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NICOTINAMIDE ADENINE DINUCLEOTIDE (NAD^+)

FORMAL POTENTIAL OF THE $NAD^+/NAD^{\cdot}$ COUPLE AND $NAD^{\cdot}$ DIMERIZATION RATE

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The rate constant, k_d, for the dimerization of the free radical ($NAD^{\cdot}$), produced on the initial one-electron reduction of NAD^+, was measured by double potential-step chronoamperometry, fast-scan cyclic voltammetry (cathodic-anodic peak current ratio) and slow-scan cyclic voltammetry (peak potential shift) for a medium in which neither NAD^+ nor its reduction products are adsorbed at the solution/electrode interface. All three methods give concordant values of k_d (approx. $3 \cdot 10^7$ $M^{-1} \cdot s^{-1}$), which are in reasonable accord with the values determined by pulse radiolysis but are considerably greater than values previously determined electrochemically. For the $NAD^+/NAD^{\cdot}$ couple, the heterogeneous rate constant ($k_{s,h}$) exceeds 1 $cm \cdot s^{-1}$ at 25°C and the formal potential (E_c^0) vs. sce is -1.155 V at 25°C and -1.149 V at 1°C at pH 9.1, with an uncertainty of about ± 0.005 V.

Introduction

Nicotinamide adenine dinucleotide (NAD^+; diphosphopyridine nucleotide, DPN^+; coenzyme I) is one of the principal coenzymes, serving as a proton and electron acceptor in a broad range of enzymatically catalyzed biological redox reactions, e.g.;

$$NAD^+_{(ox)} + SH_2 \overset{\text{enzyme}}{\rightleftharpoons} 1,4\text{-}NADH_{(red)} + S + H^+ \quad (1)$$

where S represents the oxidized form of the substrate and SH_2 its reduced form. Thermal studies on such reactions and potentiometric measurements on systems containing the NAD^+/NADH couple in the presence of an appropriate enzyme and a mediator, have led to the calculation of a formal potential, E_c^0, of -0.54 V vs. s.c.e. at pH 7 for the redox couple:

$$NAD^+ + 2e^- + H^+ \rightleftharpoons 1,4\text{-}NADH \quad (2)$$

In contrast to the enzymatic reduction, the electrochemical reduction of NAD^+ occurs via two discrete electron-transfers [1,2]. The initial one-electron (1e) addition to form the free radical $NAD^{\cdot}$, which dimerizes, is essentially insensitive to pH and buffer composition. When only weakly surface-active cations are present, the electrochemical response for the reduction is dominated by the adsorption of NAD^+ and its reduction products; in the presence of a more strongly adsorbed cation, NAD^+ is more difficult to reduce. At more negative potential, the free radical is reduced (addition of a proton and an electron) to NADH. No evidence has been reported for the existence of the NAD free radical in biological redox processes [3]; operationally, this only implies that the life span

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Abbreviations: sce, saturated calomel electrode; nhe, normal hydrogen elecrode.

of such a free radical would have to be quite short. As has been recently stated [4], "it is still not possible to rule out one-electron transfer as a mechanism for the action of NAD in enzyme systems".

Investigation of the electrochemical reduction of NAD^+ [1,2,5] has included several measurements of the rate constant (k_d) for dimerization of the initially produced free radical:

$$NAD^+ + e \rightleftharpoons NAD^\cdot \quad (3)$$

The latter reduction is a reversible charge-transfer process but may be complicated by adsorption of the reactant and/or the product and by the follow-up chemical reaction [6,7]. The electrochemically determined k_d values have generally been compared to those determined in 0.1 m sodium formate solution (pH 6.4) by Land and Swallow [8], using pulse radiolysis and spectrophotometric measurements: $5.15 \cdot 10^7$ and $5.97 \cdot 10^7$ $M^{-1} \cdot s^{-1}$ (values differed for the two wavelengths used). Recently, Bielski and Chan [9], using a similar medium (pH 7.3) and approach, reported k_d to be $(7.72 \pm 0.78) \cdot 10^7$ $M^{-1} \cdot s^{-1}$ with an activation energy of 3.4 ± 0.4 kcal $\cdot$ mol^{-1}; the radical is stabilized by attachment to an enzyme active site.

Based on an estimated free radical half-life of less than 1 ms at pH 8 (measured by cyclic chronopotentiometry), k_d was considered to exceed 10^6 $M^{-1} \cdot s^{-1}$ [10]. A k_d of $8.49 \cdot 10^6$ was obtained at pH 7 from cyclic voltammetric potential peak measurement [11]. Based on Nicholson's cyclic voltammetric peak current ratio method [12], values were obtained of $(2.2 \pm 0.6) \cdot 10^6$ $M^{-1} \cdot s^{-1}$ (mean and standard deviation for 11 measurements in 0.5 M ionic strength acetate buffer at pH 5.0) and $(2.4 \pm 2.1) \cdot 10^6$ $M^{-1} \cdot s^{-1}$ (three measurements in 0.5 M ionic strength carbonate buffer (0.4 M in KCl) at pH 9.0) [1]; in a more recent study [6], k_d was found to be $(2.7 \pm 2.3) \cdot 10^6$ $M^{-1} \cdot s^{-1}$ (eight measurements in 0.4 M $(C_2H_5)_4NCl$ and 63 mM carbonate of pH 9.1).

The difference in an order of magnitude in k_d for the NAD free radical dimerization determined by pulse radiolysis and by polarographically based technics is disturbing, especially since k_d values for the 1-methylnicotinamide (1-methyl-3-carbamoylpyridinium ion; MCP^+) free radical determined by the two approaches agree quite well [13]. Since the polarographic measurements are related to phenomena at an interface, adsorption and diffusion may affect the rate measurements. NAD^+ and its reduction products are, as noted, strongly adsorbed. While MCP^+ is only slightly adsorbed, its dimeric and dihydropyridine reduction products are strongly and moderately adsorbed, respectively, due to their hydrophobic alkyl substituent. The relative values of k_d for NAD and MCP free radicals found in the authors' laboratory ($2 \cdot 10^6$ and $6 \cdot 10^7$) are supported by the statement in another polarographic study [14] that NAD radicals have an appreciably longer lifetime than those of the *N*-alkyl models.

The present study involves the determination of k_d by three different technics for measuring the kinetics of a chemical reaction coupled to a charge-transfer, in the course of which work the formal potential and standard heterogeneous charge-transfer rate constant for the $NAD^+/NAD^\cdot$ couple (Eqn. 3) were estimated. A buffer was used, in whose presence neither NAD^+ nor its reduction products are adsorbed in the potential region involved [1,6,15].

Experimental

Chemicals, apparatus and procedures, unless otherwise indicated, are as previously described [1,13,15–17]. Potentials are reported with respect to the aqueous saturated calomel electrode (sce). Measurements were made at 1°C and 25°C. The potential of the sce vs. E_h is 0.260 V at 1°C and 0.242 V at 25°C.

Chemicals. Although the reported analytical data and spectrophotometric assay indicated sufficient purity for electrochemical examination of the NAD^+ (P-L Biochemicals), its purity was confirmed by enzymatic assay [18].

The background solution of pH 9.1 and 0.5 M ionic strength was 0.4 M in tetraethylammonium chloride ($(C_2H_5)_4$ NCl; Aldrich) and contained a $K_2CO_3/KHCO_3$ buffer (J.T. Baker, reagent grade chemicals).

Apparatus. Polarographic and other voltammetric studies were conducted in a water-jacketed one-compartment cell with a Luggin capillary (Fig. 4 of Ref. 19). A mercury drop hung on a Hg-coated platinum wire was used as the working electrode.

The potentiostat used was designed and constructed to have a minimal response time [19]. Data obtained on slow-scan voltammetric measurements were recorded on a Houston Series 2000 $X-Y$ recorder; for the high-speed potentiometric data acquisition and transient signal observation required for double-potential step chronoamperometry and fast-scan cyclic voltammetry, a Tektronix 5103N power supply/amplifier was used as the basic oscilloscope system with a C-12 camera system. Observations of voltage amplitude vs. time ($Y-t$) were made using 5A18N dual-trace amplifier and 5B10N time base/amplifier modules; for use as an $X-Y$ recorder, the 5B10N was replaced by a 5A15N amplifier.

Results and Discussion

The experimental conditions of temperature and NAD^+ concentrations used, and the range of K_d magnitude measured are summarized in Table I.

Double potential-step chronoamperometry. In this approach, the potential at a stationary electrode is set to an initial level, at which an electrode reaction does not occur; it is then stepped to a new level, at which the reaction does occur, for a period of time τ; finally, it is stepped back to the original level [20]; the cathodic-anodic faradaic current ratios at given time intervals in the pulse may be related to the rate of the coupled chemical reaction.

For a τ of 60–120 μs, the results corresponded to $k_d = (4 \pm 2.7) \cdot 10^7\ M^{-1} \cdot s^{-1}$ (mean and standard deviation) at 25°. The primary limitation in the double potential-step method was an extremely poor signal to background ratio due to the charging current following each potential step being several times greater than the faradaic current. The rapid dimerization prohibited the use of larger NAD^+ concentrations or longer τ values, either of which would have increased the ratio of the cathodic current to charging current, but would have decreased the anodic current.

Fast-scan cyclic voltammetry. Use of scan rates, v, on a time-scale competitive with the dimerization under conditions where the charge transfer is reversible, allows the determination of k_d from the variation of the cathodic-anodic peak current ratio as a function of v, switching potential, and concentration of reactant [12].

The voltammetric response for reduction of NAD^+ to $NAD^\cdot$ at v of 200–500 V/s is indicative of a reversible charge transfer within an experimental uncertainty of 5 mV in each peak potential, e.g., Fig. 1. The minimum charge-transfer rate constant, $k_{s,h}$, which would result in such peak

TABLE I

DETERMINATION OF THE RATE CONSTANT FOR DIMERIZATION OF $NAD^\cdot$ IN 0.4 M $(C_2H_5)_4NCl$ AND 0.1 M CARBONATE AT pH 9.1 AT 1°C AND 25°C

Method	Temperature (°C)	NAD^+ concn. (mM)	k_d ($M^{-1} \cdot s^{-1}$) ($\times 10^{-7}$)
DPSC [a]	25	0.12, 1.18	4 ±2.7 [b]
FSCV [c]	25	0.30, 0.50	2 ±0.3 [d]
	1	0.40, 0.60	1 ±0.2 [d]
SSCV [e]	25	0.30, 0.50	3 ±1.7 [f]
	1	0.40, 0.60	1.5 ±0.9 [f]

[a] Double potential-step chronoamperometry.
[b] Uncertainty represents one standard deviation for six determinations.
[c] Fast-scan cyclic voltammetry.
[d] Uncertainty represents one standard deviation for six determinations at 25°C and seven determinations at 1°C.
[e] Slow-scan cyclic voltammetry.
[f] Uncertainty represents the maximum and minimum values of k_d assuming a ±3 mV uncertainty in measuring the peak potential.

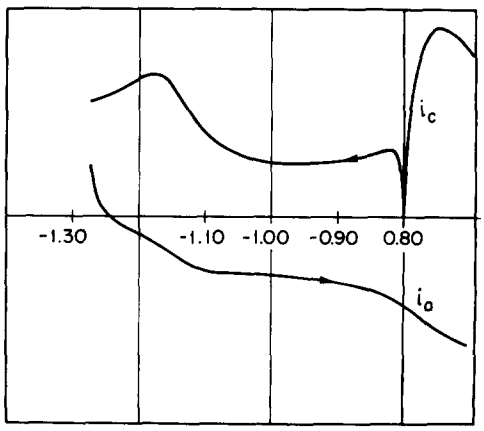

Fig. 1. Cyclic voltammogram for the initial one-electron reduction of 0.6 mM NAD^+ in 0.4 M $(C_2H_5)_4NCl$ and 0.1 M carbonate at pH 9.1 at 1°C. Scan rate is 200 V/200 V/s; arrow heads indicate scan direction. Current direction and numerical potential axis are shown.

separation at 500 V/s, is 1 cm/s at 25°C. For v of 250–500 V/s, a k_d of $(2 \pm 0.3) \cdot 10^7$ $M^{-1} \cdot s^{-1}$ was determined at 25°C and of $(1 \pm 0.2) \cdot 10^7$ $M^{-1} \cdot s^{-1}$ at 1°C.

Formal potential for $NAD^+/NAD^\cdot$ couple. The formal potential, E_c^0, for the $NAD^+/NAD^\cdot$ couple can be calculated from the voltammetric peak potential, E_p, which is related to the polarographic half-wave potential, $E_{1/2}$, for a reversible charge transfer [21] by:

$$E_p = E_{1/2} - 0.0285/n \quad (4)$$

If the diffusion coefficients, D_o and D_r, for the oxidized and reduced species are assumed to be equal, $E_{1/2}$ is equivalent to E_c^0. For the specific buffer used at pH 9.1, E_c^0 vs. sce is −1.155 V at 25°C and −1.149 V at 1°C.

As indicated, E_p was determined at scan rates v, which were sufficiently rapid, that the dimerization was outrun, i.e., the couple involves only a simple reversible charge transfer.

Use of Eqn. 5 and the experimentally measured k_d values at 25°, gave formal potentials of −1.18 V for the $MCP^+/MCP^\cdot$ couple and −1.24 V for the $NMN^+/NMN^\cdot$ couple [13].

Slow-scan cyclic voltammetry. Under the conditions that v is less than 3 k_d C_o/n mV/s, E_p is described by:

$$E_p = E_c^0 - \left(\frac{RT}{3nF}\right) \ln\left(\frac{4.78\pi 3D_o}{2D_r}\right)$$

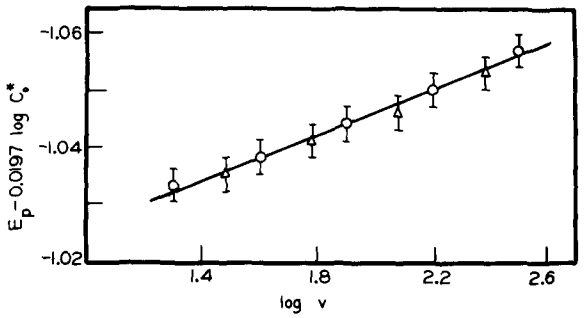

Fig. 2. Variation of cyclic voltammetric peak potential-concentration function with scan rate for initial one-electron reduction of NAD^+ in 0.4 M $(C_2H_5)_4NCl$ and 0.1 M carbonate at pH 9.1 at 25°C. Circles: 0.30 mM NAD^+; triangles: 0.50 mM NAD^+. Error bars correspond to an uncertainty of ±3 mV in the measurement of the peak potential. v is expressed in mV/s.

$$-\left(\frac{RT}{3nF}\right) \ln\left(\frac{a}{k_d C_o}\right) \quad (5)$$

where $a = nFv/RT$ [22]. From linear least-squares fit of the data for a plot of $[E_p - (2.303\ RT/3nF) \log C_0]$ vs. log v (Fig. 2), whose slope agreed with the theoretical slope of 19.7 mV/decade v at 25°C, k_d was determined to be $(3 \pm 1.7) \cdot 10^7$ $M^{-1} \cdot s^{-1}$ at 25°C and $(1.5 \pm 0.9) \cdot 10^7$ $M^{-1} \cdot s^{-1}$ at 1°C, using the previously determined E_c^0. The uncertainties in k_d are based on the maximum and minimum values for the y-axis intercept of the plot, assuming a ±3 mV uncertainty in E_p.

Evaluation of data

Potential. Recently, Anderson [3] reported the following formal potentials at pH 7 for the couples indicated (nhe scale): −0.922 V for $NAD^+/NAD^\cdot$ (determined by pulse radiolysis) and 0.282 V for $NAD^\cdot/NADH$ (calculated); the corresponding values on the sce scale are −1.164 and 0.040 V, respectively. At the same time, Farrington, Land and Swallow [4] reported the potentials for the same two couples to be −0.94 and 0.30 V (nhe scale) (−1.18 and 0.06 V on the sce scale). The value of −1.155 (sce scale), determined electrochemically, is thus in agreement with the values determined by pulse radiolysis and spectrophotometry.

In a recent theoretical analysis of the NAD system [7], calculation of the formal potential, in which account was taken of both the free radical dimerization and the protonation and reduction of the free radical, gave a value of −1.123 V vs. sce for the $NAD^+/NAD^\cdot$ couple at 25°C, which is in reasonable agreement with the values cited in the previous paragraph.

Since the potential for the reduction of NAD^+ to $NAD^\cdot$ is essentially pH-independent, the change in pH of the buffer with temperature would not be a contributing factor to the shift in potential with temperature.

Dimerization rate. The dimerization rate for the NAD free radical may be dependent on (a) pH as it controls the net charge on the free radical species, (b) ionic strength through its effect on the activity coefficient of the charged species, (c) nature and concentration of the buffer system in terms of its

effects on buffering capacity, ionic strength, interaction of buffer species with different NAD species and composition of the interfacial region, and (d) surface activity of other species present, e.g., KCl or TeaCl, through the control of the extent of adsorption of NAD species at the solution-electrode interface. The relative adsorption of NAD^+, its free radical and the corresponding dimer, and the resulting effects on $k_{s,h}$ and k_d have been considered [6].

All three electrochemical methods used gave values of k_d, which agree within experimental error (Table I). Fast-scan cyclic voltammetry demonstrated the best reproducibility and, therefore, appears to be best suited for the determination of second-order rate constants on the order of 10^7 $M^{-1} \cdot s^{-1}$. If background noise is reduced, e.g., by use of a faradaic cage, the double potential-step method might possibly show a precision comparable to that of fast-scan cyclic voltammetry. It need not be stressed that reproducibility in itself is no criterion of accuracy, since systematic error could be present.

The values for k_d are in reasonable agreement with those of $5.6 \cdot 10^7$ $M^{-1} \cdot s^{-1}$ and $7.7 \cdot 10^7$ $M^{-1} \cdot s^{-1}$ determined by pulse radiolysis at approx. 0.1 M ionic strength [8,9]. The lower rate constant measured at 0.5 M ionic strength may be attributable to a lower activity coefficient for NAD$^\cdot$. Since the NAD free radical has a net charge of -2, it is not surprising that a 5-fold increase in ionic strength significantly affects the dimerization rate of NAD$^\cdot$.

The activation energy and effective frequency factor for the dimerization reaction are 6 kcal/mol and $7 \cdot 10^{10}$ $M^{-1} \cdot s^{-1}$, respectively, when calculated from the fast-scan rates at 25°C and 1°C, and 5 kcal/mol^{-1} and $3 \cdot 10^{11}$ $M^{-1} \cdot s^{-1}$, when the slow-scan data are used. The activation energies are in reasonable agreement with those calculated for other 1-substituted nicotinamide dimerizations, e.g., 4 kcal/mol^{-1} for both 1-methylnicotinamide and nicotinamide mononucleoside [13] and 3.4 kcal/mol^{-1} for NAD$^\cdot$ [9] (the former results are based on electrochemical measurement and the latter on pulse radiolysis).

The values of k_d found in the present study are nearly an order of magnitude greater than those previously reported by the authors' laboratory on the basis of electrochemical approaches, e.g., Refs. 1 and 6, and 2- or 3-times the magnitude reported by other investigators using electrochemical approaches, e.g., Ref. 11. Possible causes for such differences may in the case of fast-scan cyclic voltammetry involve the method of measuring the anodic peak current and the choice of the initial potential, E_i, of the negative potential sweep. The proper method of measuring the cathodic and anodic peak currents is not obvious from the original paper [12], especially in respect to the extrapolation involved in correcting for the large charging current caused by the use of high scan rates. The somewhat arbitrary extrapolation and charging current subtraction could readily account for a 2- or 3-fold differences in k_d. The effect of the E_i selected has been reviewed [6]; essentially, NAD^+ must not be adsorbed at E_i. In the present study, E_i was -0.80 V, at which potential NAD^+ is not adsorbed for the solution composition used (cf. Fig. 3 in Ref. 1 and Fig. 3 in Ref. 6).

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