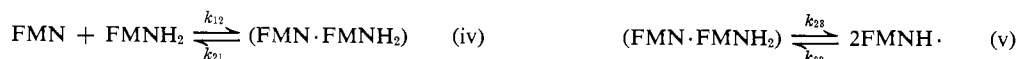# Electron Transfer in the Flavin Mononucleotide System

James H. Swinehart

Contribution from the Department of Chemistry, University of California, Davis, California. Received October 22, 1965

Abstract: A kinetic investigation of partially reduced aqueous flavin mononucleotide solutions at pH 5 is reported. On the basis of the thermodynamic and activation parameters the mechanism is formulated in terms of the equilibria

$$FMN + FMNH_2 \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} (FMN \cdot FMNH_2) \quad (iv) \qquad (FMN \cdot FMNH_2) \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} 2FMNH \cdot \quad (v)$$

where FMN, $FMNH_2$, $(FMN \cdot FMNH_2)$, and $FMNH \cdot$ refer to the oxidized, reduced, dimeric, and free-radical forms of flavin mononucleotide, respectively. The enthalpy and entropy for reaction v, $K_{23} = [FMNH \cdot]^2/[(FMN \cdot FMNH_2)]$, are 9.3 ± 0.3 kcal/mole and 15 ± 2 eu, respectively. The activation parameters, ΔH^* and ΔS^*, for k_{32} are 5.6 ± 0.5 kcal/mole and −9 ± 2 eu. The activation parameters for k_{23}, as calculated from the thermodynamic parameters for K_{23} and activation parameters for k_{32}, are in fair agreement with those experimentally observed. The value of $k_{32}(H_2O)/k_{32}(D_2O)$ is found to be 2.3, which is consistent with the transfer of charge taking place in (v) as a hydrogen-atom transfer.

In a previous report a preliminary study of the kinetics of the partially reduced flavin mononucleotide system in the pH range 3.9 to 5.2 using the temperature-jump–relaxation method was described.[1] A complete kinetic investigation of the system in this pH range is reported with special emphasis being placed on a clarification of whether or not the dimeric species, $(FMN \cdot FMNH_2)$, is involved directly in equilibria with both the oxidized and reduced forms, FMN and $FMNH_2$, and the free radical, $FMNH \cdot$. The mechanism by which the transfer of electrons takes place is also investigated to determine whether the mechanism is one of direct electron transfer or of hydrogen atom transfer.

Experimental Section

Chemicals. Flavin mononucleotide (riboflavin 5′-phosphate sodium salt) was obtained from Mann Research Laboratories, New York, N. Y., and was used without further purification. Especially pure FMN, greater than 99%, was obtained from Dr. L. L. Ingraham, Department of Biochemistry, University of California, Davis, Calif. This sample was obtained by a paper chromatographic separation using a butanol–water–acetic acid mixture (4:5:1). The kinetics proved to be independent of the sample. Sodium dithonite (Merck, reagent), acetic acid (Mallinckrodt, analytical reagent), sodium acetate (Baker and Adamson, reagent), and hydrochloric acid, 37% (Mallinckrodt, analytical reagent), were used without further purification. D_2O was purchased from Bio-Rad Laboratories, Richmond, Calif. All solutions were prepared with doubly distilled water.

Preparation of Solutions. Solution preparation is described in a previous paper and need not be repeated in detail here.[1] Acetate–acetic acid was used as the buffer and the final solutions were 0.2 M in total acetate.

Apparatus. The pH was measured with a Leeds and Northrup Model 7401 pH meter. Standard buffers were used for calibration. The spectrophotometer used was a Cary Model 14 recording spectrophotometer with a thermostated sample compartment, which was nitrogen flushed. One-centimeter matched cells were used. Temperature control was good to 0.3°.

The theory of relaxation methods and a technical description of the temperature-jump–relaxation equipment is summarized elsewhere.[1,2] The instrument used in this investigation was manufactured by the Messanlagen Studiengesellschaft m.b.H., Goettingen, Germany.

Measurements. The two absorbancy measurements important for a description of the system are the absorbancy at 840 mμ, $A_{840m\mu}$, and the corrected absorbancy at 570 mμ, $A_{570m\mu}$. The shoulder of the band which maximizes at 840 mμ makes a major contribution to the total absorbancy at 570 mμ. The corrected absorbancy at 570 mμ was calculated by assuming the 840-mμ band was symmetrical and subtracting this contribution from the total 570-mμ band. The contribution to the absorbancy at 570 mμ from the oxidized and reduced forms is small.

The error recorded in measurements is the maximum error taken from extreme measurements divided by four. This gives a reasonable estimate of the probable error.[3]

Results and Discussion

The pK values of the various species present in partially reduced flavin mononucleotide solutions are: FMN, -0.1^4 (in HCl) and 9.8; $FMNH \cdot$, 1.2 (riboflavin) and 7.3;[5] and $FMNH_2$, 6.8.

At pH 4.7 the possible equilibria are

$$2FMN \rightleftharpoons (FMN)_2 \qquad (i)$$

$$2FMNH_2 \rightleftharpoons (FMNH_2)_2 \qquad (ii)$$

$$FMNH_2 + FMNH \cdot \rightleftharpoons (FMNH_2 \cdot FMNH \cdot) \qquad (iii)$$

$$FMN + FMNH_2 \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} (FMN \cdot FMNH_2) \qquad (iv)$$

$$(FMN \cdot FMNH_2) \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} 2FMNH \cdot \qquad (v)$$

where the species in equilibria iv and v may be in other combinations (*e.g.*, FMN + $FMNH_2$ ⇌ 2FMNH·, etc.). Calculations using the data of Gibson, Massey, and Atherton[6] show that at the greatest FMN and $FMNH_2$ concentrations used less than 10% of the species are present as the dimers $(FMN)_2$ and $(FMNH_2)_2$. Thus equilibria i and ii are of minor importance.

Figure 1 is a plot of $A_{570m\mu}^2$ *vs.* $A_{840m\mu}$ at 298°K for pH values 2.7 and 5.9. The species contributing to the cor-

(1) J. H. Swinehart, *J. Am. Chem. Soc.*, **87**, 904 (1965).

(2) M. Eigen and L. deMaeyer in "Technique of Organic Chemistry," Vol. VIII, A. Weissberger, Ed., Interscience Publisher, Inc., New York, N. Y., 1963, Part II, Chapter XVIII.
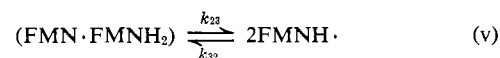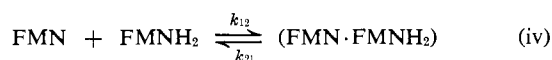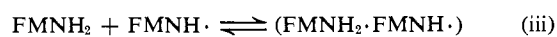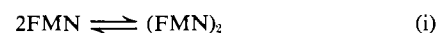
(3) R. Livingston, "Physico Chemical Experiments," 3rd ed, The Macmillan Co., New York, N. Y., 1948, p 44.

(4) Determined spectrophotometrically by the author.

(5) There is considerable disagreement on the value of this pK. L. Michaelis and G. Schwarzenbach, *J. Biol. Chem.*, **123**, 527 (1938), reported a value of 6.5 for riboflavin. B. Holmström, *Photochem. Photobiol.*, **3**, 97, (1964), reported the value recorded here. Other workers have reported values as high as 8.3 (riboflavin): R. D. Draper and L. L. Ingraham, private communication.

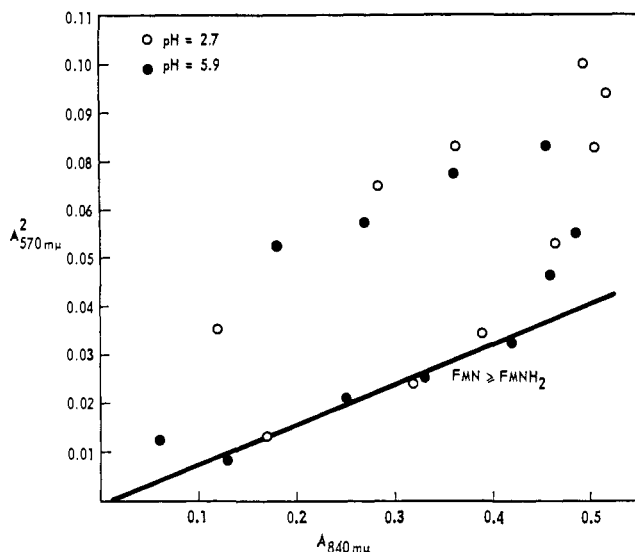(6) Q. H. Gibson, V. Massey, and N. M. Atherton, *Biochem. J.*, **85** 369 (1962).

Figure 1. Plot of absorbancy at 570 mμ squared vs. absorbancy at 840 mμ at pH values 2.7 and 5.9 (298°K).

Figure 2. Plot of the relaxation time, $1/\tau$ vs. $4A_{570m\mu}$ at 300, 292, and 282°K (accuracy of temperature, ± 1°C).

rected absorbancy at 570 mμ are FMNH· and (FMNH$_2$·FMNH·), and at 840 mμ the absorbancy is attributed to (FMN·FMNH$_2$).[6,7] There is a linear relation between $A_{570m\mu}^2$ and $A_{840m\mu}$ in the region in which *the [FMN] is greater than or equal to the [FMNH$_2$]*. This indicates that equilibrium iii is of minor importance compared to equilibria iv and v.

Values of $A_{570m\mu}^2/A_{840m\mu}$ as a function of temperature are contained in Table I.

Table I. Equilibrium Data for Reaction v at pH 4.7

Temp, °K	10^2, $\dfrac{A_{570m\mu}^2}{A_{840m\mu}}$	Concn conditions
314	17.5 ± 1.0	b
313	15.0 ± 1.0	c
299	8.5 ± 1.0	a
293	6.1 ± 0.6	c
291	5.1 ± 0.5	a
283	3.7 ± 0.3	c
282	2.9 ± 0.3	b

a [FMN] > [FMNH$_2$] and [FMN] = [FMNH$_2$]. b [FMN] > FMNH$_2$]. c [FMN] = [FMNH$_2$].

When the ln $(A_{570m\mu}^2/A_{840m\mu})$ is plotted as a function of the reciprocal of the absolute temperature, ΔH is found to be 9.3 ± 0.3 kcal/mole.

It has been shown in previous work that a linear relation exists between the relaxation time, $1/\tau$, and $A_{570m\mu}$.[1] Figure 2 shows a plot of $1/\tau$ vs. $4 \times A_{570m\mu}$ at three temperatures. It is clear from the accumulated data that there is not a linear relation between $1/\tau$ and $A_{840m\mu}$ or ([FMN] + [FMNH$_2$]). Experiments where the ([FMN] + [FMNH$_2$]) was made five times the concentration normally used, while $A_{570m\mu}$ was kept small, led to no abnormal behavior when $1/\tau$ was plotted against $4 \times A_{570m\mu}$ (point 1 in Figure 2). Thus it can be concluded that the mechanism which relates FMN, FMNH$_2$, FMN·FMNH$_2$, and FMNH· must lead to a relationship in which $1/\tau$ increases linearly with $A_{570m\mu}$ or [FMNH·]. Such a mechanism,
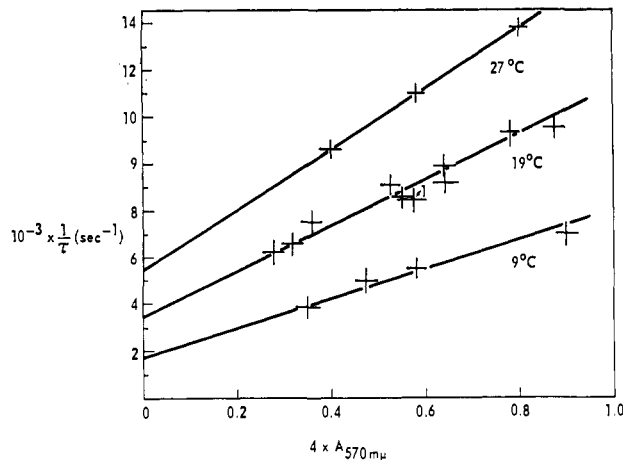
(7) H. Beinert, J. Am. Chem. Soc., 78, 5323 (1956).

as discussed in a previous paper, is one in which reaction iv equilibrates rapidly compared to (v).[1] The form of $1/\tau$ for such a mechanism is

$$\frac{1}{\tau} = \frac{k_{23}K_{12}([FMN] + [FMNH_2])}{1 + K_{12}([FMN] + [FMNH_2])} + 4k_{32}[FMNH\cdot]$$

where $K_{12} = [FMN\cdot FMNH_2]/([FMN][FMNH_2])$.[8] If $K_{12}([FMN] + [FMNH_2])$ is much greater than 1, $1/\tau = k_{23} + 4k_{32}[FMNH\cdot]$. The slope of $1/\tau$ vs. 4[FMNH·] yields k_{32} and the extrapolated intercept is k_{23}. The only other reasonable mechanism which would lead to a linear relationship between $1/\tau$ and $A_{570m\mu}$, [FMNH·], is one in which the equilibria are

$$FMN\cdot FMNH_2 \rightleftharpoons FMN + FMNH_2 \quad (vi)$$

$$FMN + FMNH_2 \underset{k_{54}}{\overset{k_{45}}{\rightleftharpoons}} 2FMNH\cdot \quad (vii)$$
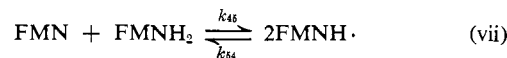
and (vi) equilibrates rapidly compared to (vii). This mechanism leads to a relationship

$$\frac{1}{\tau} = \frac{k_{45}K_{12}([FMN] + [FMNH_2])}{\{K_{12} + ([FMN] + [FMNH_2])\}} + 4k_{54}[FMNH\cdot]$$

The problem becomes a question of whether FMN·FMNH$_2$ is a precursor to FMNH·. The results of Gibson, et al., indicate that the dimer is a precursor to the free radical.[6] The values for the activation parameters of the rate constants obtained lead to the same conclusion.

The values of $k_{32}/\epsilon_{570m\mu}$, where $\epsilon_{570m\mu}$ is the extinction coefficient of FMNH· at 570 mμ obtained from Figure 2, are (12.8 ± 1.0) × 10^3 sec^{-1} cm (300°K), (10.0 ± 1.0) × 10^3 sec^{-1} cm (292°K), and (6.3 ± 1.0) × 10^3 sec^{-1} cm (282°K). As determined from Figure 3 the value of ΔH^* for k_{32} is 5.6 ± 0.5 kcal/mole. The enthalpy of activation for k_{23}, as calculated from ΔH for reaction v, is 14.9 ± 0.8 kcal/mole. Fair agreement is obtained between this value and ΔH^* determined directly using extrapolated k_{23} values from Figure 2. The values of k_{23} are (5.5 ± 1.0) × 10^3 sec^{-1} (300°K), (3.5 ± 1.0) × 10^3 sec^{-1} (292°K), and (1.5 ± 1.0) × 10^3 sec^{-1} (282°K). A line as drawn through ln (k_{23}/T) vs. $1/\tau$ values in Figure 3 corresponds to 14.9 kcal. Since the values for k_{23} are obtained from a long extrapolation, the error will be large.

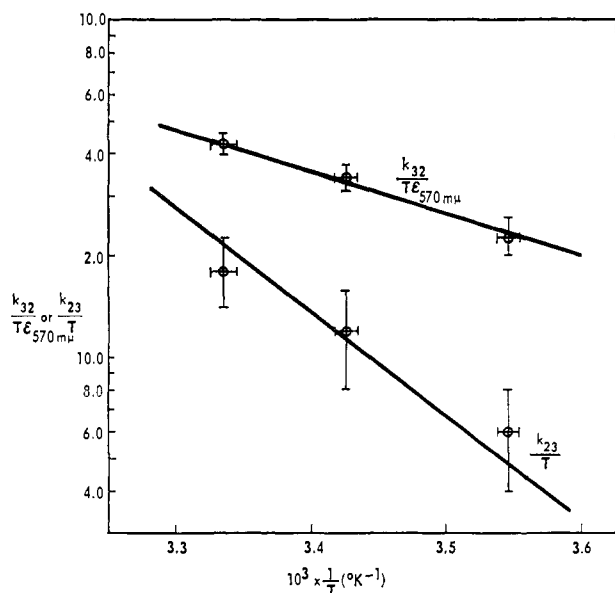(8) The form for $1/\tau$ is incorrect in ref 1 and is corrected here.

Figure 3. Plots of ln (k_{23}/T) and ln $(k_{32}/T\epsilon_{570m\mu})$ vs. $1/T$.

If the extinction coefficient of FMNH· at 570 mμ is assumed to be 3050 M^{-1} cm^{-1},[5] the value of ΔS^* for k_{32} is -6 ± 2 eu. This value is consistent with the mechanism proposed. [(FMN·FMNH$_2$)] can be calculated from the equilibrium constant K_{23} and [FMNH·], which equals $A_{570m\mu}/3050$ M^{-1} cm^{-1}. Values of K_{12} calculated from the initial concentrations of FMN and FMNH$_2$ and the concentrations of (FMN·FMNH$_2$) and FMNH· are inconsistent. The values of K_{12} decrease with increasing total flavin mononucleotide indicating that the concentrations of (FMN·FMNH$_2$) and FMNH· subtracted from the initial concentration of FMN and FMNH$_2$ are too small. It is quite clear that an extinction coefficient which will satisfy the experimental conditions must be considerably smaller than 3050 M^{-1} sec^{-1}. A wide variety of extinction coefficients for FMNH· are reported in the literature (700^9 to 13,600 M^{-1} cm^{-1}).[6] An extinction coefficient at the lower end of this range (about 500 M^{-1} cm^{-1}) yields both consistent values of K_{12} and values greater than 10^3 M^{-1} which are necessary in order to satisfy the condition that K_{12}([FMN] + [FMNH$_2$]) is greater than 1. A calculation of K_{12} is very sensitive to the value of $\epsilon_{570m\mu}$ in this region, so the value selected is accurate to ± 200 M^{-1} cm^{-1}. The value of ΔS^* for k_{32} calculated using $\epsilon_{570m\mu} = 500$ M^{-1} cm^{-1} is -9 ± 2 eu.

Since reaction iv is rapid compared to (v) and cannot be observed experiential, $k_{21} > 10^6$ sec^{-1}. As a result of K_{12} being greater than 10^3, k_{12} is greater than 10^9 M^{-1} sec^{-1}.

The value of K_{23} calculated from the ratio of the rate

(9) B. Holmström, Bull. Soc. Chem. Belges, 71, 869 (1962).

constants k_{23} and k_{32} at 298°K is 8×10^{-4} M. ΔS is calculated to be 15 ± 2 eu. The value of K_{23} is in fair agreement with the value of about 10^{-4} determined by Michaelis, et al., for glucoroalloxazene.[10,11] The values of 10^{-6} M determined by Gibson, et al.,[6] are at pH 6.3, which is near the pK of FMNH$_2$, and thus no valid comparison can be made.

Table II summarizes the results obtained at pH 4.7. Previous work has shown the same mechanism to be operative over a pH range from 2.9 to 5.5. The data in

Table II. Rate and Equilibrium Information at pH 4.7 and 298°K

	Value at 298°K	ΔH^* or ΔH, kcal/mole	ΔS^* or ΔS, eu
k_{32}	$(6.0 \pm 0.2) \times 10^6$ M^{-1} sec^{-1}	5.6 ± 0.5	-9 ± 2
k_{23}	5×10^3 sec^{-1}	14.9 ± 0.8^a	6 ± 2^a
K_{23}	8×10^{-4} M	9.3 ± 0.3	15 ± 2
k_{12}	$>10^9$ M^{-1} sec^{-1}		
k_{21}	$>10^6$ sec^{-1}		
k_{12}	$>10^3$ M^{-1}		

^{a} From a combination of K_{23} and k_{32} values.

Table II are consistent with the mechanism postulated: reactions iv and v. The entropy of activation for a reaction in which the dimerization of uncharged species takes place is generally negative. Such a reaction is represented by the rate constant k_{32} which has associated with it an entropy of activation of -9 ± 2 eu. For the over-all equilibrium v, ΔS is 15 ± 2 eu which is reasonable for the process of this type. The assumption is made that interactions between the solvent and the species involved, (FMN·FMNH$_2$) and FMNH·, are small. This assumption seems reasonable since the molecules are uncharged and flavins are generally very insoluble in water (10^{-4} M for riboflavin) unless a polar group is added.

The question as to the point at which electron transfer takes place (reaction iv or v) and by what mechanism it proceeds (electron or hydrogen atom transfer) is pertinent. From experiments in D$_2$O at 292°K, the ratio of k_{32}(H$_2$O)/k_{32}(D$_2$O) is found to be 2.3 and k_{23}(H$_2$O)/k_{23}(D$_2$O) = 1.5. The latter result is less reliable than the former because of the extrapolation necessary to obtain k_{23}. This evidence is consistent with a hydrogen atom transfer which takes place in equilibrium v.

Acknowledgment. This investigation was supported by Public Health Service Research Grant GM 11767-02 from the National Institutes of Health.

(10) L. Michaelis, M. P. Schubert and C. V. Smythe, J. Biol. Chem., 116, 507 (1936).
(11) L. Michaelis and G. Schwarzenbach, ibid., 123, 527 (1938).