$ was added in small portions over a period of 10 min. The solution turned dark red, a precipitate started to form and stirring was continued for 30 min. The mixture was kept at 4 °C over night. The pale yellow precipitate was collected and washed with small portions of water three times. Yield of desiccator-dried product was 290 mg (75%) contaminated by traces of starting material. Recrystallisation from water in the dark, containing 2 mM dithionite, results in a colourless product that is free of chromatographically or spectrophotometrically detectable traces of compound Ia. m.p. 256 °C. λ_{max} (ε) at pH 5, 0.1 M acetate (neutral species) 320 (14.1) at pH 9 (anion), 316 (11.5) nm ($mM^{-1}\,cm^{-1}$). NMR (^2H_2O) = 7.20–6.56 (4-H), 3.16 (5-CH_2), 2.93 (10-CH_3), 3.70, 2.84, 2.20–1.60 [3-$(CH_2)_3$] ppm; *cf.* also [28] for ^{1}H-NMR of 5-deaza-1,5-dihydro-3,10-dimethyl-isoalloxazine.

1,4-Dihydronicotinamide Derivatives IIb–IVb. Nicotinamide (II), ethylnicotinate (III) and 3-acetylpyridine (IV) were *N*-methylated with methyl iodide and converted to the corresponding chlorides as described by Suelter and Metzler [13]. The nicotinamidium salts could be isolated in almost quantitative yield after recrystallisation from ethanol. IIa: m.p. 237 °C (*cf.* [22]: 240 °C) λ_{max} (ε) pH 8, 0.1 M Tris (cation), 265 (4.25) nm ($mM^{-1}\,cm^{-1}$). IIIa: not crystalline, λ_{max} (ε) 265 (3.7) nm ($mM^{-1}\,cm^{-1}$). IVa: m.p. 198 °C; λ_{max} (ε) 265 (3.9) nm ($mM^{-1}\,cm^{-1}$). Reduction to the corresponding 1,4-dihydro derivatives with dithionite in Na_2CO_3 solution (IIa, IIIa) or $NaHCO_3$ solution (IVa) was achieved according to Wallenfels *et al.* [23]. In contrast to compound IIb, compounds IIIb and IVb cannot be extracted into $CHCl_3$. Hence they were extracted into 1-butanol (five extractions totalling 250 ml), the combined butanol extracts were dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to yield compounds IIIb and IVb as orange-brown oils. They could not be stored (even at −18 °C) without substantial decomposition to darkly coloured unidentified products. Hence, IIIb and IV had to be freshly prepared on the day of use. Spectrophotometric properties are very similar as those reported for the corresponding *N*-benzyl-dihydronicotinamides by Wallenfels *et al.* [23]. λ_{max} (ε) pH 8.0, 0.1 M Tris: IIb, 360 (7.2); IIIb, 362 (7.7); IVb, 376 (10.5) nm ($mM^{-1}\,cm^{-1}$). Monodeuterated IIb was obtained using ^2H_2O as solvent for reduction of IIa by dithionite.

Spirocyclohexylporphyrexide (VI). This was synthesized from cyclohexanone as described by Porter and Hellerman [29]. m.p. 189 °C (*cf.* [29] 177–180 °C) λ_{max} (ε) pH 4, 0.1 M acetate (cation) 400 (1.8);

pH 8, 0.1 M Tris (neutral species) 415 (0.73) nm (mM^{-1} cm^{-1}).

7,8-Dimethyl-10-[3-(3-carbamoyl-1-pyridinium)-propyl]isoalloxazine (VIII). This was synthesized from 1-(3-bromopropyl)nicotinamide and 4,5-dimethyl-*o*-phenylenediamine as described previously [19,25].

3-Carboxymethyl-7,8-dimethyl-10-[3-(3-carbamoyl-1-pyridinium)propyl]isoalloxazine (IX). 1.29 g *N*-[3-(3-carbamoyl-1-pyridinium)propyl]-4,5-dimethyl-*o*-phenylenediamine was added in portions to an anaerobic solution of 968 mg 3-ethylcarboxymethyl-5,5-dichlorobarbituric acid [26] in pyridine and the resulting solution was heated to reflux for 30 min. After cooling, pyridine was evaporated under reduced pressure, 150 ml of 6 N HCl was added and the resulting solution was heated to reflux for 2 h. HCl was evaporated under reduced pressure, the oily residue taken up in 100 ml H_2O, and Dowex 50 WX2 (30 ml) was added to absorb products containing the positively charged nicotinamides; the supernatant was discarded. A column was packed (2.5 × 20 cm) to a height of 5 cm with Dowex 50 WX2 resin on top of which the dark brown resin was packed that contained the absorbed nicotinamides. Elution with water yielded at first two slightly yellow unidentified precipitates, followed by the product which could easily be recognized by its greenish-yellow colour. The fractions showing an absorbance ratio $A_{445}:A_{368}$ of more than 1.1 were collected and evaporated to a small volume (10 ml). An orange precipitate formed which was collected after standing in the refrigerator for 12 h; yield 60 mg (3%) orange crystals of chromatographically pure Nic^+_{ox}-$(CH_2)_3$-Fl_{ox}-3-CH_2COO^- biscoenzyme (IX). m.p. 225 °C; λ_{max} (ε) pH 8.0, 0.1 M Tris (zwitterion) 445 (12.5), 368 (10.4) nm (mM^{-1} cm^{-1}). NMR: (^2H_2O) flavin protons: δ = 2.4 (7-CH_3), 2.56 (8-CH_3), 3.59 (10-CH_3), 4.51 (3-CH_2), 7.67 (6-H), 7.67 (9-H); nicotinamide protons: δ = 8.11 (5-H), 8.87 (4-H), 9.02 (6-H), 9.23 (2-H) ppm.

Instrumentation and Assays

^{1}H-NMR spectra were measured with a Varian A 60 or a Bruker HFX-90 spectrometer, operating temperature 296 K. Chemical shifts δ are given relative to tetramethylsilane as internal standard. Electronic spectra were recorded with a Cary 118C or Varian 635M spectrophotometer. Stopped-flow kinetics were obtained using a Gibson-Durrum stopped-flow spectrophotometer, equipped with a logarithmic amplifier and a Haake NBS R 22 thermostat unit.

Anaerobiosis of solutions was achieved by adding to 10-ml volumes in the stopped-flow reservoir syringe 50 µl of an aqueous saturated glucose solution, 10 µl of catalase (20 mg/ml) and 10 µl of glucose oxidase (24 mg/ml). Reactions were measured at 20 °C, in 0.01 M Tris-HCl buffer pH 8.0 containing 0.5 M KCl unless indicated otherwise. Rate constants k and dissociation constants K_d derived from these measurements represent the average of at least three experiments. The mean error was determined as ± 10%.

Dissociation constants of intermolecular $HNic_{red}$/Nic^+_{ox} complexes (at 420 nm) and Nic^+_{ox}/HFl^-_{red} complexes (at 500 nm) were determined spectrophotometrically: 2 ml of $HNic_{red}$ derivative or HFl^-_{red} derivative (2 mM) in the appropriate buffer (0.1 M) was titrated with small increments of Nic^+_{ox} solution (1 M). Dissociation constants were determined from plots of the inverse of Nic^+_{ox} concentration *versus* the inverse of the corresponding long-wavelength absorbance change.

Intramolecular complexes between reduced flavin and oxidized nicotinamide in compounds VIII and IX (0.1 mM) were prepared by anaerobically photoreducing the flavin in a Thunberg-type cuvette with a 5-fold excess of IIb. Illumination with a tungsten-halogen projector lamp (250 W/24 V) for 10 s produced the desired complex quantitatively. For ^{1}H-NMR investigations Nic^+_{ox}/HFl^-_{red} complex of IX was quantitatively obtained as follows: in an NMR test tube that had been sealed *in vacuo*, 0.5 ml of a solution containing deuterated phosphate (0.1 M, p^{2}H 7.0), oxalate (0.2 M), and compound IX (5 mM) in ^2H_2O was illuminated with the projector lamp for 60 min.

Formation of cyanide adduct for oxidized nicotinamide analogs was measured by difference spectrophotometry at 340 nm (compounds IIa–Va) or at 390 nm (compound Ia). To maintain constant ionic strength solutions contained 3 M KCl. A 0.1 mM solution (2 ml) of nicotinamide analog was titrated with a 3 M KCN solution. After each cyanide addition it took about 15 min till equilibrium was obtained. Spectrophotometric properties of *N*-methyl-nicotinamide cyanide adducts were identical with those reported by Wallenfels and Diekmann for the corresponding *N*-dichloro-benzyl derivatives [5]. λ_{max} (ε) II: 339 (7.6), III: 344 (8.3), IV: 355 (11.0), V: 342 (9.1) nm (mM^{-1} cm^{-1}). Cyanide adduct of the 5-deazaflavin derivative Ia does not absorb at 386 nm; it is characterized by two absorption peaks: λ_{max} (ε) 296 (9.7), 305 (9.8) nm (mM^{-1} cm^{-1}).

pK values were either determined spectrophotometrically (for compound VI) or by potentiometric titration (compound Ib). pH-dependence of the reaction of 5-deaza-1,5-dihydro-10-methyl-3-sulfopropyl-isoalloxazine (Ib) with lumiflavin (VII) was measured by following the decrease in absorbance at 450 nm. Solutions of the desired pH values were obtained by mixing 1 ml of an aqueous solution of compound Ib (5 mM) with 9 ml of the appropriate buffer. 1.5 ml of this solution was anaerobically mixed with 1.5 ml lumiflavin (0.05 mM).

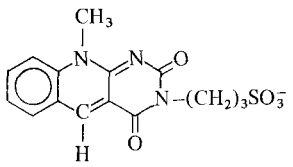
Half-wave potentials for $1e^-$ nicotinamide reduction at pH 7 were determined polarographically using a Metrohm E 354 instrument equipped with a model

E 261 recorder. A combined saturated Ag/AgCl or calomel electrode was used as reference against the dropping mercury electrode. The temperature was 20 °C. Midpoint potentials for $2e^-$ nicotinamide reduction were determined potentiometrically using a combined saturated calomel electrode and a Philips PW 9414 digital ion activity meter. Varying fractions of oxidized and reduced nicotinamide, (5 mM) total concentration in a 10-ml volume of Tris/HCl buffer (0.1 M, pH 8), were mixed to which 0.1 ml of an $HFMN^-_{red}$ solution had been added that was prepared by dithionite reduction of an FMN solution (0.1 M) in Tris/HCl buffer (0.1 M). After a constant potential reading was obtained, 0.5 ml of FMN solution (0.5 mM) was added anaerobically. If the potential increased after this addition equilibrium was proved to be reached and composition of the mixture was redetermined spectrophotometrically, diluting the solution anaerobically with anaerobic buffer. Alternatively, a solution of compound Ia (2 mM, 10 ml) in buffer (0.1 M, pH 8) containing FMN (0.4 mM) was anaerobically titrated with 1-ml increments of $Na_2S_2O_4$ solution (2 mM) in buffer (0.1 M, pH 8). Equilibration for every single step took about 30 min.

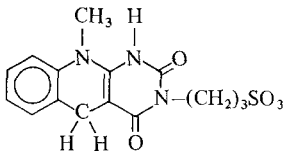
RESULTS

Syntheses

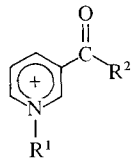
Flavin-nicotinamide oxidoreduction has been shown to follow saturation kinetics [20] due to flavin-nicotinamide complex formation, rates being linearly dependent on differences in oxidation reduction potentials. Hence, the dihydronicotinamide analogs used in this study were selected by three criteria: (a) high water solubility, in order to obtain significant amounts of the weakly associated intermolecular flavin-nicotinamide complexes; (b) variation in oxidation reduction potential, in order to measure free energy relationships; (c) variation in the ability to form complexes with acceptor molecules.



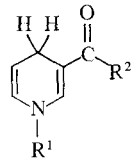
(Ia)



(Ib)



(IIa-Va)



(IIb-Vb)

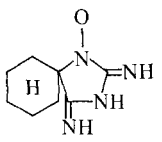
(II) $R^1=CH_3$, $R^2=-CO-NH_2$

(III) $R^1=CH_3$, $R^2=-CO-OC_2H_5$

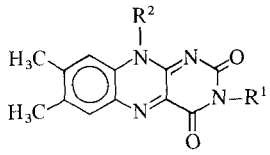
(IV) $R^1=CH_3$, $R^2=-CO-CH_3$

(V) R^1=Rib-*P*-*P*-Rib-Ade, $R^2=-CO-NH_2$

Derivatives Ib–Vb were found to possess the desired properties (see also Tables 6, 7).



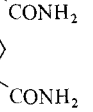
(VI)



(VII-IX)

(VII) $R^1=H$, $R^2=CH_3$

(VIII) $R^1=H$, $R^2=(CH_2)_3-N^+$ 

(IX) $R^1=CH_2-COO^-$, $R^2=(CH_2)_3-N^+$ 

Spirocyclohexylporphyrexide (VI) was selected as a $1e^-$ dihydronicotinamide oxidant because it has been shown to oxidize NADH rapidly with almost quantitative formation of NAD^+ and only minor formation of possible side products, such as NAD radical dimer, which is formed with a bimolecular rate constant of $2.4 \times 10^6\ M^{-1}\ s^{-1}$ [30] at pH 8.

Compound IX, a water-soluble derivative of the known N(3)-unsubstituted flavin-nicotinamide biscoenzymes VIII [19,25], could not be obtained by direct alkylation of VIII. Even under mild conditions, using dry dimethylformamide/K_2CO_3, a solution of VIII turned black within a few minutes and no starting material could be recovered. The total synthesis reported here is a variation of the well known Tishler synthesis [31]. Although compound IX was obtained only in a very poor yield, the described synthesis was the only successful one of three different approaches that have been investigated.

Complexes between Oxidized and $2e^-$-Reduced Coenzyme Analogs

Oxidation reduction equilibria of flavin coenzymes involve formation of charge transfer complexes between oxidized and reduced forms [32,33]. The rate of equilibration between Fl_{ox}/H_2Fl_{red} complex and flavin semiquinone as well as the magnitude of equilibrium constants involved are consistent with the interpretation that Fl_{ox}/H_2Fl_{red} charge transfer complexes are on the pathway of the dismutation reaction [34,35].

$Nic^+_{ox}/HNic_{red}$ complexes, like their flavin counterparts, could also be a means of catalyzing equilibration between oxidized and reduced forms; flavin-nicotinamide charge transfer complexes have been claimed as essential intermediates in flavin-nicotinamide oxidoreduction [19,20]. The characteristic physicochemical and structural properties of these complexes have therefore been investigated in some detail.

In Table 1 K_d values and long-wavelength absorption of oxidized and $2e^-$-reduced coenzyme analogs, as far as they could be determined experimentally, are summarized. Since thermodynamic stability of this type of complex is due to hydrophobic interactions, it should be roughly proportional to the overlap area between the individual compounds. $Nic^+_{ox}/HNic_{red}$ complexes, therefore, are the least stable ones. Fl_{ox}/HFl^-_{red} complexes are the most stable for the series reported and 5-deaza-Fl_{ox}/5-deaza HFl^-_{red} complexes are somewhat less stable than their flavin counterparts. All complexes show a broad long-wavelength absorption band, in addition to the absorptions of constituents, characteristic for a charge transfer transition. Energies are lowest for complexes with HFl^-_{red} as the donor molecule, increase when 5-deaza-HFl^-_{red} is substituted for HFl^-_{red} and further increase with $HNic_{red}$ as donor molecule. IIa/IIb and Ia/Ib complexes do not show a distinct charge transfer absorption maximum, because it is hidden under the long-wavelength chromophore of the constituents. Instead, long-wavelength tailing is observed, extending far into the visible region (Fig. 1). Similarly absorbing complexes have been observed by Ludowieg and Levi with NAD^+/NADH and $PrNic^+_{ox}/HPrNic_{red}$ complexes [36]. Thus, the dark orange colour initially observed when nicotinamides are reduced by dithionite, may arise not only from covalent sulfinate addition [37–39] but also from $Nic^+_{ox}/HNic_{red}$ complex formation. The lowest K_d values for lumiflavin-dihydronicotinamide analog complexes were obtained, as expected, for Ib (4 mM). Surprisingly, however, NADH forms a stronger complex than compounds IIb–IVb (Table 7).

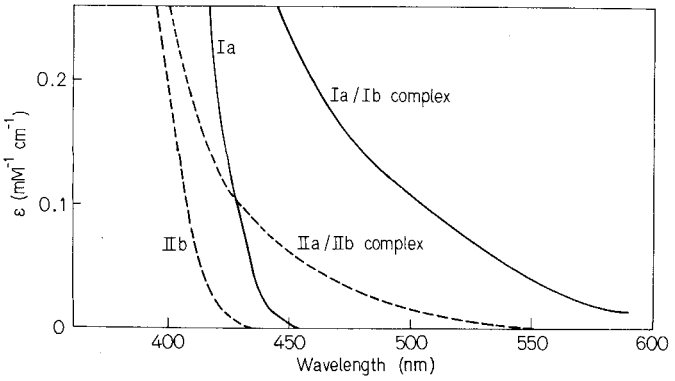


Fig. 1. *Long-wavelength absorption of oxidized $2e^-$ reduced nicotinamide analogs.* (----) IIa/IIb complex relative to IIb; total concentration of complex, 0.1 mM (0.1 M Tris/HCl pH 8.0). (——) Ia/Ib complex relative to Ia; total concentration of complex and Ia, 0.1 mM (0.1 M Tris/HCl pH 8.5)

The charge transfer transition energy of the zwitterionic Nic^+_{ox}/HFl^-_{red} complexes is strongly dependent on solvent polarity, in analogy to the results described by Kosower for the Nic^+_{ox}/I^- charge transfer transition [40]. Intermolecular Nic^+_{ox}/HFl^-_{red} complexes could not be investigated because the high concentrations needed for their formation could not be attained in non-aqueous solvents. Intramolecular Nic^+_{ox}/HFl^-_{red} complexes, however, can be measured at much lower concentrations. Results summarized in Table 2 show that the charge transfer transition energy of intramolecular Nic^+_{ox}/HFl^-_{red} complexes decreases with decreasing polarity (Fig. 2). However, complex formation (long-wavelength absorbance) could only be observed in protic solvents. No complex formation could be observed in the highly polar solvents dimethylformamide and acetonitrile but it could be observed in the less polar, but protic solvent cyclohexanol.

Table 1. *Properties of oxidized/$2e^-$-reduced coenzyme analog complexes*

Analog	pH	K_d	λ_{max}	Colour	Reference
		M	nm		
FMN/$FMNH^-$	8.9	$\approx 10^{-4}$	1010	greenish yellow	[32, 33, 35]
FMN/$FMNH_2$	2.4	$\approx 10^{-3}$	840	greenish brown	[32, 33, 35]
Ia/Ib	9.0	4×10^{-3}	< 500	red	this paper
Ia/Ib	4.0	4×10^{-2}	< 500	yellow	this paper
IIa/IIb	8.0	2.5×10^{-1}	< 500	orange	this paper
FMN/IIb	8.0	1.3×10^{-1}	575	green	[20]
Ia/IIb	8.0	1.7×10^{-1}	525	brownish red	[20]
Ia/$FMNH^-$	8.0	–	810	greenish brown	this paper
Ia/$FMNH_2$	4.0	–	640	green	this paper
IIa/$FMNH^-$	8.0	2×10^{-1}	505	brownish red	this paper

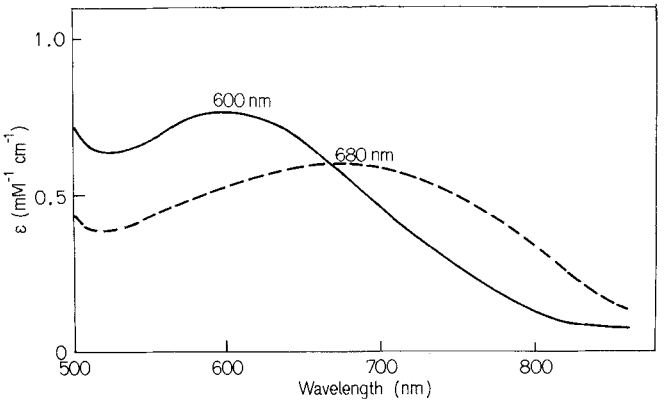


Fig. 2. *Long-wavelength absorption of intramolecular* Nic^+_{ox}/HFl^-_{red} *complex (0.07 mM).* (——) In butanol, (- - -) in 0.1 M Tris/HCl pH 8.0

Table 2. *Long-wavelength absorption maxima of intermolecular, intramolecular, and enzymic* Nic^+_{ox}-HFl^-_{red} *complexes*

Compounds	λ_{max}	Solvent	Reference
	nm		
$NAD^+/FMNH^-$	527	water	[68]
IX	550	water	[25]
VIII	600	water	this paper
VIII	630	formamide	this paper
VIII	635	methanol	this paper
VIII	670	ethanol	this paper
VIII	680	butanol	this paper
VIII	680	cyclohexanol	this paper
Lipoic dehydrogenase	700	water	[69]
Old yellow enzyme	600	water	[41]
Melilotate hydroxylase	750	water	[70]

In an attempt to obtain more information on the geometry of flavin-nicotinamide complexes, the water-soluble flavin-nicotinamide complex IX was synthesized, allowing ^{1}H-NMR spectroscopic analysis of complex relative to free components. Chemical shifts of flavin protons of IX (Nic^+_{ox}/Fl_{ox} complex) relative to those of its free flavin constituent 3-carboxymethyllumiflavin (Table 3) show that largest shifts are observed for methine protons at C-6 and C-9; methyl protons at positions 7α and 8α are shifted to a lesser degree and methylene protons at position 3α are virtually unshifted.

In the Nic^+_{ox}/HFl^-_{red} complex IX only the absorptions of methyl protons in flavin positions 7α (2.05 ppm) and 8α (2.14 ppm) were resolved well enough to allow determination of their chemical shifts. The differences relative to oxidized flavin (0.2 ppm) are in the range expected [42] indicating that no significant change in complex geometry occurred upon reduction of flavin.

Intramolecular $Nic_{red}H/Fl_{ox}$ complexes are very short-lived ($t_{0.5} < 10$ ms). Their geometry could therefore not be investigated by ^{1}H-NMR spectroscopy.

Table 3. ^{1}*H-NMR chemical shifts for flavin protons in flavin-nicotinamide biscoenzyme complex (IX)* (Fl_{ox}-Nic^+_{ox}) *relative to free 3-carboxymethyllumiflavin* (Fl_{ox}) *in deuterated phosphate, 0.1 M, pH 7*

Proton position at flavin	δ for		
	Fl_{ox}	Fl_{ox}-Nic^+_{ox}	Δδ
	ppm		
7-CH_3	2.278	2.400	+0.122
8-CH_3	2.411	2.555	+0.144
3-CH_2	4.522	4.511	−0.011
6-CH	7.377	7.666	+0.298
9-CH	7.455	7.666	+0.211

Addition of cyanide at nicotinamide C-4 should provide a more stable model complex which is isoelectronic to the $HNic_{red}/Fl_{ox}$ complex. However, attempts to synthesize the more stable isoelectronic complex Nic-CN/Fl_{ox} were unsuccessful.

Oxidation Reduction Potentials of Nicotinamide Analogs

Studies on oxidation reduction equilibria of nicotinamide analogs suffer from the fact that only the upper $1e^-$ shuttle (E_1) $Nic^+_{ox} \rightleftharpoons Nic^{\cdot}$ among the three redox shuttles of potential biological significance represents a reversible, pH-independent, redox system; $2e^-$ oxidoreduction (E_m) as well as the lower $1e^-$ shuttle (E_2) $Nic^{\cdot} \rightleftharpoons HNic_{red}$ involve transfer of hydrogen from a kinetically stable bond and thus represent, if uncatalyzed, pH-dependent oxidation reduction couples which are not rapidly equilibrated. Potentiometric determination of E_m therefore requires use of mediators, catalyzing equilibrium with the electrode and the equilibrium between oxidized and reduced nicotinamide.

Wallenfels and Diekmann [5] have shown that free energy changes in CN^- binding for different NAD^+ analogs, correspond well with their free energy change upon binding to alcohol dehydrogenase. Hence, the linear free energy relationship for CN^- binding can be used to calculate nicotinamide oxidation reduction potentials, thus providing an independent control of values obtained by potentiometric titration. Equilibrium and rate constants for CN^- addition to compounds Ia–Va were determined from the absorbance decrease at 390 nm (Ia) or the absorbance increase at 340 nm (IIa–Va) (Table 4). Since reaction rates do not increase linearly with CN^- concentration it is unlikely that a simple bimolecular mechanism according to $Nic^+_{ox} + CN^- \rightleftharpoons NicCN$ is operating. The biphasic nature of the NAD-CN complex formation (Table 4) further substantiates existence of a yet unknown intermediate. For these reasons, free energy relationships of CN^- dissociation, which should pro-

Table 4. *Nicotinamide analog equilibrium constants for CN^- binding and relative rates of formation of C(4)-CN adduct*
Aqueous solution of dihydronicotinamide analog (0.1 mM) was mixed with KCN solution (3 M)

Compound	K_a	Half-life
	M^{-1}	s
Ia	250	3
IIa	0.3	15
IIIa	0.2	15
IVa	60	17
Va	250	7 (slow phase) 0.02 (burst phase

Table 5. *Nicotinamide analog $2e^-$ oxidation reduction potentials*
E_m was determined directly by potentiometry (with 5 mM nicotinamide analog 1 mM FMN mediator, 0.1 M Tris-HCl buffer pH 8) or it was calculated from differences in CN^- binding (see also Table 6), based on a NAD^+/NADH potential (pH 8) of −350 mV [43]

Compound	E_m	
	potentiometrically	from CN^- binding
	mV	
Ia	−340	−350
IIa	−398	−436
IIIa	–	−441
IVa	–	−367
Va	−320	−350

vide a model for hydride ion transfer from 1,4-dihydronicotinamides, cannot be determined from the respective rates of association and equilibrium constants.

The potentiometrically measured differences in oxidation reduction potential between individual compounds and those calculated from cyanide binding agree very well; the deviation for compound II is probably caused by the instability of IIb, leading to products of higher oxidation reduction potential. However, to obtain correct values, as measured by Rodkey [43] or Burton and Wilson [44] (Table 6), NAD^+/NADH concentrations of at least 20 mM and 10 mM $FMNH^-$ are required. At lower nicotinamide (5 mM) and $FMNH^-$ (1 mM) concentrations, E_m values are obtained that are 30 mV lower than expected (Table 5).

Polarographic reduction of compounds Ia–Va at pH 8 is characterized by two $1e^-$ waves, showing a ratio in diffusion current of 1 : 2 (Fig. 3). The wave at higher potential corresponds to radical formation, the one at lower potential probably to reduction of radical dimer. At pH 10 polarographic reduction of compound Ia shows only one wave (Fig. 3) showing an increased slope and diffusion current relative to the high-potential $1e^-$ wave at lower pH. This change in mechanism, possibly radical disproportionation in favour of dimerization, may be caused by N(1)-deprotonation of Ib radical.

E_1 potentials of nicotinamide analogs Ia–Va are characteristically high in energy (−640 mV up to −860 mV) reflecting the high thermodynamic instability of the respective radical states (Table 6). Due

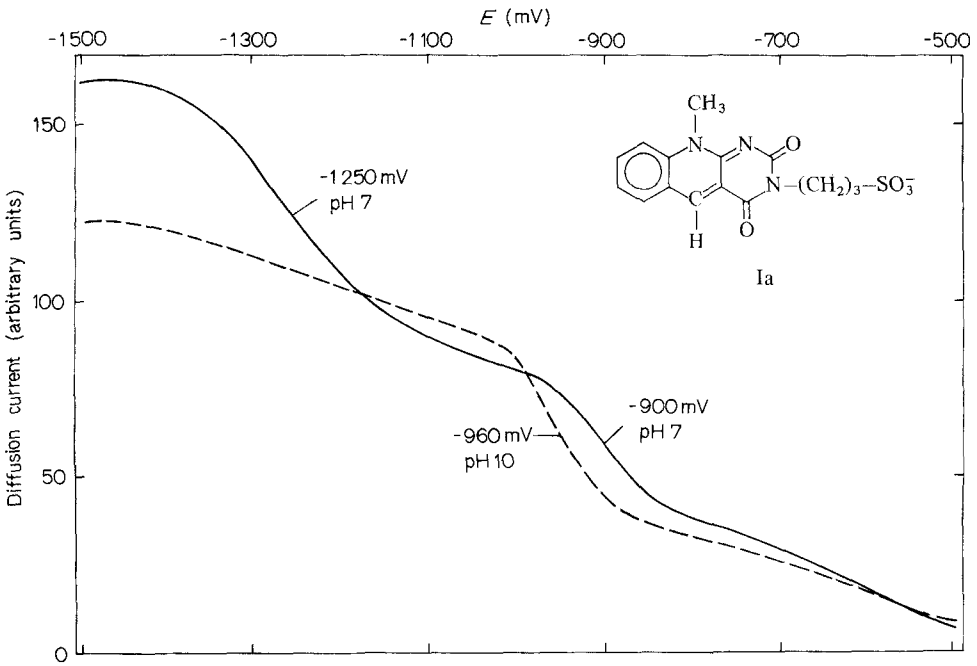


Fig. 3. *Polarographic reduction of compound Ia (0.05 mM).* (——) 0.1 M phosphate pH 7.0; (– – –) 0.1 M borate pH 10; a mercury dropping electrode was used against a saturated calomel electrode as reference

Table 6. *$1e^-$ and $2e^-$ oxidation reduction potentials (E_0', pH 7) of coenzyme analogs*
E_1 values were determined polarographically, E_2 values were calculated from the differences between E_1 and E_m values; E_m values were determined potentiometrically and calculated from CN^- equilibrium data, based on a value for NAD^+ of -320 mV [43]. Since E_1-potentials are kinetic potentials, the listed values for E_1 and E_2 represent upper and lower limits respectively, relative to the corresponding thermodynamic oxidation reduction potentials

Compound	E_1	E_2	E_m
	mV		
Ia	− 650	+ 10	− 320
IIa	− 845	+ 33	− 406
IIIa	− 860	+ 38	− 411
IVa	− 640	− 34	− 337
Va	− 730	+ 90	− 320
FMN	− 238	− 172	− 205

to dimerization of the respective radicals [30] kinetic E_1-potentials rather than thermodynamic potentials are obtained. Values for thermodynamic E_1-potentials are either equal to or more negative than the respective values for kinetic E_1-potentials, depending on the rates of radical dimerization. Furthermore, the differences in electron affinity between compounds Ia–Va can clearly be seen from the respective differences of the corresponding E_1 potentials.

Kinetics of $1e^-$ Dihydronicotinamide Oxidation

Our results of $1e^-$ oxidation reduction potentials of nicotinamides show that an efficient $1e^-$ oxidant for dihydronicotinamide oxidation should possess an oxidation reduction potential of +100 mV. Furthermore, reactivity of the $1e^-$ oxidant with nicotinamide radical should be very high to compete effectively with radical dimerization. Only if this condition is met can quantitative formation of *N*-alkyl nicotinamide cation be expected rather than a mixture of dihydronicotinamide oxidation products. Ferric ion (E_0' = + 430 mV) [24, 45, 46] does not show these required properties, in contrast to spirocyclohexylporphyrexide (VI) (E_0' = +690 mV). Since compound VI absorbs at 500 nm it has the further advantage that its reduction by dihydronicotinamide analogs Ib–Vb can be followed at this wavelength. Oxidation of compounds IIb–Vb could be followed by the decrease in absorbance at 350 nm, that of compound Ib by increase in absorbance at 390 nm due to formation of Ia. The reaction proved to be first order in both $HNic_{red}$ and spirocyclohexylporphyrexide in the concentration range that could be investigated (0.1–2 mM). At higher concentrations reaction was too fast to be followed by the stopped-flow method. Complex formation (saturation kinetics) between dihydronicotinamide analogs Ib–Vb and VI prior to oxidoreduction could not therefore be investigated. Rate constants were identical if calculated from stopped-flow traces at either 350 nm or 500 nm; they are summarized in Table 7.

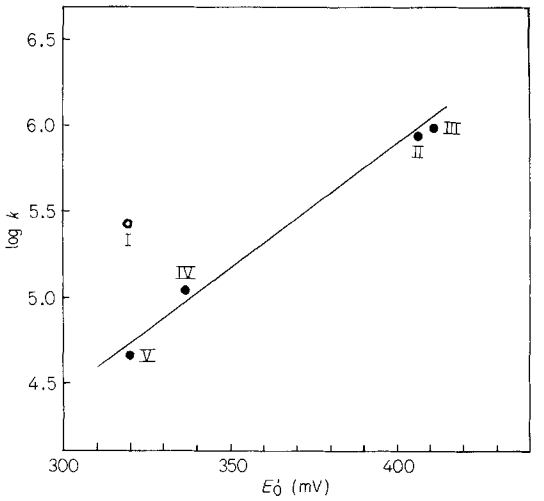


Fig. 4. *Free energy relationship for the $1e^-$ oxidation of analogs by spirocyclohexylporphyrexide (VI).* (See also Table 7.) Experimental conditions: 0.04 mM $HNic_{red}$ analog in 0.1 M Tris/HCl pH 8.0, containing 0.5 M KCl was mixed with 0.4 mM compound VI, 0.1 M Tris/HCl pH 8.0, containing 0.5 M KCl. k has the units of $M^{-1}\,s^{-1}$

Table 7. *Second-order rate constants for $1e^-$ oxidation of dihydronicotinamide analogs by spirocyclohexylporphyrexide; first-order rate constants for $2e^-$ oxidation by lumiflavin and dissociation constants of the respective flavin-dihydronicotinamide analog complexes*

Compound	k for		K_d of lumiflavin complex
	$1e^-$ oxidation	$2e^-$ oxidation	
	$M^{-1}\,s^{-1}$	s^{-1}	M
Ib	2.7×10^5	0.3	0.004
IIb	9.1×10^5	7.0	0.1
IIIb	9.5×10^5	10.0	0.1
IVb	1.1×10^5	0.3	0.1
Vb	4.55×10^4	0.02	0.025

NADH oxidation is not sterically hindered by intramolecular nicotinamide/adenine complexing. No rate increase could be observed when the adenine diphosphate moiety of NADH was cleaved by phosphodiesterase. The free energy relationship log k *versus* E_0' is linear for compounds IIb–Vb, whereas Ib reacts significantly faster than expected from its oxidation reduction potential (Fig. 4). A difference of 68 mV in nicotinamide oxidation reduction potential causes a change in rate of about 10-fold. Hydrogen transfer is rate-limiting as demonstrated from the primary isotope effect k_H/k_{2H} of 1.5 obtained for compound IIb monodeuterated at C-4, corresponding to a

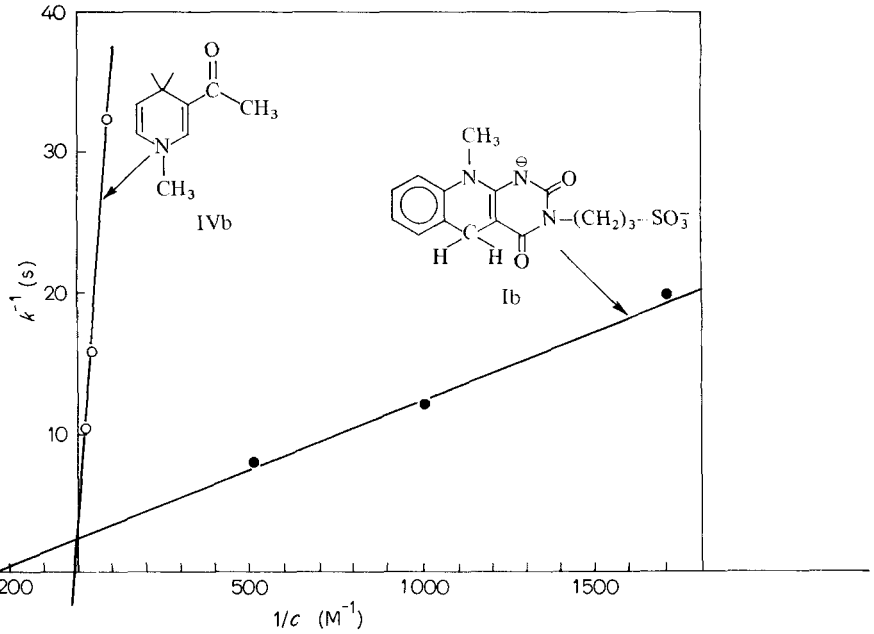


Fig. 5. *Plot of the inverse of the observed first-order rate constant of lumiflavin reduction (0.1 mM, 0.1 M Tris/HCl pH 8, containing 0.5 M KCl)* versus *the inverse $HNic_{red}$ analog concentration*, c

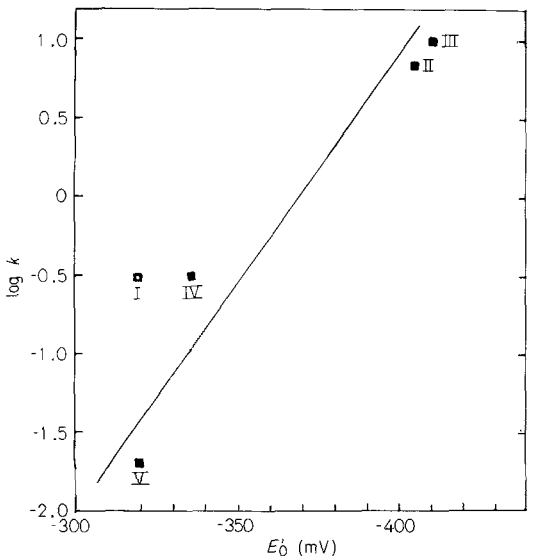


Fig. 6. *Free energy relationship for the $2e^-$ oxidation of $HNic_{red}$ analogs by lumiflavin.* (See also Table 6.) Experimental conditions were as cited in Fig. 4

value of 3 + 0.5 for dideuterated IIb. This value is very similar to that found for nicotinamide-dependent flavin reduction [13] or the enzyme-catalyzed reduction of NAD^+ by 1,5-dihydrodeazariboflavin [47]. The Arrhenius activation energy of NADH--$1e^-$ oxidation by compound VI has been determined as 7.5 ± 0.5 kcal/mol (31.4 ± 2.1 kJ/mol), which is very similar to the value reported for NADH oxidation by FMN [14,15].

Kinetics of $2e^-$ Dihydronicotinamide Oxidation

Oxidation of dihydronicotinamide analogs Ib–Vb by lumiflavin shows saturation kinetics (Fig. 5). As in $1e^-$ $HNic_{red}$ oxidation a linear free energy relationship is obtained, reaction of 1,5-dihydro-5-deazaflavin anion also being much faster than expected for a neutral dihydronicotinamide of the same potential (Fig. 6). A difference of 33 mV in nicotinamide oxidation reduction potential causes a rate change of about 10-fold. Hydrogen transfer is rate-limiting [7,13] and Arrhenius activation energies are 8.3 ± 0.5 kcal/mol (34.7 ± 2.1 kJ/mol) for NADH [14,15], 5.8 ± 0.5 kcal/mol (24.3 ± 2.1 kJ/mol) for IIb and 3.9 ± 0.5 kcal/mol (16.3 ± 2.1 kJ/mol) for Ib.

Reaction rates of Ib oxidation are pH-dependent due to ionization at N(1). From the pH-dependence of lumiflavin reduction rates a pK value of 7.4 is obtained which is somewhat more acidic than the value of 7.6 obtained by potentiometric titration. Neutral Ib reacts at least 100 times more slowly than its anion.

DISCUSSION

Oxidation Reduction Potentials

$2e^-$ oxidation reduction potentials of nicotinamides can be determined potentiometrically with flavin as mediator. Due to the low stability of Nic^+_{ox}/$HNic_{red}$ and flavin/nicotinamide complexes, which are considered as the equilibrium species, correct potentials are only obtained at rather high flavin and nicotinamide concentrations. Data reported in the

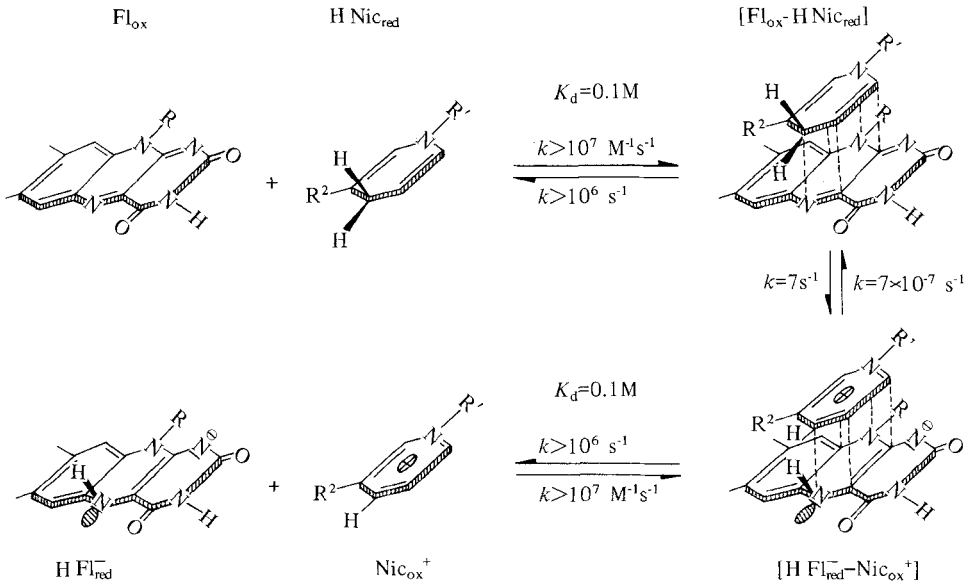
literature were either determined from free energy changes, with yeast alcohol dehydrogenase [44], or by potentiometry, with xanthine oxidase and benzyl viologen as mediators [43]. Equilibration times in potentiometric experiments are quite long (up to 43 h). Hence, oxidation reduction potentials calculated from free energy changes appear to be more reliable, in view of the instability of dihydronicotinamides. Polarographic results obtained for NAD^+ and $MeNic^+_{ox}$ potentials are in good agreement with values reported in the literature [30,48–50]. Polarographic reduction of 5-deazaflavin (Ia) at pH 8 follows the same pattern as that of nicotinamides. The change in the reduction pattern observed at pH 9 is probably due to N(1) deprotonation of the radical. Thus the pK_a of 5-deazaflavin semiquinone can be estimated as 8.5 ± 0.5.

In contrast to nicotinamide E_1 potentials, which are well outside the range of naturally occurring $1e^-$ reductants, the corresponding E_2 potentials are within the physiological range. Consequently, $1e^-$ NADH oxidation by a variety of $1e^-$ oxidants is feasible (see also below). $1e^-$ oxidation by oxygen, however, in contrast to the $1e^-$ oxidation by $\dot{O}_2^-$-radical, is thermodynamically unfavourable since the $O_2 \rightleftharpoons \dot{O}_2^-$ oxidation reduction potential has been determined as $-330/-330$ mV [51].

Flavin-Nicotinamide Charge Transfer Complexes

The evidence reported in this investigation supports a catalytic role of flavin-nicotinamide charge transfer complexes: (a) for two analogs (Ib and IVb) showing identical reaction rates at infinite reductant concentration, rates at finite reductant concentration were found to be proportional to their complex stability with flavin (Fig. 5, Table 7); (b) the pK value for 5-deaza H_2Fl_{red}, obtained from kinetic measurements (pH-dependence of lumiflavin reduction) was found to be slightly more acidic than that obtained by potentiometric titration. We have shown that dihydroflavin acidity in intramolecular Nic^+_{ox}/HFl^-_{red} complexes increases with increasing proximity of the nicotinamidium ion. Consequently, the lower pK value for 5-deaza-H_2Fl_{red} had to be expected, representing dissociation of the proton at N(1) in the active complex with oxidized flavin. Thus, dihydronicotinamide charge transfer complexes may be precursors of both $1e^-$ oxidoreduction by ferric ion [45,46] and $2e^-$ oxidoreduction by flavin, depending only on the type of acceptor present in the oxidation reduction process.

The most striking physical property of enzymic Nic^+_{ox}/HFl^-_{red} complexes consists of the large variation of their long-wavelength charge transfer transition ($\lambda_{max} = 600-800$ nm) [41]. Our results demonstrate that these differences do not only reflect differences in oxidation reduction potential but also reflect differences in polarity of the environment. If one accepts that flavin-nicotinamide complexes linked by three methylene groups through positions N(1) of nicotinamide and N(10) of flavin are good models for the respective enzymic species [19,41], possible geometries of the complex become limited. The experiments reported here do not agree with a conformation where the pyrimidine subnucleus of flavin and the nicotinamide are in close proximity, because the methylene protons in position N(3)α are not shifted relative to free flavin. The conformation proposed in Scheme 1 does agree with the evidence presently available; however, its validity awaits further experimental proof. An X-ray structural determination is presently under way (David Porter, personal communication).



Scheme 1. *Proposed mechanism for flavin-nicotinamide oxidoreduction.* Data are given for IIb/FMN reaction [20]

Free Energy Relationships and the Mode of Hydrogen Transfer in Nicotinamide-Dependent Oxidoreduction

Oxidation of NADH analogs by two subsequent $1e^-$ steps yielding a stoichiometric amount of NAD^+ has been described for ferricyanide [22], ferric ion [45,46], spirocyclohexylporphyrexide [24], Br_2^- radical [53], hyperoxide radical [54] and recently an enzyme-catalyzed stereospecific hydrogen atom transfer from NADH to a dicarboxylate radical [55] has been reported. The obvious question, therefore, is whether the above-cited examples of nicotinamide-dependent $1e^-$ transfer are chemical artefacts of only limited or no biological importance or whether the Michaelis concept [56] of $1e^-$ oxidoreduction applies obligatorily in biological redox systems. The biological importance of $1e^-$ oxidoreduction has very recently been strongly emphasized, mainly by the laboratories of Kosower [57], Bruice [58] and Walsh [47].

In the present investigation, free energy relationships of defined $1e^-$ and $2e^-$ dihydronicotinamide analog oxidation reactions were investigated, because it could be expected that they may provide the desired answers. From the basic free energy equations

$$\Delta G = -RT \ln K \quad \text{and} \quad \Delta G = -n\,E\,F$$

one obtains the equation which is the basis of the present investigation:

$$E = \frac{RT}{nF} \ln K$$

Since $K = k_1/k_{-1}$ and $\frac{RT}{F} \ln 10 = 0.059$ V at 20 °C,

this can also be written as

$$E = -\frac{0.059}{n} (\log k_1 - \log k_{-1})$$

where E represents the difference in potential between donor and acceptor, K the corresponding overall equilibrium constant, k_1 and k_{-1} the corresponding rate constants. Independent of mechanism, a linear free energy relationship for $n = 2$ has to be obtained with a slope changing by 30 mV per unit of log K, when dihydronicotinamide potentials (or their difference to lumiflavin potential) are plotted *versus* the logarithm of the overall equilibrium constant. This condition is found to be fulfilled for dihydronicotinamides reported here. Keeping in mind that for $1e^-$ and $2e^-$ dihydronicotinamide oxidation a hydrogen species is transferred in the rate-limiting step, it becomes obvious that a linear free energy relationship of log k_1 *versus* E may allow one to distinguish between H^- transfer and H· transfer, as follows.

a) If the energy of the transition state is relatively independent of nicotinamide substituent effects, the different energies of the nicotinamide ground states

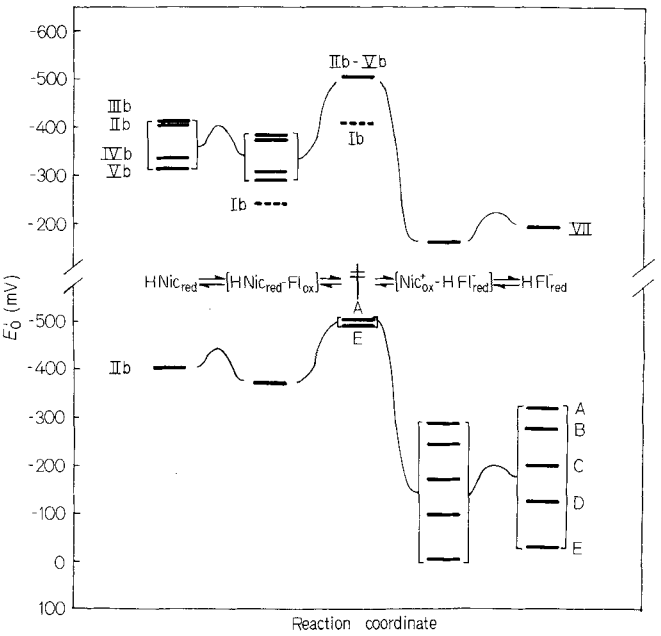


Fig. 7. *Free energy diagrams illustrating flavin nicotinamide oxidoreduction.* Dependence on nicotinamide free energy changes is shown in the upper part. Free energy differences of free nicotinamides are conserved upon formation of the respective charge transfer complexes (indicated by square brackets) with lumiflavin with the exception of compound Ib. The energy of the transition state is constant, again with the exception of Ib. Thus, with the exception of Ib, differences in log K are conserved in differences of log k_1, the reverse reaction being independent of nicotinamide free energy changes. Dependence on flavin free energy changes is shown in the lower part. Although free energy differences are conserved upon complex formation with nicotinamide, differences in log K are not conserved in differences of log k_{-1} because the energy of the transition state is increasingly stabilized with increasing flavin oxidation reduction potential. Data are taken from [20]: (A) 10-methyl-5-deazaisoalloxazine; (B) 8-nor-8-morpholinolumiflavin; (C) lumiflavin (VII); (D) *S*-methyl-2-thiolumiflavin; (E) 8α-oxo-3-methyllumiflavin

will lead to different free energies of activation (Fig. 7). A linear free energy relationship will result, with a slope of 30 mV per unit of log k_1 for H^- transfer and 60 mV per unit of log k_1 for H· transfer. The respective rates k_{-1} for the reverse reactions are consequently found to be almost independent of changes in nicotinamide oxidation reduction potential.

b) However, if the energy of the transition state depends to a significant extent on dihydronicotinamide substituents, rate changes will differ from 30 mV per unit of log k_1 and consequently the respective rates for the reverse reactions will no longer be independent of changes in nicotinamide oxidation reduction potential.

The linear free energy relationship obtained from dihydronicotinamide oxidation by nitroxide radical (VI) shows a slope of 68 mV per unit of log k_1, while the respective value for lumiflavin (VII) shows a slope of 33 mV. Rate changes for the corresponding reverse reactions are almost independent of changes in

nicotinamide potentials. These values are very close to theoretical values, demonstrating that nicotinamide substituents in positions N(1) and C(3) do not significantly influence the energy of the transition state. Furthermore, it is demonstrated that dihydronicotinamide oxidation by lumiflavin does not proceed *via* hydrogen atom transfer but rather by a hydride ion transfer mechanism.

5-Deaza-$Fl_{red}H^-$ (Ib) reacts significantly faster than expected for dihydronicotinamides of the same oxidation reduction potential (Fig. 5 and 7). This catalytic effect reflects the increased lability of the C(5)-H bond involved in oxidoreduction, due to deprotonation at N(1). Upon protonation, reduction rates are decreased at least 100-fold.

Flavin substituents, in contrast to nicotinamide substituents, do significantly influence the energy of the transition state of flavin-nicotinamide oxidoreduction. With increasing flavin oxidation reduction potential, energy of the transition state is increasingly stabilized (Fig. 7) leading to increased oxidation reduction rates [20]. Consequently it takes a larger difference than 30 mV to effect a 10-fold rate change. The observed difference of 40 mV per unit of log k_{-1} still does not deviate from the expected value for a transition state energy independent of substituents to such an extent that a mechanism other than H^- transfer has to be considered.

The evidence leading several authors to propose that nicotinamide-dependent oxidoreduction might proceed by subsequent $1e^-$ steps will therefore be discussed at this point. Williams *et al.* [58] have shown that $2e^-$ reduction of formaldehyde and related carbonyl compounds by $Fl_{red}H^-$ might well proceed *via* an $H^\cdot$ transfer mechanism. Based on calculations showing that free energy changes involved in $1e^-$ carbonyl reduction by $Fl_{red}H^-$ were consistent with $H^\cdot$ transfer, they concluded that carbonyl reduction by dihydronicotinamide might proceed by the same mechanism. They did not take into account, however, that nicotinamide radicals are much higher in energy than flavin radicals, the difference in E_1 being about 490 mV (see Table 6). Hence, $1e^-$ alcohol reduction by oxidized nicotinamide is thermodynamically less favourable by 10^8-fold than the respective flavin reaction, eliminating this type of mechanism as a feasible route.

Fisher *et al.* [47] base their arguments on the finding that several flavoenzymes substituted with 5-deazaflavin coenzyme analogs still catalyze electron transfer. They take this result as 'strong circumstantial evidence for an identical mechanism operating, not only between deazaflavins and flavins but as well between flavins and dihydronicotinamides'. $H^\cdot$ transfer was suggested as the most feasible route for dihydronicotinamide-deazaflavin and dihydronicotinamide-flavin reduction. This postulate does not take into account that flavoenzymes, substituted by deazaflavins, only catalyze $2e^-$ oxidoreduction, but no $1e^-$ transfer reactions, as demonstrated by Fisher *et al.* themselves and by other workers [47, 59, 60]. The real reason for this behaviour becomes evident if one compares the respective oxidation reduction potentials: $2e^-$ oxidation reduction potentials differ by about 120 mV (Table 6), hence $2e^-$ oxidoreduction may still proceed when deazaflavin is substituted for flavin, although significantly higher substrate concentrations will be needed to achieve full reduction of coenzyme. $1e^-$ oxidation reduction potentials differ by about 420 mV in E_1, a difference that will not be smaller in the enzyme-bound state (see also below). Changes in oxidation reduction potential upon binding should point in the same direction for both coenzyme and coenzyme analog and there is no reason to believe that changes might be larger for the latter one. Thus, the equilibrium position for $1e^-$ oxidoreduction is 10^7-fold less favourable in deazaflavins relative to flavin. Results reported in this investigation as well as those reported by Fisher *et al.* should not be taken as evidence that similar mechanisms operate in flavin, deazaflavin, and dihydronicotinamide oxidoreduction. On the contrary, the evidence suggests that dihydronicotinamide and deazaflavin oxidoreduction operate by similar $2e^-$ processes in contrast to flavin which may participate in both $1e^-$ and $2e^-$ oxidoreduction. Similar conclusions as those reported here have also been drawn by Hemmerich [52] and Fenner *et al.* [61]. The high energy of nicotinamide radicals also is the cause for the absence of direct interaction of nicotinamides with $1e^-$ oxidoreductants in biological redox systems. In photosynthetic or mitochondrial electron transport flavin is the compulsory mediator because both its $1e^-$ and $2e^-$ oxidation reduction shuttles are in the physiological range [62].

Radical Stabilization in the Enzyme-Bound State

The question of whether reversible nicotinamide-dependent $1e^-$ oxidoreduction can be biologically important may be reduced to the question of whether proteins can stabilize nicotinamide radicals sufficiently such that both E_1 and E_2 are in the physiological range, a question that also applies to deazaflavins. Flavodoxin from *Azotobacter* has been shown to stabilize FMN radicals by shifting E_2 by -250 mV relative to free FMN thus producing a thermodynamically stable radical state [59]. Deaza-FMN bound to flavodoxin, however, does not show a stable radical. Knowing deazaflavin $1e^-$ oxidation reduction potentials (Table 6), this result would have been predictable: in order to obtain a stable semiquinone a shift of E_2 by at least -350 mV, at constant E_m, would have been necessary. In order to obtain thermodynamically stable NAD radicals an even larger shift in E_2, by about -400 mV would be necessary. No evidence has been reported so

far indicating that such a large shift could be effectuated by an apoprotein.

One apparent exception, a 'stable' deazaflavin radical bound to D-amino acid oxidase, has very recently been reported [63]. It was obtained by irradiating deaza-FAD, bound to the oxidase, in the presence of EDTA. The interpretation by the authors, that deazaflavin semiquinones are potential intermediates in deazaflavoprotein-catalyzed reactions, cannot be accepted on the basis of their evidence. The fact that deazaflavin semiquinone could not be observed on dithionite reduction of the enzyme, a reaction that is known to proceed *via* $1e^-$ transfer in flavoenzymes [64, 65], indicates that dithionite is not a strong enough $1e^-$ reductant (E_0' around -440 mV) to reduce deazaflavin (E_0' in the unbound state -650 mV, Table 6). At the same time, however, the enzyme-bound deazaflavin is $2e^-$ reduced by dithionite ($E_0' = -527$ mV), demonstrating the ambivalent nature of $S_2O_4^{2-}$ as a $1e^-/2e^-$ reductant: with flavin it reacts preferably by the $1e^-$ mode, with deazaflavin and nicotinamides in the $2e^-$ mode (G. Blankenhorn and P. Hemmerich, unpublished observations).

The data provided by Hersh *et al.* establish convincing evidence against $1e^-$ transfer, showing that deazaflavin semiquinone bound to D-amino acid oxidase is kinetically stabilized due to its protection from intermolecular contact at the enzyme binding site, thus preventing radical disproportionation. It has to be considered, therefore, as a special case and chemical artefact unrelated to the thermodynamical radical stabilization observed in the native enzyme and many other flavoproteins.

Mechanism

On the basis of the reported results, a detailed reaction mechanism can be described (Scheme 1, Fig. 7). Flavin-nicotinamide charge transfer complexes are seen as precursors of oxidoreduction, catalyzing transfer of hydride between dihydrinicotinamide and flavin. Their catalytic function may consist in providing pre-orientation of the reactants in a geometry similar to that of the transition state, leading to prepolarization of the C(4)-H dihydronicotinamide bond, to be broken in the rate-limiting step of the reaction. Catalysis by electron-withdrawing flavin substituents would then be interpreted as a consequence of increased prepolarization in the charge transfer complex. Further support for the catalytic role of flavin-nicotinamide charge transfer complexes comes from the fact that reaction rates decrease with increasing ethanol concentration in the aqueous solvent [13]. This effect is readily explained as a consequence of decreasing complex stability.

Charge transfer complexes *per se* are not considered as catalytically important because complex conformations not leading to the transition state may allow charge transfer interaction. On the other hand, complexes not showing charge transfer interactions can effectively catalyze electron transfer [66]. The possibility of synchronous ($H^+ + 2e^-$) transfer involving formation and fission of a covalent bond between flavin and nicotinamide [52] cannot be excluded.

The primary kinetic isotope effects k_H/k_{2_H} of 3.0 ± 0.5, observed for both $1e^-$ and $2e^-$ oxidoreduction, demonstrate that in either case fission of the dihydronicotinamide C(4)-H bond is rate-limiting. The intensity of the isotope effect cannot serve as a tool to distinguish $H^\cdot$ transfer from H^- transfer.

In nicotinamide-dependent alcohol/carbonyl oxidoreduction [67], π complexation can hardly be assumed to be a driving force. Prepolarization of substrate bonds at the active site might be achieved by metal ion complexation and/or hydrogen bonding.

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Note Added in Proof (July 12, 1976). In a recent study, Klinman [Klinman, J. (1976), *Biochemistry, 15*, 2018–2026] reported on rates and equilibria involved in the oxidoreduction of aromatic aldehydes, catalyzed by yeast alcohol dehydrogenase. Rearranging the reported data in the fashion described in this paper, a linear free energy relationship is obtained, when the rate of aldehyde reduction ($\log k_R$) is plotted versus the free energy difference of the bound aldehydes (ΔE). A change of 34 mV in free energy causes a tenfold rate change (1 unit of $\log k_R$) indicating that hydrogen is transferred as a hydride rather than a hydrogen atom in this enzymic reaction. The energy of the transition state is stabilized to the same degree by substituents as the energy of the corresponding ground states. This in contrast to flavin nicotinamide oxidoreduction where transition state energies can be considered as independent of substituent effects in a first approximation.