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ENERGETICS OF THE ONE-ELECTRON REDUCTION STEPS OF RIBOFLAVIN, FMN AND FAD TO THEIR FULLY REDUCED FORMS

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The one-electron reduction potentials (E_7^1) of riboflavin, FMN and FAD have been determined using pulse radiolysis from the position of the electron-transfer equilibria between flavins and reference quinones in aqueous solution. The average value for all three flavins $E_7^1(F/FH^{\cdot}) = -314 \pm 8$ mV is used to calculate the second-electron reduction potential of the flavins $E_7^2(FH^{\cdot}/FH_2(FH^-)) = -124$ mV.

Introduction

The properties of flavosemiquinones have been investigated by ESR [1], potentiometric titrations [2–4], flash photolysis [5] and pulse radiolysis [6,7]. Pulse radiolysis has been shown to be a powerful method for studying equilibrium reactions occurring between short-lived radicals [8,9] and can be used to measure one-electron reduction potentials, E^1, of an electron acceptor/radical one-electron couple, e.g., $A/A^{\cdot-}$ [10]. Recently, the E^1 at pH 7.0 (E_7^1) for the $NAD^+/NAD^{\cdot}$ couple has been determined using this method [11,12].

The basis of the method is to measure the position of the one-electron transfer equilibrium between a reference compound Q (e.g., DQ or AQS^- [10]) and the compound under study (e.g., a flavin, F):

$$F^{\cdot-} + Q \rightleftharpoons F + Q^{\cdot-} \quad (1)$$

The semiquinone radicals can be conveniently produced by pulse radiolysis of an oxygen-free solution containing propan-2-ol (0.4 mol · dm^{-3}):

$$H_2O \rightarrow e_{aq}^-, H, OH, H_2O_2, H_2, H_3O^+ \quad (2)$$

$$OH(H) + (CH_3)_2CHOH \rightarrow H_2O(H_2) + (CH_3)_2\dot{C}OH \quad (3)$$

$$(CH_3)_2COH + F/Q \rightarrow (CH_3)_2CO + H^+ + F^{\cdot-}/Q^{\cdot-} \quad (4)$$

$$e_{aq}^- + F/Q \rightarrow F^{\cdot-}/Q^{\cdot-} \quad (5)$$

By choosing suitable concentrations of F and Q, reactions 2–5 will be completed sufficiently fast to enable the approach to, and establishment of, equilibrium 1, to be followed. The position of equilibrium 1 may also be influenced by the ionic strength of the solution if both reactants or both products are charged.

An earlier measurement of E^1 for riboflavin using this technique [13] showed approx. 20 mV discrepancy between the measured and calculated values at high pH. Further, an estimate of the E_7^1 of FAD [7] from measurements at high ionic strengths was approx. 56 mV higher than the value reported [13] for riboflavin. The equilibria at different ionic strengths (I) were therefore investigated to help determine whether these differences were real.

Abbreviations: E_7^1, E_7^2 and E_7^0 the first-, second- and two-electron reduction potentials at pH 7; DQ, duroquinone; AQS^-, 9,10-anthraquinone-2-sulphonate; TQ^{2+}, 1,1′-propano-2,2′-bipyridinium.

Experimental Procedure

Materials and preparation of solutions

Duroquinone, DQ (Sigma) and AQS^- (monohydrate) (BDH) were recrystallised from ethanol and water and three times from water, respectively. Benzyl viologen (Sigma), propan-2-ol (BDH Aristar), phosphate buffers and NaCl (BDH AnalaR) were used as supplied. TQ^{2+} (dibromide) was prepared as described in the literature [14]. Stock concentrations of riboflavin, FMN, FAD (Sigma) were determined from literature extinction coefficients [15]. Solutions of the compounds containing NaCl (0–80 mmol · dm^{-3}) were adjusted to the required pH using phosphate buffers (2 mmol · dm^{-3}) and purged free of oxygen using N_2 prior to pulse radiolysis.

Apparatus and treatment of results

Pulse radiolysis was carried out using a 1.8 MeV linear accelerator; details of the principles of the kinetic spectrophotometer and dosimeter used have been published [16,17]. Typically, a 2.0 Gy dose was delivered in a 0.2 μs pulse and equilibrium 1 was established and measured within a few tens of microseconds after the pulse, with a total radical concentration of approx. 1.2 μmol · dm^{-3}.

Both the rate of approach to, and the position of, an equilibrium between charged species will be influenced by the ionic strength, I, of the solution. The influence of I on the rate is predicted by the Bronsted-Bjerrum equation and taking the ratio of the forward and back reactions to obtain an expression for the dependence of the equilibrium constant (K_I^1) on I yields:

$$\log K_I^1 = \log K_0^1 - 1.02\left[\left(z_1^p z_2^p\right) - \left(z_1^r z_2^r\right)\right] \times I^{1/2}\left(1 + 3.29 a_i I^{1/2}\right)^{-1} \quad (6)$$

where z_1 and z_2 are the charges on the reactants r and products p, K_0^1 the equilibrium constant at zero ionic strength and a_i the ion size parameter. Eqn. 6 can be rewritten as:

$$\log K_I^1 = \log K_0^1 - Cfn(I) \quad (7)$$

where $fn(I) = I^{1/2}(1 + I^{1/2})^{-1}$ using the Guntelberg value of $a_i = 0.31$ nm which gives a good approximation up to $I = 0.1$ [26]. By measuring K_I^1 at various values of I, K_0^1 is determined by linear-regression analysis using Eqn. 7.

The difference in one-electron reduction potential, ΔE^1, between F and Q is related to the free energy change, ΔG^1 by:

$$\Delta G^1 = nf\Delta E^1 = RT \ln K_0^1 \quad (8)$$

hence

$$\Delta E^1\ (\text{mV}) = 59 \log K_0^1 \text{ at } T = 295 \pm 2\ \text{K} \quad (9)$$

(At low ionic strengths, Eqn. 6 should be adequate to permit extrapolation to $I = 0$ from measurements at a single, low ionic strength. In the case of FMN and FAD, the interesting question is raised as to whether the overall net charge on the molecule controls the ionic strength dependence of rates or equilibria, since the reaction centre is somewhat remote from the phosphate groups. In the present work, therefore, I was varied. The results below indicate some difference between the data when plotted according to Eqn. 7 and the slope calculated from the net charges. The implications of these results will be discussed in a subsequent paper describing measurements of the effects of I on the disproportionation of flavosemiquinones where these differences between theory and experiment are more marked.)

Results

Reference quinone compounds were used in this study in preference to bipyridinium compounds as their semiquinones do not absorb in the accessible spectral region (where the ground-state flavins do not absorb) [9]; further, the spectra of flavosemiquinones [6,7] and the bipyridinium radical cations overlap [7,18].

One-electron reduction potentials of the reference quinones

The E_7^1 values of AQS^- and DQ were checked by the above method using benzyl $viologen^{2+}$ as the reference compound with added NaCl (0–80 mmol · dm^{-3}) as the inert electrolyte and measuring the absorption of the benzyl $viologen^{+}$ radical

TABLE I

EQUILIBRIUM AND E_7^1 DATA FOR REFERENCE QUINONES

Redox couple	K_0^1 [a]	C [a]	ΔE (mV)	$E_7^1(Q/Q^{\cdot-})$ (mV)
Benzyl viologen^{2+}/AQS^-	0.64 ± 0.03	−2.07 ± 0.06	−12 ± 2	−366 ± 8
Benzyl viologen^{2+}/DQ	65.9 ± 6.1	−2.79 ± 0.24	107 ± 3	−247 ± 7
TQ^{2+}/AQS^-	670 ± 120 [b]	-	167 ± 5	−381 ± 7
AQS^-/DQ	166 ± 14 [b]	-	131 ± 2	-

[a] Parameters as in Eqn. 7.
[b] Equilibrium constants corrected by use of Eqn. 6.

at 600 nm [18]. Corrected equilibrium constants (using Eqn. 6) were also obtained for four mixtures of both AQS^-/TQ^{2+} (observing at 900 nm [7]) and AQS^-/DQ (at 480 nm [10,19]). Equilibrium was observed between benzyl viologen^{2+} (0.15 mmol · dm^{-3}) and AQS^- (0.25 mmol · dm^{-3}), between benzyl viologen^{2+} (2.5 mmol · dm^{-3}) and DQ (80 μmol · dm^{-3}), and between AQS^- (2 mmol · dm^{-3}) and DQ (20–60 μmol · dm^{-3}). The E_7^1 of benzyl viologen^{2+} (−354 ± 5 mV) and TQ^{2+} (−548 ± 5 mV) (potentials on the normal hydrogen electrode scale) have been well established electrochemically [18].

The data obtained from these experiments are presented in Table I. The value E_7^1 ($DQ/DQ^{\cdot-}$) = −247 ± 7 mV is slightly lower than that reported by Meisel and Czapski [20] but agrees well with the results of other workers [19,21]. The mean value of E_7^1 ($AQS^-/AQS^{\cdot-}$) = −374 ± 7 mV is in good agreement with previously determined values [10,19,21].

One-electron reduction potentials of the flavins

The E_7^1 values of riboflavin, FMN and FAD were determined by the described method using both DQ and AQS^- as the reference compounds. Equilibrium was observed at 540 nm where the flavosemiquinone species absorb.

Solutions contained FMN or FAD (1 mmol · dm^{-3}) and DQ (125 μmol · dm^{-3}) or AQS^- (2.25 mmol · dm^{-3}). Riboflavin is less soluble and solutions of riboflavin (80 μmol · dm^{-3}) with DQ (26 μmol · dm^{-3}) or AQS^- (2 mmol · dm^{-3}) were used. Five solutions of increasing ionic strength (I = 0.004–0.087) were studied for each flavin/quinone combination. The results from these experiments are presented in Table II. Low ionic strengths had little effect on the position of the radical equilibria between riboflavin and the quinones at pH 7. This is expected as both riboflavin and its semiquinone are virtually uncharged at this pH.

Within experimental error, E_7^1 values for all

TABLE II

EQUILIBRIUM AND E_7^1 DATA FOR FLAVINS

Flavin	Quinone [a]	K_0^1 [b]	C [b]	ΔE (mV)	$E_7^1(F/F^{\cdot-})$ (mV)
Riboflavin	DQ	16.0 ± 1.7	0.47 ± 0.39	71 ± 3	−318 ± 8
Riboflavin	AQS^-	0.11 ± 0.01	0.01 ± 0.59	−57 ± 3	−317 ± 8
FMN	DQ	13.1 ± 1.8	1.32 ± 0.35	66 ± 3	−313 ± 8
FMN	AQS^-	0.08 ± 0.01	0.64 ± 0.14	−65 ± 3	−309 ± 8
FAD	DQ	10.7 ± 0.3	0.56 ± 0.05	61 ± 1	−308 ± 7
FAD	AQS^-	0.12 ± 0.01	0.66 ± 0.10	−55 ± 3	−317 ± 8

[a] Reference couples used $E_7^1(AQS^-/AQS^{\cdot-})$ = −374 ± 7 mV, $E_7^1(DQ/DQ^{\cdot-})$ = −247 ± 7 mV from Table I.
[b] Parameters as in Eqn. 7.

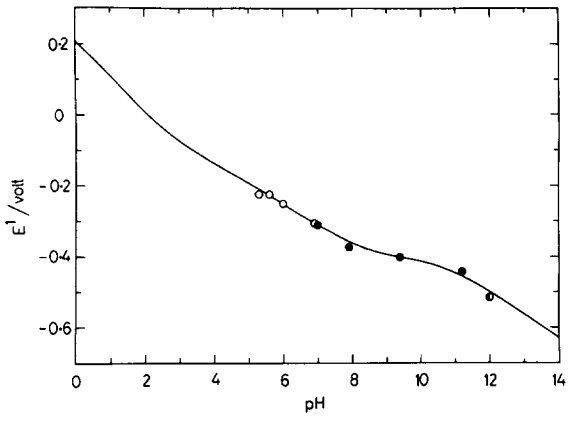

Fig. 1. Dependence of the one-electron reduction potential of FMN on pH. Experimental points were determined by the method described in the text using DQ (○), AQS^- (●) or TQ^{2+} (◐) as reference compounds. The solid line was calculated using Eqn. 10.

three flavins were similar; the average value $E_7^1 = -314 \pm 8$ mV at 295 ± 2 K.

The one-electron reduction potentials of FMN at various pH values were also measured by the same method. These experimental results can be compared with the calculated values obtained from the expression [10]:

$$E^1_{pH} = E^1_7 + \frac{RT}{F}\ln\left\{\left(\frac{K_1'K_2' + K_1'[H^+] + [H^+]^2}{K_1'K_2' + 10^{-7}K_1' + 10^{-14}}\right) \times \left(\frac{1 + \frac{K_3}{10^{-7}} + \frac{10^{-7}}{K_2} + \frac{10^{-14}}{K_1K_2}}{\frac{K_3}{[H^+]} + \frac{[H^+]}{K_2} + \frac{[H^+]^2}{K_1K_2}}\right)\right\} \quad (10)$$

where the protropic equilibrium constants of the isoalloxazine ring and its flavosemiquinone are K_1, K_2 and K_3 (respective pK_a values -8, 0.25 and 10.35) and K_1' and K_2' (pK_a values 2.3 and 8.5, respectively) [4,6]. The experimental data and the calculated dependence are presented in Fig. 1.

Discussion

The values of C calculated from Eqn. 7 using the appropriate net charges on the reactants and products may be compared with the experimental values. In every case the effect of I was of the direction expected; some quantitative difference between theory and practice was found, but did not influence the extrapolations to $I = 0$ used in the present work. This difference is most marked for FAD where two negative charges on the pyrophosphate group are present on both the parent compound and its flavosemiquinone. The calculated slope in Eqn. 7 for equilibrium 1 involving FAD and the quinones is 2 while experimentally 0.6 is found. This result indicates that the influence of the charges carried by the pyrophosphate group on equilibrium 1 is less than stoicheiometric.

The E_7^1 value of the flavins determined in this work has to be compared with the original value of -270 mV at 30°C determined electrochemically for riboflavin by Michaelis et al. [2]. This value was subsequently revised to be more positive [3] and Draper and Ingraham [4] using the same method reported E_7^1 for riboflavin and FMN near -240 mV at 20 ± 1°C.

The present value is significantly lower than these values but is more reliable, since the experimentally verified redox equilibrium is established before there is any significant bimolecular disappearance of the flavosemiquinone species (for the neutral flavosemiquinone of riboflavin $2k = 1.14 \cdot 10^9$ $mol^{-1} \cdot dm^3 \cdot s^{-1}$ [6], which under the present pulse conditions leads to a first half-life in decay of nearly two orders of magnitude greater than the time required to establish equilibrium). Complications due to electron transfer at a solid surface, absorption and hydrogen gas evolution that are inherent in polarography are avoided using pulse radiolysis. Further, the good agreement found between our experimentally determined one-electron reduction potentials at various pH values and the calculated dependence (based on our E_7^1 value and the known prototropic parameters) give support to our figure.

Meisel and Neta [13] measured $K^1 = 9.4$ for riboflavin/DQ at $I > 0.1$ and pH 7; at pH 11 their experimental one-electron reduction potentials were 20 mV higher than calculated values. These differences may arise from the high ionic strength used in the earlier work. In the previous pulse radiolysis measurement of E_7^1(FAD/FADH·) [7]

the ionic strength correction (-25 mV) calculated using Eqn. 6 was subtracted from the apparent E_7^1 in error instead of being added. The revised estimate becomes -286 ± 7 mV but as $I > 0.2$ in these experiments the validity of using Eqn. 6 at such a high ionic strength is questionable.

Using the literature value for the two-electron reduction potential of the flavins $E_7^0(F/FH_2\text{-}(FH^-)) = -219$ mV [15] the calculated value for E_7^2 ($FH^{\cdot}/FH_2(FH^-)$) becomes -124 mV.

The free-energy change for FH_2 (FH^-) to act as a one-electron donor in reducing a substrate S will only be favourable if $E_7^2(FH^{\cdot}/FH_2(FH^-))$ is less positive than $E_7^1(S/S^{\cdot-})$. Hence, FH_2 (FH^-) cannot function as a one-electron donor if the $E_7^1(S/S^{\cdot-})$ is appreciably less than -124 mV. The $E_7^1(O_2/O_2^{\cdot-}) = -155$ mV when referred to a standard state of 1 mol · dm^{-3} $O_2/O_2^{\cdot-}$ [22,23] and hence the formation of superoxide ions upon the oxidation of fully reduced flavins is unfavourable for pH < 7.5. At higher pH values $E^1(O_2/O_2^{\cdot-})$ remains unchanged while $E^1(F/FH\ (F^{\cdot-}))$ and hence $E^2(FH^{\cdot}(F^{\cdot-})/FH^-)$ both decrease, favouring the formation of superoxide. These findings support the contention that below pH 8 the decay of the complex formed between fully reduced flavins and oxygen proceeds without the formation of flavosemiquinone and superoxide ions [24,25] while at higher pH these radical species are formed [24].

In enzyme reactions involving FMN and FAD other factors will also determine the relative importance of one- and two-electron reductions. The local pH may be different from 7 and raising the pH will increase the likelihood of one-electron reduction. In a less polar environment, ionic species such as the deprotonated fully reduced flavin (FH^-) may be destablized more than the neutral flavosemiquinone species thus lowering $E_7^2(FH^{\cdot}/FH^-)$ and increasing the likelihood of one-electron transfer to substrates. Steric considerations for molecular interactions in which electron transfer is more facile than hydride ion transfer may also favour one-electron reduction.

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