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THE BIMOLECULAR DECAY RATES OF THE FLAVOSEMIQUINONES OF RIBOFLAVIN, FMN AND FAD

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The bimolecular decay rates ($2k$) of the flavosemiquinones ($FH^{\cdot}/F^{\overline{\cdot}}$) of riboflavin, FMN and FAD have been determined using pulse radiolysis. The rates (defined as $d[FH^{\cdot}/F^{\overline{\cdot}}]/dt = -2k[FH^{\cdot}/F^{\overline{\cdot}}]^2$) for the neutral flavosemiquinones at zero ionic strength and pH 5.9 are (in units of $mol^{-1} \cdot dm^3 \cdot s^{-1}$): $(1.2 \pm 0.1) \cdot 10^9$, $(5.0 \pm 0.2) \cdot 10^8$ and $(1.4 \pm 0.1) \cdot 10^8$; and for the anionic flavosemiquinones at pH 11.2 $(5.4 \pm 0.9) \cdot 10^8$, $(4.5 \pm 0.3) \cdot 10^7$ and $(8.5 \pm 1.3) \cdot 10^6$, respectively. The kinetic salt effect has been used to formulate rate equations for each flavin to adjust for ionic strength effects.

Introduction

The bimolecular decay of the flavosemiquinones ($FH^{\cdot}/F^{\overline{\cdot}}$) of free flavins in solutions of low flavin concentration is known to proceed through a dismutation reaction to yield equimolar oxidised flavin (F) and fully reduced flavin (FH_2/FH^-)

$$2FH^{\cdot}/F^{-} \rightarrow F + FH_2/FH^{-} \tag{1}$$

It has been shown for riboflavin that reaction 1 proceeds slower in basic solution where the flavosemiquinone carries a negative charge than in neutral solution [1]. The dismutation rates of the flavosemiquinones of FMN and FAD at pH 9 0 where both the neutral ($FH^{\cdot}$) and anionic (F^-) forms are present ($pK_a(FH^{\cdot}/F^-) = 8.5$ [1–3]), are slower than for the neutral form [4] This slower rate at high pH is not unexpected as electrostatic repulsion between like-charged molecules will occur. The lack of influence of increasing ionic strength on the dismutation rate of riboflavin flavosemiquinone at pH 5.1 [1] showed that it does not carry a net charge at this pH Other studies on the three flavins have been carried out at a variety of ionic strengths and pH [1,4,5] making comparisons between the flavins difficult

In this study we have measured the dismutation rates for both the neutral and anionic flavosemiquinones of riboflavin, FMN and FAD as a function of ionic strength This should yield information on the relative influence of charge on the isoalloxazine ring (and in the case of FMN the charges on the phosphate groups) on the rates of flavosemiquinone dismutation.

Experimental Procedure

Materials and preparation of solutions

Stock concentrations of riboflavin, FMN (99%) and FAD (95%) (Sigma) were determined from literature extinction coefficients [6]. AnalaR phosphate buffers, NaCl and Aristar propan-2-ol (BDH) were used as supplied.

Solutions of the flavins (0.25 $mmol \cdot dm^{-3}$)

Abbreviations F, oxidised flavin, $FH^{\cdot}/F^-$, flavosemiquinone, FH_2/FH^-, fully reduced flavin containing NaCl (0–80 mmol · dm^{-3}) and propan-2-ol (0.2 mol · dm^{-3}) were adjusted to pH 5.9 with NaH_2PO_4 (1 mmol dm^{-3}) or to pH 11.2 with Na_3PO_4 (2 mmol dm^{-3}). Solutions were purged free of oxygen using N_2 and on pulse radiolysis (approx 60 Gy in 1 μs) the following reactions are completed in a few microseconds

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

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

$$(CH_3)_2\dot{C}OH + F \rightarrow (CH_3)_2CO + H_3O^+ + F^{\cdot -} \quad (4)$$

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

$$FH^{\cdot} \rightleftharpoons F^{\cdot -} + H_3O^+ \quad (pK_a = 8\ 5\ [1\text{–}3]) \quad (6)$$

The total concentration of flavosemiquinone radicals at this time was approx. 36 μmol · dm^{-3}. The subsequent decay of the neutral and anionic flavosemiquinones of each flavin were studied in buffered solutions at pH 5 9 and 11.2, respectively

Apparatus and treatment of results

Pulse radiolysis was carried out using a 4 MV Van de Graaff accelerator. Kinetic spectroscopy was made using a SPEX monochromator and an ultraviolet enhanced solid-state photodiode (RCA type C30842) in conjunction with a wide-band virtual-earth amplifier. Transients were recorded on a Tektronix 7612D digitizer with a 7A16P plug-in and kinetic parameters were determined with the aid of a PDP 11/34 computer A toroidal magnetic device which monitored the beam charge per pulse was calibrated by irradiating aerated KSCN (10 mmol · dm^{-3}) assuming the $(SCN)_2^{\cdot -}$ radical produced had *G* (molecules formed per 100 eV absorbed dose) = 2.8 and ϵ = 7600 dm^3 mol^{-1} · cm^{-1} at 480 nm [7]

The bimolecular rate of reaction of charged species is influenced by the ionic strength, *I*, of the solution and can be described by the Bronsted-Bjerrum relationship

$$\log 2k_I = \log 2k_0 + 1\ 02(z_A z_A) I^{1/2}\left(1 + 3\ 29 a_i I^{1/2}\right)^{-1} \quad (7)$$

where z_A is the charge on the reactants, $2k_0$ and $2k_I$ the bimolecular rate constants of the species at ionic strength zero and *I*, respectively, and a_i the ion size parameter.

Eqn. 7 can be rewritten as:

$$\log 2k_I = \log 2k_0 + 1\ 02(z_A z_A) f(I) \quad (8)$$

where $f(I) = I^{1/2}(1 + 2\ 6 I^{1/2})^{-1}$ taking $a_i = 0\ 8$ nm. (In the transition state $a_i = a_{\pm} = \sqrt{2(a_r + a_e)^2}$ [16], where a_r and a_e are the mean radii of the reactants (taken as the isoalloxazine ring, 0 4 nm) and electrolyte (Na^+, 0.2 nm), respectively.) By measuring $2k_I$ at various values of *I*, $2k_0$ is determined by linear regression analysis of Eqn 8. The slope of the line yields the product of the effective ionic charges

Results

Determination of extinction coefficients

Although the spectral characteristics of flavosemiquinones have been known for some time there is disagreement as to their extinction coefficients [1,5,8,9]. We have redetermined the extinction coefficient of the neutral flavosemiquinone at 560 nm and the anionic flavosemiquinone at 525 nm of FMN by comparing the changes in absorbance with the bleaching of the $Fe(CN)_6^{3-}$ absorbance at 420 nm following a normalised dose of 5 Gy. To ensure equal radical scavenging at $I = 0.1$, the rate of e_{aq}^- scavenging by FMN (four solutions containing 0–18 μmol · dm^{-3} FMN, 0.1 mol · dm^{-3} NaCl and 0.4 mol · dm^{-3} *tert*-butanol saturated with N_2) and the rate of electron transfer from $CO_2^{\cdot -}$ to FMN (four solutions containing 0–24 μmol dm^{-3} FMN and 0.1 mol · dm^{-3} NaCOOH saturated with N_2O) were first measured at both pH 5.9 and 11 2. The rate constant of e_{aq}^- scavenging by $Fe(CN)_6^{3-}$ at $I = 0.1$ has been reported [10] and we also determined the rate of electron transfer from $CO_2^{\cdot -}$ to $Fe(CN)_6^{3-}$ (five solutions containing 0–150 μmol · dm^{-3} $Fe(CN)_6^{3-}$ and 0.1 mol · dm^{-3} NaCOOH). All these rate constants are summarised in Table I.

Concentrations of FMN (140 μmol dm^{-3}) and $K_3Fe(CN)_6$ (500 μmol dm^{-3}) were selected for the comparison of absorbance study on the basis of the data in Table I. The ratios of absorbance change for FMN at 560 nm to $K_3Fe(CN)_6$ at 420 nm following pulse radiolysis of eight solutions of each scavenger were 4.98 ± 0 20 at pH 5.9 and 0.91 ± 0.02 at pH 11.2 Using a value for the

TABLE I

REACTION RATE CONSTANTS OF e^-_{aq} AND THE CO_2^- RADICAL ANION WITH FMN AND $Fe(CN)_6^{3-}$ AT $I = 0.1$

Estimated error of rate constants ± 10%

Scavenger	pH	Rate constant ($mol^{-1}\ dm^3\ s^{-1}$)	
		e^-_{aq}	CO_2^-
FMN	6.0	$3.3\ 10^{10}$	$3.0\ 10^9$
	11.0	$2.5\ 10^{10}$	$1.0\ 10^9$
$Fe(CN)_6^{3-}$	6.0	$9.5\ 10^{9\,a}$	$7.0\ 10^8$
	11.0	$9.5\ 10^{9\,a}$	$7.0\ 10^8$

[a] From Ref 10

extinction coefficient of $Fe(CN)_6^{3-}$ at 420 nm of 1027 ± 2 [11], we find that the extinction coefficient of the neutral flavosemiquinone ($FH^\cdot$) at 560 nm is equal 5115 ± 200 $mol^{-1} \cdot dm^3 \cdot cm^{-1}$ and that of the anionic flavosemiquinone (F^-) at 525 nm equal to 1050 ± 20 $mol^{-1} \cdot dm^3 \cdot cm^{-1}$, including the correction for the bleaching of the small absorbance of the ground state at 525 nm (116 $mol^{-1}\ dm^3 \cdot cm^{-1}$). Our figure for the extinction coefficient of the neutral flavosemiquinone of FMN agrees well with the value for riboflavin determined by Land and Swallow [1]

Products of the bimolecular decay of flavosemiquinones

The oxidised and fully reduced forms of FMN absorb negligibly at 560 nm However, full restoration to the pre-pulse light level was not achieved at the end of the decay of the absorbing flavosemiquinones at pH 5.9 (Fig. 1). This final absorbance was small at 560 nm (less than or equal to 4% of the initial flavosemiquinone absorbance) when 0.25 $mmol \cdot dm^{-3}$ FMN was used (the FMN concentration was chosen to ensure greater than 95% scavenging of the radicals). The asymptotes for second-order kinetic analysis are drawn to this final absorbance level (A_∞).

The decay of the absorbing flavosemiquinones ($FH^\cdot$) to an absorbing product (P) is described by the equation

$$\frac{1}{A_t - A_\infty} = \frac{\epsilon_P}{A_\infty(2\epsilon_{FH} - \epsilon_P)} + \frac{2k}{(\epsilon_{FH} - \epsilon_P/2)}\ t \qquad (9)$$

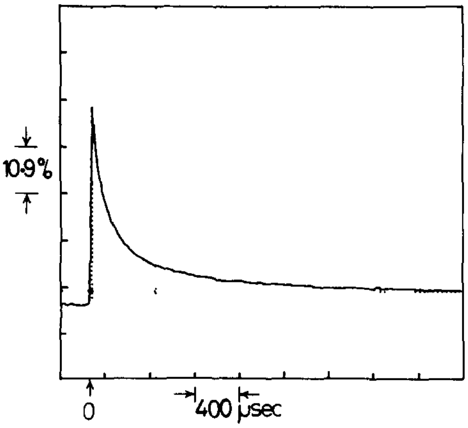

Fig 1 Oscilloscope trace showing the change in percentage absorption at 560 nm (ordinate) against time (abscissa) following a 1 μs 60 Gy pulse at time 0 of an N_2-saturated solution containing FMN (0.25 mmol dm^{-3}), propan-2-ol (0.2 mol dm^{-3}) and NaH_2PO_4 (1.0 mmol dm^{-3}) at pH 5.9. The horizontal and vertical dotted lines are the asymptote and time period of analysis used for the second-order kinetic plot (Fig 2)

where A_t is the absorbance at time t and ϵ_P the apparent extinction coefficient of the final absorbing species The second-order rate constant, $2k$, is determined from the slope of the plot $1/(A_t - A_\infty)$ vs. time t (Fig. 2). The slope $= 2k/(\epsilon_{FH} - \epsilon_P/2)l$, where l is the optical path length. A value of 4910 $mol^{-1} \cdot dm^3 \cdot cm^{-1}$ for ($\epsilon_{FH} - \epsilon_P/2$) was used to determine $2k$. The extinction coefficients of oxidised and fully reduced FMN at 525 nm in pH 11.2 solution were determined as 116 and 15 $dm^3 \cdot$

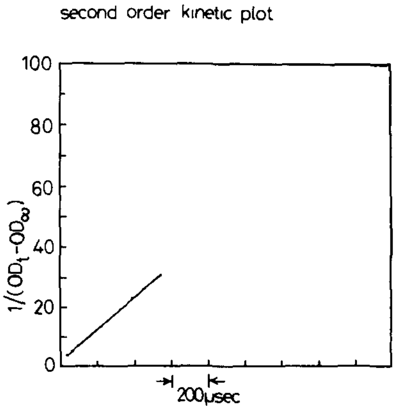

Fig 2 Second-order kinetic plot of decay of the transient in Fig 1

$mol^{-1} \cdot cm^{-1}$, respectively. However, restoration to the pre-pulse light level, and not a small bleaching was observed at the end of the decay of the absorbing flavosemiquinone. This indicates that there is some absorbance of a product and a value of 985 $mol^{-1} \cdot dm^3 \cdot cm^{-1}$ for $(\epsilon_{F\dot{\cdot}} - \epsilon_P/2)$ was used to calculate $2k$.

The final absorbance is most likely that of a small amount of the charge-transfer complex known to be formed between fully reduced and oxidised flavins [12,8].

$$FH + FH \rightleftharpoons (FH_2 \rightarrow F) \rightleftharpoons F + FH_2 \tag{10}$$

Decay rates of the flavosemiquinones

Solutions for each flavin at five ionic strengths and pH 5.9 or 11.2 were irradiated. The decay rate constants of the flavosemiquinones were determined from second-order plots using the effective extinction coefficients described above. The observed decay kinetics of the flavosemiquinones fit second-order decay plots up to approx 2.5 half-lives after which a small positive deviation is seen (Fig. 2). At low concentrations of oxidised flavin the second equilibrium in Eqn. 10 is mainly to the right [9] and is quickly established relative to the decay of the flavosemiquinone. Initially, $[FH] \gg [(FH_2 \rightarrow F)]$ so that $k_{obs} = 2k_I$, but when $[FH^{\cdot}] \cong [(FH_2 \rightarrow F)]$ k_{obs} increases as the reverse reaction of the left-hand equilibrium will be incorporated into the kinetics. The initial decay kinetics were uninfluenced when the concentration of oxidised flavin was halved (0.125 $mmol \cdot dm^{-3}$).

Each series of rate constants (means of at least three measurements at each ionic strength) are presented in Fig. 3. These data were analysed using Eqn. 8 and the second-order rate constants at zero ionic strength $2k_0$ together with the slopes of the plots in Fig. 3 (divided by 1.02) are presented in Table II.

TABLE II

SECOND-ORDER DECAY RATES OF FLAVOSEMIQUINONES

Parameters were taken from Eqn. 8

Flavin	pH	$2k_0$ (mol^{-1} dm^3 s^{-1})	slope/1.02
Riboflavin	5.9	$1.2 \pm 0.1 \cdot 10^9$	0.07 ± 0.07
	11.2	$5.4 \pm 0.9 \cdot 10^8$	0.81 ± 0.24
FMN	5.9	$5.0 \pm 0.2 \cdot 10^8$	1.63 ± 0.13
	11.2	$4.5 \pm 0.3 \cdot 10^7$	4.35 ± 0.10
FAD	5.9	$1.4 \pm 0.1 \cdot 10^8$	2.20 ± 0.16
	11.2	$8.5 \pm 1.3 \cdot 10^6$	6.32 ± 0.43

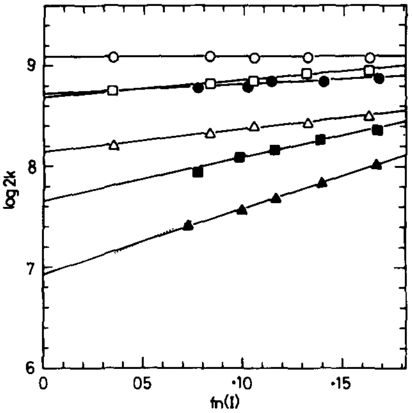

Fig. 3. Log plots of the means of the second-order decay rate constants against $f(I)$ as described in the text. Open and closed symbols are data at pH 5.9 and 11.2 for riboflavin (○, ●), FMN (□, ■) and FAD (△, ▲), respectively. Solid lines are derived from regression analysis of the data using Eqn. 8 and the dotted line results from similar treatment but with $a_i = 1.1$ nm.

Discussion

The bimolecular rate constants determined in this study are in the expected order on the basis of molecular size and charges carried by the various flavosemiquinones. It is interesting that whereas the rate constants at low ionic strengths and both pH values differ between the three flavins as much as a factor of approx. 150, at isotonic strength ($I = 0.154$) it is extrapolated that the rates are much less pH dependent (factor of 8).

A comparison between the rate constants determined in this study with those of previous workers can be made using the data in Table II and Eqn. 8. The rate constants of the neutral and anionic flavosemiquinones of riboflavin at $I = 0.1$ in this work, $1.2 \cdot 10^9$ and $7.4 \cdot 10^8$ $mol^{-1} \cdot dm^3 s^{-1}$, agree well with the rates $1.14 \cdot 10^9$ and $7 \cdot 10^8$ $mol^{-1} \cdot dm^3 \cdot s^{-1}$ found by Land and Swallow [1]. The rate constant for the neutral flavosemiquinone of FMN at $I = 0.1$, $9\ 4 \cdot 10^8$ mol^{-1} · dm^3 · s^{-1}, is similar to that calculated from the data of Holmstrom [5] $7 \cdot 10^8$ mol^{-1} dm^3 · s^{-1}, when the present extinction coefficient is used. The rate constants for the decay of the neutral flavosemiquinones of FMN and FAD at $I = 0\ 05$ found in the present study are considerably lower than the values $2.6 \cdot 10^9$ and $1.9 \cdot 10^9$ mol^{-1} · dm^3 · s^{-1} reported by Vaish and Tollin [4] A comparison between the rate constants for the decay of the anionic flavosemiquinones of both FMN and FAD with previous studies [4,5] cannot be made as the previous studies were carried out at pH 9.0 with a mixture of flavosemiquinones

The values of $(z_A z_A)$ from Eqn. 7 using the appropriate net charges on the flavosemiquinones may be compared with the experimentally determined slopes (Table II) The flavosemiquinones of riboflavin gave the predicted kinetic salt effect. However, the charges on the phosphate groups of FMN and FAD have some influence on the decay rates. Whilst both pK_a values of the pyrophosphate of FAD and the first pK_a of the orthophosphate of FMN are less than 2 the second pK_a for FMN is in the region 6.2–6.6 [13]. At pH 5.9 the second proton of the orthophosphate group (FMN) is only partially ionized, but clearly the effective charge of both phosphate groups is less than stoicheiometric (Table II). At pH 11.2 both the ortho- and pyrophosphate groups carry two negative charges and the isoalloxazine ring of the flavosemiquinones also carry a negative charge The predicted kinetic salt effect for both FMN and FAD is $(z_A z_A) = 9$ whereas slopes of approx 4.4 and 6.3, respectively, are found. This difference between FMN and FAD would be greater if a larger value for the ion size parameter a_i was used for FAD. In fact, using an a_i value of 1.1 nm, i e., approaching the dimension of a nucleotide in Eqn 7, yields a slope close to 9 (the dotted line in Fig. 3) The greater influence of ionic strength for the pyrophosphate (FAD) is understood in terms of the negative charge being spread over a greater number of atoms in the hairpin-like structure of FAD [14] which may keep the pyrophosphate group in closer proximity to the radical centre on the isoalloxazine ring compared to the orthophosphate group of FMN

In a recent study [15] it was found that the charges on the phosphate groups of FMN and FAD have a far from stoicheiometric influence on the electron-transfer equilibria between flavosemiquinones and quinones. The larger effects of overall charge on the bimolecular reactions studied here indicates differences between the activated complexes of the two cases. The activated complex formed on the bimolecular reaction of flavosemiquinones is likely to be energetically similar to the charge-transfer complex that is stable enough to be observed in high flavin concentrations The pyrophosphate group may have a greater influence than the orthophosphate group on the formation of inner sphere complexes whereas the influence of the phosphate groups is less in outer-sphere electron-transfer reactions.

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