Chemistry of Peroxidic Tetrahedral Intermediates of Flavin

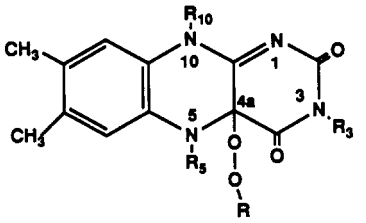
Gábor Merényi* and Johan Lind

Contribution from the Departments of Physical Chemistry and of Nuclear Chemistry, Royal Institute of Technology, S-10044 Stockholm, Sweden. *Received June 13, 1990. Revised Manuscript Received November 26, 1990*

Abstract: By means of pulse radiolysis 4a-peroxy intermediates of normal and 5-alkylated flavins were produced and the kinetics of their decay into flavin and the corresponding hydroperoxide was investigated as a function of the pH. The neutral and proton-catalyzed breakdown of the 4a-intermediates of 5-alkylated flavinium cations on the one hand and of 5-protio flavins on the other was very similar. It was concluded that the rate-determining step in the neutral decomposition of normal flavin 4a-peroxides is a heterolysis along the C(4a)–O bond which is catalyzed by water as a general acid. The species initially produced consist of a N(5)-protonated flavinium cation, a neutral hydroperoxide, and a hydroxide ion. The process is completed by rapid deprotonation of the flavinium cation to yield the neutral flavin. By combination of kinetic and thermodynamic data determined in this and other laboratories, the energetics of the autoxidation of 1,5-dihydroflavin was resolved into individual steps. The proton-catalyzed breakdown of flavin 4a-peroxides is initiated by a proton-assisted expulsion of neutral hydroperoxide leaving behind the N(5)-protonated flavinium cation. The attenuation of proton catalysis with decreasing pH indicates thermodynamic protonation of the 4a-intermediates around pH 3. The site of protonation is presumably the N(5) or the N(10) atom. The hydroxide ion catalyzed breakdown of the 4a-species is best interpreted by assuming the rate-determining step to be deprotonation of the N(5)-H site followed by rapid expulsion of the hydroperoxide anion and neutral flavin. This picture demands the microscopic pK_a of the N(5)-H group to be below 17. The possible role of enzymes in stabilizing the 4a-intermediates against breakdown into flavin and hydroperoxide is discussed. It is suggested that an apolar, hydrophobic pocket may be the chief stabilizing factor. In such an environment, the transition state for heterolysis and homolysis may approach each other. Finally, the bond strength of the peroxidic O–O bond was calculated from recent thermodynamic data. This bond turns out to be weaker (<26 kcal/mol) than the O–O bond in any known linear peroxide. From the finding that the O–O bond is weaker than the C(4a)–O bond it is argued that, in sufficiently hydrophobic enzymes, monooxygenation may be initiated by homolysis of the O–O bond. It is suggested that the comparable strengths of the C(4a)–O and O–O bonds may be the prime reason for the versatility of flavin enzymes.

Introduction

Many vital functions of flavin enzymes involve the transient presence of intermediates characterized by a C(4a)–O bond. For definitions, see formula FlR_5OOR. The most important inter-



FlR_5OOR

mediate is a flavin C(4a)-hydroperoxide, which operates in monooxygenases and hydroxylases.[1,2] Bound to the *Vibrio Harveyi* luciferase enzyme, this species has been positively identified by ^{13}C NMR spectroscopy.[3] Bacterial luminescence is generally believed to arise from the decomposition of a flavin C(4a)-peroxyhemiacetal[4,5] bound to a luciferase enzyme. Although strongly implicated by kinetic evidence,[6,7] this species has never been isolated or even characterized unequivocally. The decomposition of the peroxyhemiacetal is believed to produce the enzyme-bound pseudobase[7,8] in an excited state from whence chemiluminescence occurs. The isolation of the pseudobase and the finding that its fluorescence (albeit weak) is superimposable on the bioluminescence spectrum lends strong support to this view.[7,9] In many monooxygenase enzymes the bound 4a-hydroxide has been observed[2] (judging from spectral properties) prior to its reverting to flavin and water.

With an eye to understanding the role of flavin enzymes, much effort has been expended in the past to investigate the chemistry of model C(4a)-hydroperoxides.[10-14] These studies necessitated alkylation of the N(5) position of the flavin, a process that apparently entails thermodynamic stabilization of the tetrahedral species in relation to the parent flavinium cation. What is still lacking is a comparative study to pinpoint the factors that affect the breakdown kinetics of the peroxidic species. The present work encompasses the kinetic investigation of several flavin C(4a)-peroxy species as a function of site alkylation and the pH of the water solvent.

Results

C(4a)-hydroperoxides of N(5)-H Flavins. In a series of papers Anderson[15,16] has demonstrated the facile generation, by means of pulse radiolysis, of C(4a)-hydroperoxides of normal flavins (Fl), such as riboflavin and flavin mononucleotide (FMN). Although these species could not be positively identified, spectral comparison with enzyme-bound C(4a)-hydroperoxides and 5-alkylated model

(1) Massey, V.; Hemmerich, P. In *The Enzymes*, 3rd ed.; Boyer, P. D., Ed.; Academic Press: New York, 1975; Vol. XII, pp 191-252.
(2) Ballou, D. P. In *Flavins and Flavoproteins*; Bray, R. C., Engel, P. C., Mayhew, S. G., Eds.; de Gruyter: Berlin, 1984; pp 605-618.
(3) Vervoort, J.; Müller, F.; Lee, J.; van den Berg, W. A. M.; Moonen, Ch. T. W. *Biochemistry* **1986**, *25*, 8062.
(4) Eberhard, A.; Hastings, J. W. *Biochem. Biophys. Res. Commun.* **1972**, *47*, 348.
(5) Kurfürst, M.; Ghisla, S.; Hastings, J. W. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 2990.
(6) Macheroux, P.; Ghisla, S.; Kurfürst, M.; Hastings, J. W. In *Flavins and Flavoproteins*; Bray, R. C., Engel, P. C., Mayhew, S. G., Eds.; de Gruyter: Berlin, 1984; pp 669-672.
(7) Ghisla, S.; Eckstein, J.; Macheroux, P. In *Flavins and Flavoproteins*; Edmondson, D. E., McCormick, D. B., Eds.; de Gruyter: Berlin, 1987; pp 601-612.
(8) Kurfürst, M.; Hastings, J. W.; Ghisla, S.; Macheroux, P. In *Flavins and Flavoproteins*; Bray, R. C., Engel, P. C., Mayhew, S. G., Eds.; de Gruyter: Berlin, 1984; pp 658-667.
(9) Lee, J. In *Chemi- and Bioluminescence*; Burr, J. G., Ed.; Marcel Dekker: New York, 1985; pp 401-437.
(10) Kemal, C.; Bruice, T. C. *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73*, 995.
(11) Kemal, C.; Chan, T. W.; Bruice, T. C. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 405.
(12) Bruice, T. C. In *Flavins and Flavoproteins*; Bray, R. C., Engel, P. C., Mayhew, S. G., Eds.; de Gruyter: Berlin, 1984; pp 46-55, and references cited therein.
(13) Bruice, T. C. *Isr. J. Chem.* **1984**, *24*, 54, and references cited therein.
(14) Keum, S.-R.; Gregory, D. H.; Bruice, T. C. *J. Am. Chem. Soc.* **1990**, *112*, 2711.
(15) Anderson, R. F. In *Oxygen and Oxy-radicals in Chemistry and Biology*; Rodgers, M. A. J., Powers, E. L., Eds.; Academic Press: New York, 1981; pp 597-600.
(16) Anderson, R. F. In *Flavins and Flavoproteins*; Massey, V., Williams, C. H., Eds.; Elsevier: Amsterdam, 1982; pp 278-283.

0002-7863/91/1513-3146$02.50/0

Table I. Yields per Pulse of Flavin C(4a)-Peroxides Relative to their Maximum Yields as a Function of the pH[a]

pH	C(4a)-OOH	pH	C(4a)-OOR
5.0	86		
5.5	100		
5.8	100	5.9	100
6.0	99		
6.4	88		
6.8	88		
7.1	76	7.4	67
7.7	71	7.7	47
7.9	65	7.8	41
8.1	61	8.0	35
8.4	44	8.3	28
9.1	39	9.1	17
9.3	33	9.3	<10

[a] The dose per pulse was 200 Gy. R denotes $(CH_3)_2C(OH)CH_2$.

C(4a)-hydroperoxides, as well as a similarity in chemical behavior in several enzymes of authentic C(4a)-hydroperoxide and radiolytically generated intermediate,[17] leaves little doubt as to the nature of these species. A brief description of the experimental technique is appropriate. Irradiation of an oxygenated aqueous formate solution rapidly generates $CO_2^{\bullet-}$ and $O_2^{\bullet-}$. The former reduces Fl to $Fl^{\bullet-}$ and O_2 to $O_2^{\bullet-}$, and these radical anions protonate according to their pK_a's. By tailoring the system a desired ratio $FlH^\bullet/O_2^{\bullet-}$ can be obtained. The hydroperoxide forms according to reactions 1–3 with $k_1 \approx 10^9$, $k_2 \approx 5 \times 10^8$, and $k_3 \approx$ 2×10^8 M^{-1} s^{-1}.

$$FlH^\bullet + HO_2^\bullet \rightarrow FlHO_2H \quad (1)$$

$$FlH^\bullet + O_2^{\bullet-} \xrightarrow[-OH^-]{H_2O} FlHO_2H \quad (2)$$

$$Fl^{\bullet-} + O_2^{\bullet-} \xrightarrow[-OH^-]{H_2O} FlHO_2H \quad (3)$$

At 460 nm, where Fl absorbs but $FlHO_2H$ is almost transparent, the kinetics of the breakdown of $FlHO_2H$ into Fl and H_2O_2 has been measured[15,16] in the pH interval 5–8. Using a similar technique, we remeasured these rates as well as the corresponding rates with 3-methylated tetraacetylriboflavin (3Me-TARF), extending the pH range from pH 1 to ca. 10. It will be recalled[18] that $FlH^\bullet$ undergoes the dismutation reaction 4. In order to

$$2FlH^\bullet \rightleftharpoons Fl + FlH_2 \quad (4)$$

promote reactions 1–3 at the expense of 4, $O_2^{\bullet-}$ was produced in a 5–10-fold excess over $FlH^\bullet$. With aerated solutions, this required the Fl concentration to be well below 10^{-4} M. Apart from the competition of reaction 4 with 1–3, which is independent of the radical concentration generated in a single pulse, i.e. the dose, there are additional reactions, to be mentioned below, that may further reduce the yield of $FlHO_2H$.

(a) At very high doses the initial reductant, $CO_2^{\bullet-}$, can reduce the semiquinone radical, $FlH^\bullet$, to fully reduced flavin in reaction 5.

$$CO_2^{\bullet-} + FlH^\bullet(Fl^{\bullet-}) \xrightarrow{H_2O} CO_2 + FlH_2(FlH^-) \quad (5)$$

(b) When the pH is raised above ca. 7.5, the $FlH^\bullet$ radical with a pK_a of ca. 8.3[19-21] starts to dissociate into $Fl^{\bullet-}$. $Fl^{\bullet-}$ reacts rapidly with O_2 in competition with its reaction with $O_2^{\bullet-}$.

$$Fl^{\bullet-} + O_2 \rightarrow Fl + O_2^{\bullet-} \quad (6)$$

k_3 and k_6 have comparable magnitudes ($(2–3) \times 10^8$ M^{-1} s^{-1})[22,23]

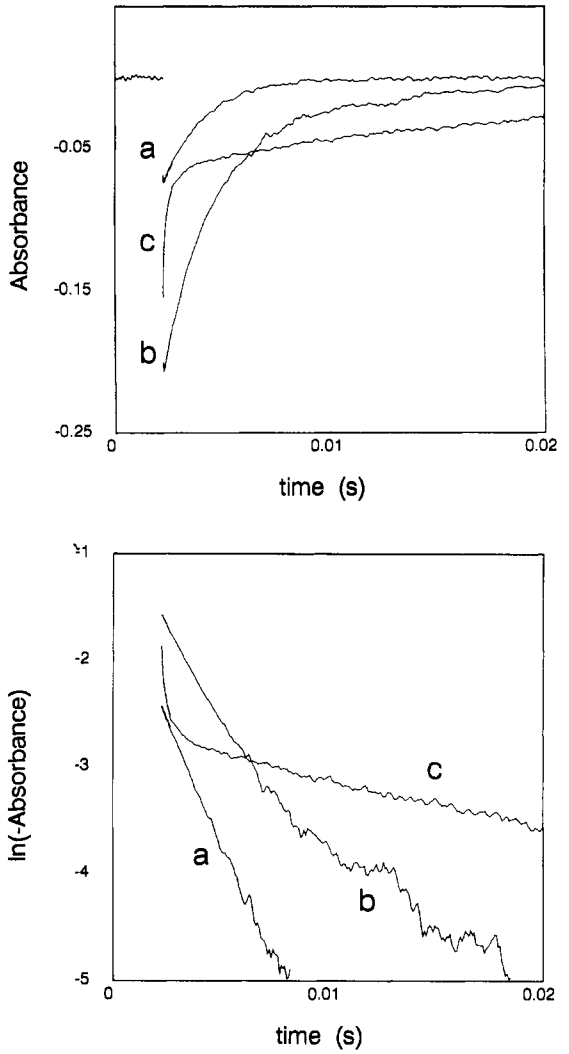
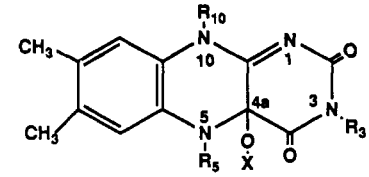


Figure 1. Kinetic traces representing the change of absorbance at 460 nm with time. The upper curves show the measured signal while the lower ones represent the same data as log linear plots. In all cases the solutions contain 5×10^{-5} M riboflavin, 2.6×10^{-4} M O_2, and 1.2×10^{-2} M HCO_2Na. Traces: (a) pH 7.73, initial radical concentration 7×10^{-5} M; (b) pH 7.73, initial radical concentration $\sim 2 \times 10^{-4}$ M; (c) pH 9.05, initial radical concentration $\sim 2 \times 10^{-4}$ M.

and therefore a substantial portion of $Fl^{\bullet-}$ reverts to Fl instead of forming $FlHO_2H$. Indeed, in order for reaction 3 to compete efficiently with reaction 6, the $O_2^{\bullet-}$ concentration must equal or exceed that of oxygen. Consequently, very high doses are needed, which, however, also brings reaction 5 into play. Obviously, the yield of the hydroperoxide per pulse will always be smaller at high than at low pH. Thus, the signal at 460 nm will be reduced whence the rate measurements become more difficult. Table I shows the initial yield of $FlHO_2H$ as a function of the pH at a dose of 200 Gy/pulse, which produces a total radical concentration of ca. 1.2×10^{-4} M. From the above points *a* and *b* it transpires that, generally, the bleaching observed at 460 nm by ca. 2×10^{-4} s after the pulse (the time during which all semiquinone radicals, $FlH^\bullet$ ($Fl^{\bullet-}$) have essentially disappeared) is due to the presence of $FlHO_2H$ and $FlH_2(FlH^-)$. The rate of reformation of fully oxidized flavin should then be given by the sum of two exponentials. Figure 1 presents kinetic traces that demonstrate the features to be described forthwith. Below pH 7.5 the kinetic traces fit to a single exponential irrespective of the dose, flavin, and O_2 concentration. This is not shown in the figure as the low-pH traces are similar to trace a. Above pH 7.5 a biphasic process is observed at high doses (see trace b in Figure 1). The rate of the rapid

(17) Massey, V.; Schopfer, L. M.; Anderson, R. F. In *Oxidases and Related Redox Systems*; King, T. E., Mason, H. S., Morrison, M. M., Eds.; Alan Liss: New York, 1988; pp 147–166.
(18) Lind, J.; Merényi, G. *Photochem. Photobiol.* **1990**, *51*, 21.
(19) Ehrenberg, A.; Müller, F.; Hemmerich, P. *Eur. J. Biochem.* **1967**, *2*, 286.
(20) Draper, R. D.; Ingraham, L. L. *Arch. Biochem. Biophys.* **1968**, *125*, 802.
(21) Land, E. J.; Swallow, A. J. *Biochemistry* **1969**, *8*, 2117.
(22) Vaish, S. P.; Tollin, G. *Bioenergetics* **1971**, *2*, 61.
(23) Faraggi, M.; Hemmerich, P.; Pecht, I. *FEBS Lett.* **1975**, *51*, 47.

Table II. Rate Constants of Decomposition of Flavin C(4a)–O–X species into Fully Oxidized Flavin and XOH[e]



X	R_3	R_5	R_{10}	k_{H_2O}, s^{-1}	k_{H1}, $M^{-1}\,s^{-1}$	k_{H2}, $M^{-1}\,s^{-1}$	k_{OH}, $M^{-1}\,s^{-1}$	k_{H_2O}/k_{D_2O}
H	CH_3	C_2H_5	TAR[a]	0.01	3×10^3	3×10^2		
OH	H	H	ribose	300	10^6	4×10^4	5×10^8	3.0 ± 0.2
OH	CH_3	H	TAR	36	5×10^5	6×10^3	5×10^7	2.5 ± 0.2
OH	H	C_2H_5	RP[b]	27	1.1×10^5	nm	no	nm
OH	CH_3	C_2H_5	TAR	28	1×10^5	1.6×10^3	no	2.2 ± 0.2
X_1[c]	H	H	ribose	820	10^6	nm	3×10^8	nm
X_2[d]	H	H	ribose	520	nm	nm	10^8	nm

[a] Tetraacetyl ribose. [b] Ribose 5′-phosphate. [c] $CH_3CH(OH)O$. [d] $(CH_3)_2C(OH)CH_2O$. [e] nm, no measured.

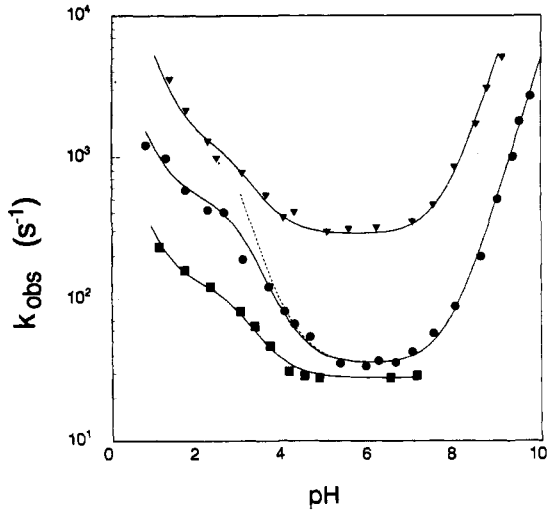


Figure 2. Measured rates of breakdown into flavin and hydrogen peroxide of three flavin 4a-OOH species in water as a function of the pH. ▼, FlH-4a-OOH; ●, 3MeTARFH-4a-OOH; ■ 5TARF-4a-OOH.

reformation of Fl increases with increasing pH while the rate of the slow component decreases in the pH 7.5–9 range, above which it assumes a pH-independent value (cf. traces b and c in Figure 1). By varying the dose in the pH range $7.5 < pH < 10$ it was possible to extract the rate constant of reaction 7 from the rapid

$$FlHOOH \rightarrow Fl + H_2O_2 \qquad (7)$$

component of the kinetic trace. Above pH 10 the rate of formation of FlHOOH is lower than that of reaction 7, making measurements impossible.

Figure 2 presents the measured rate of reaction 7 as a function of the pH for three flavin C(4a)-hydroperoxides. Apart from a small factor, the rates of the hydroperoxides of normal and 3-methylflavin show parallel pH dependences in the whole pH regime. This holds equally for the hydroperoxide derived from the 3-methyl-5-ethyltetracetylriboflavinium cation (5Et-TARF$^+$) at pH <6.5, to be discussed in the next section. All species display proton catalysis which, however, levels off at still lower pH. This suggests protonation at ca. pH 3 of the N(5) or N(10) site of the 4a-hydroperoxide to form $FlH_2O_2H^+$ or $FlEtHO_2H^+$. Since both of these sites resemble electron-deficient anilinic species, such a process is reasonable. The full lines in Figure 2 are calculated according to eq I, where the equilibrium constant, K_8, is set to

$$k_{obs} = K_8(k_{H_2O} + k_{H1}[H^+] + k_{OH}[OH^-])/(K_8 + [H^+]) + k_{H2}[H^+]^2/(K_8 + [H^+]) \qquad (I)$$

10^{-3} M for each of the three hydroperoxides and the uncatalyzed breakdown rate of FlH_2OOH^+ or $FlEtHO_2H^+$ is assumed negligible. For the 5-alkylated species, K_{OH} is set equal to zero (see below).

$$FlH_2OOH^+ \rightleftharpoons FlHOOH + H^+ \qquad (8)$$

The dotted line is computed with eq II, i.e., by neglecting the

$$k_{obs} = k_{H_2O} + k_{H1}[H^+] + k_{OH}[OH^-] \qquad (II)$$

protonation equilibrium 8. The rate constants extracted from Figure 2 and similar plots are compiled in Table II. A general-acid catalysis was observed when k_{H_2O} rates were measured at buffer concentrations of $>10^{-2}$ M. The presented values refer to low buffer concentrations where this effect is negligible.

We have made some qualitative study of the slow component in the regeneration of oxidized flavin, in order to gain insight into the reoxidation of FlH_2. The observed rates are too high to be ascribed to direct oxidation[18,24,25] of FlH_2 by molecular O_2. Above pH 9 the measured rates varied from ca. 30 to 100 s^{-1}. The rates appeared to increase with initial Fl concentration, dose, and O_2 concentration but the sensitivity toward these parameters was low. The low sensitivity is not unexpected as in these experiments the radical concentration is comparable to that of dissolved O_2, a situation favoring second-order radical reactions and hence an attenuation of linearity. The observed reoxidation process can ascribed to a combination of reaction 9 and the equilibration reaction 4 rapidly followed by $O_2/O_2^{\bullet-}$-mediated oxidation of $Fl^{\bullet-}$.

$$FlH^- + O_2^{\bullet-} \rightarrow Fl^{\bullet-} + HO_2^- \qquad (9)$$

From the data in ref 18 the rate constant k_{-4} is estimated to be between 10^5 and 10^6 $M^{-1}\,s^{-1}$, which at a typical flavin concentration of 8×10^{-5} M would result in rates between 8 and 80 s^{-1}. These values may account for all or part of the observed rates. In all events k_9 must be lower than 10^6 $M^{-1}\,s^{-1}$.

The increase of the rate of FlH^- oxidation below pH 9 is ascribed to reaction 10. From the behavior of the slow component

$$FlH^- + HOO^\bullet \rightarrow FlH^\bullet + HO_2^- \qquad (10)$$

between pH 7.5 and 9, we estimate the rate constant of reaction 10 to be 3×10^9 $M^{-1}\,s^{-1}$.

The observation of an undistorted single exponential even at $pH < 2$ puts a lower limit of 10^8 $M^{-1}\,s^{-1}$ to reaction 11. Kemal

$$FlH_2 + HOO^\bullet \rightarrow FlH^\bullet + H_2O_2 \qquad (11)$$

et al.[24] have shown that superoxide must oxidize FlH^-/FlH_2 at pH 6.4 with a rate significantly higher than that of the autoxidation. Their simulated rate constant at pH 6.4 was 5×10^7 $M^{-1}\,s^{-1}$. The present work demonstrates that $HO_2^\bullet$ is a much more

(24) Kemal, C.; Chan, T. W.; Bruice, T. C. *J. Am. Chem. Soc.* **1977**, *99*, 7272.
(25) Favaudon, V. *Eur. J. Biochem.* **1977**, *78*, 293.

efficient oxidant than $O_2^{\bullet-}$, and indeed, at pH 6.4, our estimated k_{10} yields an effective rate constant of 3×10^7 M^{-1} s^{-1}, close to the simulated value.[24]

5-Alkylated Flavin Species. The chemistry of 5-alkylated flavinium cations is different from N(5)H flavins in that the former exist as pseudobases[26,27] at sufficiently high pH. This introduces some complications in the pulse radiolysis experiments. As a preliminary to these investigations, equilibrium 12 was established

$$5EtFl^+ + H_2O \rightleftharpoons 5EtFl\text{-}4a\text{-}OH + H^+ \quad (12)$$

with 5Et-TARF$^+$ as the parent. The measurements were done by spectrophotometric titration. K_{12} was found to be 1.6×10^{-4} M (pure liquid, standard state for H_2O). This is very close to 10^{-4} M, the K_{12} value reported for the 3-Me,5-Et lumiflavinium cation (5Et-LUMF$^+$).[26] In stopped-flow experiments, both the formation and the breakdown of the pseudobase were investigated as a function of the pH. At pH values above ca. 3 k_{-12} was estimated to be $(2.5\text{–}5) \times 10^3$ M^{-1} s^{-1} and hence $k_{12} = 0.4\text{–}0.8$ s^{-1}. The uncertainty in these values derives from the buffer catalysis in these experiments. At still lower pH k_{-12} displays a significant leveling off. This indicates protonation of the pseudobase in the same way as was suggested above for the C(4a)-OOH intermediates. At higher pH the formation of the pseudobase according to eq 13 could be observed. From $K_{12} = 1.6$

$$5Et\text{-}TARF^+ + OH^- \rightleftharpoons 5Et\text{-}TARF\text{-}4a\text{-}OH \quad (13)$$

$\times 10^{-4}$ M and $K_w = 10^{-14}$ M^2, K_{13} is calculated to be 1.6×10^{10} M^{-1}. k_{13} was measured to be 2×10^8 M^{-1} s^{-1} and thus $k_{-13} = 10^{-2}$ s^{-1}. 5-Ethylflavinium mononucleotide cation (5Et-FMN$^+$) was found to have the same thermodynamics and rates of pseudobase formation as 5Et-TARF$^+$. Within small factors these rates agree with the corresponding values reported for 5Me-LUMF$^+$.[27]

In a study varying amounts of H_2O_2 were added to 5Et-TARF$^+$ samples with the pH as parameter. From spectrophotometric titration the equilibrium constant K_{14} was measured to be 2.5×10^{-4}.

$$5Et\text{-}TARF^+ + H_2O_2 \rightleftharpoons 5Et\text{-}TARF\text{-}4a\text{-}OOH + H^+ \quad (14)$$

The closeness of K_{12} and K_{14} is expected, given the ease with which Bruice et al.[10] produced the C(4a)-OOH species by treating 5Me-LUMF$^+$ with aqueous H_2O_2.

In pulse radiolysis experiments between pH 1 and ca. 5, the breakdown of 5TARF-4a-OOH was observed as the restoration of the absorbance at 600 nm, due to reformation of 5Et-TARF$^+$.

As the flavinium cation diminishes at the expense of the pseudobase above pH 4 the yield of the 5Et-TARF$^\bullet$ radical at constant dose decreases. This reflects the fact that the flavinium cation is reduced by e^-_{aq} much more efficiently than the pseudobase. However, as was confirmed in argon-purged 5Et-TARF-4a-OH solutions between pH 6 and 8, the pseudobase is also reduced to the 5Et-TARF$^\bullet$ radical, albeit to only a small extent (5–10%). This occurs presumably through the dissociative electron-transfer reaction 15. Apparently, the major reaction

$$5Et\text{-}TARF\text{-}4a\text{-}OH + e^-_{aq} \rightarrow 5Et\text{-}TARF^\bullet + OH^- \quad (15)$$

between the pseudobase and e^-_{aq} forms some unidentified reducing carbon-centered radical(s), which ultimately reduces O_2 to $O_2^{\bullet-}$. The net effect is a strong decrease of the yield of 5Et-TARF-4a-OOH at high pH. Even so, the signals are sufficiently strong to allow a reliable determination of rate constants. The operation of reaction 15 is further confirmed by the finding that at pH > 4 the breakdown of 5Et-TARF-4a-OOH results in a small increase of the absorbance at 600 nm. This shows that at the end of this process more 5Et-TARF$^+$ is present than corresponds to eq 12. Indeed, were this not so, the rate measurements could not have been made at all at pH 7.3. At later times, the small extra absorbance at 600 nm decays to the base line with rates that are exactly identical with the k_{13} values as measured by stopped flow. As $k_{13}[OH^-]$ eventually becomes larger than the breakdown rate of the hydroperoxide, k_{obs} could not be extracted above pH ca. 7.3. Even so, up to this pH no OH^- catalysis could be observed for this process (see Figure 2).

Deuterium Effect. The solvent deuterium isotope effects on the k_{H_2O} of the C(4a)-OOH species of Fl, 3Me-TARF, and 5Et-TARF$^+$ were obtained by comparing the breakdown rates of these species in H_2O and D_2O at pH 5 where both OH^- and H^+ catalyses are negligible. The pH (pD) was adjusted by titrating formate (5×10^{-3} M) with H_2SO_4 or D_2SO_4. The values obtained are compiled in Table II.

4a-Alkylperoxide of Riboflavin. When a 8×10^{-5} M riboflavin solution, purged with a mixture of N_2O/O_2 (9/1, v/v), is pulse irradiated in the presence of a large excess of *tert*-butyl alcohol (0.1 M) the β-hydroxy alkyl radical $^\bullet CH_2C(CH_3)_2OH$ is produced with a yield corresponding to ca. 85% of all radicals. The remainder consists of ca. 10% $O_2^{\bullet-}$ and 5% FlH$^\bullet$, the riboflavin semiquinone radical. The alkyl radical rapidly reacts with O_2 according to reaction 16.

$$\underset{(R^\bullet)}{^\bullet CH_2C(CH_3)_2OH} + O_2 \rightarrow \underset{(RO_2^\bullet)}{^\bullet O_2CH_2C(CH_3)_2OH} \quad (16)$$

Due to the excess of $RO_2^\bullet$, FlH$^\bullet$ will react predominantly through reaction 17, forming a C(4a)-alkyl peroxide. The

$$FlH^\bullet + RO_2^\bullet \rightarrow FlH\text{-}4a\text{-}O_2R \quad (17)$$

breakdown of FlH-4a-O_2R into Fl and RO_2H was studied in the pH interval 5–9 and the extracted rate constants are presented in Table II. Upon inspection of Table I it is found that the yield of FlH-4a-O_2R per pulse decreases more with the pH than can be accounted for by reaction 6, i.e., the oxidation of $Fl^{\bullet-}$ by O_2. The plausible reason is that $Fl^{\bullet-}$, in contrast to FlH$^\bullet$ ($pK_a = 8.3$), may react with $RO_2^\bullet$ mainly through electron transfer (reaction 18) rather than through radical coupling (reaction 17). The

$$Fl^{\bullet-} + RO_2^\bullet \rightarrow Fl + RO_2^- \quad (18)$$

rationale is that the rates of electron-transfer processes are much more sensitive toward the thermodynamic driving force than those of radical coupling reactions and that the latter are favored at comparable exothermicities. Assuming the redox potential of the $RO_2^\bullet/RO_2^-$ couple to be close to that of $HO_2^\bullet/HO_2^-$, i.e., ca. 0.75 V vs NHE, we can calculate ΔG^0 for reaction 18 to be ca. −27 kcal/mol. On the other hand, the presumptive electron-transfer reaction 19 would have a ΔG^0 of only ca. −10 kcal/mol. In this

$$FlH^\bullet + RO_2^\bullet \rightarrow FlH^+ + RO_2^- \quad (19)$$

context it can be envisaged that, in solvents (or enzymes such as the oxidases) where, contrary to the case in water, $HO_2^\bullet$[28] is a weaker acid than FlH$^\bullet$,[25] FlH-4a-O_2H may never form as a transient during the autoxidation of 1,5-dihydroflavin.

Chemistry of FlH 4a-Peroxyhemiacetals. As enzyme-bound flavin 4a-peroxyhemiacetals are generally accepted[4,5] as critical intermediates in bacterial luminescence, we thought it of interest to produce and study them in free form.

The species were produced by rapid coupling of the flavin semiquinone radical, FlH$^\bullet$, with an excess of α-hydroxymethyl- or -ethylperoxy radicals, $RCH(OH)O_2^\bullet$.

$$FlH^\bullet + RCH(OH)O_2^\bullet \rightarrow FlH\text{-}4a\text{-}O_2CH(OH)R \quad (20)$$

$CH_2(OH)O_2^\bullet$ was produced by pulse radiolysis of an N_2O/O_2 (4/1 or 9/1, v/v) purged riboflavin solution (5×10^{-5} M) containing 0.1–1 M CH_3OH, where the main reactions are

$$CH_3OH + OH^\bullet \rightarrow {}^\bullet CH_2OH + H_2O$$

$$^\bullet CH_2OH + O_2 \rightarrow (OH)CH_2O_2^\bullet$$

$$^\bullet CH_2OH + Fl \rightarrow CH_2O + FlH^\bullet$$

(26) Ghisla, S.; Hartmann, U.; Hemmerich, P.; Müller, F. *Liebigs Ann. Chem.* **1973**, 1388.

(27) Kemal, C.; Bruice, T. C. *J. Am. Chem. Soc.* **1976**, *98*, 3955.

(28) Sawyer, D. T.; Valentine, J. S. *Acc. Chem. Res.* **1981**, *14*, 393.

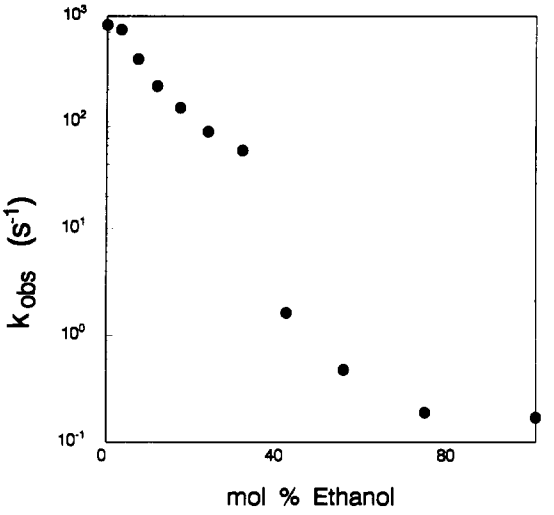


Figure 3. Measured rate of breakdown into TARF and $CH_3CH(OH)OOH$ of TARFH-4a-$O_2CH(OH)CH_3$ as a function of the molar percentage of ethanol in water.

The corresponding ethylperoxy radical was generated in aerated solutions containing, apart from riboflavin, 0.5 M CH_3CH_2OH and 0.05 M acetaldehyde. The main reactions are as follows.

$$e^-_{aq} + CH_3CHO + H_2O \rightarrow CH_3(OH)CH^\bullet + OH^-$$

$$OH^\bullet + CH_3CH_2OH \rightarrow CH_3(OH)CH^\bullet + H_2O$$

$$e^-_{aq} + Fl + H_2O \rightarrow FlH + OH^-$$

$$CH_3(OH)CH^\bullet + Fl \rightarrow CH_3CHO + FH^\bullet$$

$$CH_3(OH)CH^\bullet + O_2 \rightarrow CH_3(OH)CHO_2^\bullet$$

The protons produced during the primary radiolysis of the solvent exactly balance the OH^- ions formed in the above reactions.

The net result in both types of experiment is the eventual generation of $FlH^\bullet$ and $RH(OH)O_2^\bullet$ in a ratio of ca. 1/10.

The above processes are concluded by the radical coupling reaction 20. The breakdown of the 4a-peroxyhemiacetals into Fl and the corresponding hydroperoxide was monitored at 460 nm similarly to the case of FlH-4a-O_2H. Besides optical detection, some solutions were also monitored by means of pulsed conductometry. In a typical experiment an N_2O/O_2- (9/1, v/v) saturated aqueous solution (pH ≈ 6.3, unbuffered) containing 1 M methanol and 5×10^{-5} M riboflavin was irradiated with a pulse of ca. 130 Gy, producing a total radical concentration of ca. 7×10^{-5} M. Both the optical absorption and the conductivity were monitored. Between 7×10^{-4} and 1.6×10^{-3} s after the end of the pulse, ~6 $\times 10^{-7}$ M FlH-4a-O_2CH_2OH had broken down into oxidized flavin, as was evidenced by the optical measurement at 460 nm. During the same time the conductivity was constant. Allowing for the uncertainty in the latter measurement, it can be calculated that the breakdown of the 4a-peroxyhemiacetal produces less than 3×10^{-8} M formic acid, which is less than 5% of the total product yield. Thus reaction 21 operates while reaction 22 is undetectable.

$$FlH\text{-}4a\text{-}O_2CH(OH)R \rightarrow Fl + RCH(OH)O_2H \quad (21)$$

$$FlH\text{-}4a\text{-}O_2CH(OH)R \rightarrow Fl + H_2O + RCO_2^- + H^+ \quad (22)$$

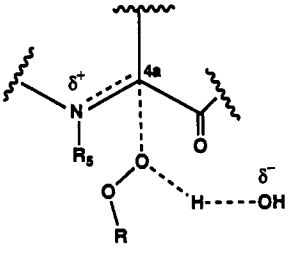
Reaction 21 (with R = CH_3) was investigated between pH 2 and 8 and was found to have almost the same kinetics as the breakdown of the corresponding 4a-O_2H species, including protonation around pH 3. For rate constants, see Table II. Just as with the 4a-O_2R species studied above, the yield of the peroxyhemiacetal per pulse diminished with increasing pH. The reasons are probably the same. At pH > 8 the peroxy radical breaks down through reaction 23. Therefore, the study of reaction 21 cannot be extended beyond ca. pH 8.

$$RCH(OH)O_2^\bullet + OH^- \rightarrow RCHO + H_2O + O_2^{\bullet-} \quad (23)$$

Solvent Effect. The rate of neutral breakdown of the 4a-peroxyhemiacetals was also probed as a function of the solvent. For sufficient solubility tetraacetylriboflavin (TARF) instead of riboflavin was used. An increase of the percentage of methanol or ethanol in water was found to dramatically increase the stability of the tetrahedral intermediate. This is displayed in Figure 3. The lowering of the breakdown rate of flavin 4a-OOH in less polar solvents than water has previously been noted.[24,29] An interesting feature of Figure 3 is the abrupt lowering of the decay rate around 35 mol % ethanol. This indicates that at this composition the transition state undergoes a qualitative change in terms of solvation. In a follow-up experiment the FlH-4a-$O_2CH(OH)CH_3$ intermediate, produced in pure ethanol, was admitted into a luminometer. No chemiluminescence above the background could be detected, although the decay rate of the 4a-species is convenient (0.17 s^{-1}) for such measurements. On the basis of a calibration against luminol, we estimate the chemiluminescence quantum yield of FlH-$O_2CH(OH)CH_3$ to be below 10^{-5}.

Discussion

Neutral and Proton-Catalyzed Breakdown of the 4a-Peroxy Species. The results have revealed that the breakdown rates of the 4a-O_2H species are only slightly affected by alkylation of the N(5) atom. In particular, the essential identity of the neutral breakdown rates of the 3-Me, 5-Et, and 3-Me, 5-Et species is evidence that the proton on the N(5) site in 5-unalkylated flavin intermediates is not moved during the rate-determining step. This is further confirmed by the closeness of the solvent deuterium isotope effects. It seems that the main effects that the solvent exerts are protonation and solvation of the HO_2^- anion in the transition state. We note that the deuterium effect for 5Et-TARF-4a-OOH is close to that found for the pseudobases of *N*-methyl heterocyclic cations.[30] In the latter case the neutral breakdown of the pseudobases was suggested to involve H_2O as a general acid protonating OH^- in the rate-determining step. Adopting a similar view, we picture the transition state of the breakdown of the 4a-peroxides according to A. We can thus



A

safely conclude that the breakdown of both alkylated and unalkylated 4a-species is initiated by a heterolysis leaving behind an N(5)-alkylated or N(5)-protonated flavinium cation. The ratio between the breakdown rates of the 4a-OOH and 4a-OH intermediates of 5Et-TARF$^+$ is ca. 3×10^3, which is very close to 10^4, the ratio of the acid dissociation constants (on molar basis) of H_2O_2 and H_2O. This is in keeping with heterolysis. The small differences in the deuterium effects reflect that the nonalkylated flavinium cation is solvated somewhat better than the alkylated one through hydrogen bonding. In ref 16 the neutral breakdown rate of riboflavin 4a-O_2H was shown to have a large negative entropy of activation (ca. −22 eu). This is consistent with the active participation of several water molecules in the transition state. The large drop in the rate constant of heterolysis of C(4a)-FlH-$O_2CH(OH)CH_3$ at 35 mol % ethanol (see Figure 3) is also indicative of the involvement of more than one water molecule in the elementary step. A simplified view would be to assume that three H_2O molecules participate in the solvation of the transition state. Once the water content falls below two-thirds of the solvent

(29) Kemal, C.; Bruice, T. C. *J. Am. Chem. Soc.* **1979**, *101*, 1635.
(30) Bunting, J. W.; Meathrel, W. G. *Can. J. Chem.* **1973**, *51*, 1965.

composition an ethanol molecule may replace a H_2O in the solvation shell.

The decomposition of 4a-peroxides has been studied in several solvents and it was concluded that the solvent proticity was the main factor affecting the reaction.[31]

Perusal of Table II discloses that the breakdown rates of FlH-4a-OOH, FlH-4a-OOR, and FlH-4a-OOCH(OH)R are very close. Recalling that the pK_a of alkyl hydroperoxides are all in the vicinity[32] of 12, this observation further supports the heterolysis model.

It may seem surprising that the 4a-species breaks down into an N(5)-protonated flavinium cation as at no pH is this species thermodynamically stable. It is known that the first protonation of flavins occurs at the N(1) site.[33] Using reasonable assumptions based on the observed acid–base properties of different alkylated flavins, Eberlein and Bruice[34] utilized a thermochemical cycle to calculate the microscopic pK_a of the N(5)-protonated lumiflavinium cation to be −3.8. In view of the sound assumptions made, this value is probably accurate within 0.5 pH unit. Given the fact that acid–base equilibria with N-acids are rapid in water,[35] the amount of the N(5)-protonated flavinium cation, never predominant, should be dictated by the pH in agreement with its microscopic pK_a value. In the sense of the above discussion, it is also easy to understand the parallel response of normal and 5-alkyl flavin peroxides to proton catalysis. Here, the proton acts as a general acid bringing about the expulsion of neutral H_2O_2 or an other hydroperoxide

$$\text{FlH-4a-OOH} + H^+ \rightleftarrows \text{Fl-N(5)-H}^+ + H_2O_2$$

$$\text{Fl-N(5)-H}^+ \rightleftarrows \text{Fl} + H^+$$

This mode of catalysis ceases to hold once the 4a-hydroperoxide becomes thermodynamically protonated. The weakening of proton catalysis makes sense as the overall positive charge should block the expulsion of the hydroperoxide. Although we believe the protonation site of the 4a-species to be the N(5) atom we cannot be sure and therefore refrain from proposing a special model for the weak proton catalysis at low pH.

Hydroxide Ion Catalysis. With all the 5-unalkylated flavin 4a-peroxy species the operation of OH^- catalysis is observed. In the case of the hydroperoxides, up to pH 10 the OH^--catalyzed reaction does not level off. We feel that, once the OOH group is deprotonated, the rate of OH^- catalysis should plummet owing to O_2^{2-} being a poor leaving group. In this sense the above observations imply that the pK_a of the 4a-OOH group is above 10 although it is probably below 11.7, the pK_a of H_2O_2. Our estimate is thus significantly higher than previous ones.[25,36]

The rate constants k_{OH} range from 5×10^7 to 5×10^8 M^{-1} s^{-1} (see Table II), irrespective of 3-alkylation or substitution of OOH for OOR. 5-Alkylation on the other hand suppresses OH^- catalysis. Thus, OH^- operates on the N(5) proton. Whether this is done by sequential proton abstraction from N(5)–H, in accordance with the latter's microscopic pK_a, followed by expulsion of HO_2^-, or whether OH^- acts as a general base in a concerted process cannot be decided. The latter mode of reaction was suggested in ref 37 for the OH^--catalyzed breakdown of C(4a)-maleimidyl flavins. However, in this case the bond to be broken was a C–C bond and the measured rates were only ca. 10^4 M^{-1} s^{-1}. As in the sequential model $k_{obs} \leq k_{deprotonation}$ it necessitates a microscopic $pK_a < 17$ for the N(5) proton. It is known that an OH or OOR group in α position lowers the pK_a of alcohols by ca. 3–4 units.[38] Such an effect would be expected for amines as well although this issue has not been explored in the literature. Recently,[39] the second pK_a of 1,5-dihydroflavin was determined by means of ^{15}N NMR to be ca. 25. The pK_a of the N(5)H group in 4a,5-dihydroflavin should be lower, the latter species being a neutral molecule. If, in addition, the 4a-peroxy substituent further increases the acidity, the microscopic pK_a of the N(5)H group in FlH 4a-peroxides may well drop below 17.

Table III. Equilibria and Rates[a] of the Autoxidation of Reduced N(5)-H Flavin

	pH independent			at pH 7		
equilbrm	K	k_f	k_r[b]	K[c]	k_f	k_r
26[d]	10^{-3}	2×10^2	2×10^5	7×10^{-2}	3×10^3	4×10^4
27[e]	10^{15}	10^{9e}	10^{-6}	5×10^{12}	5×10^{8e}	10^{-4}
25[f]	10^8	3×10^2	3×10^{-6}	10^8	4×10^2	4×10^{-6}

[a] K_{26} is dimensionless, while K_{27} and K_{25} have the units of M^{-1} and M, respectively. k_{f25} and k_{r27} have units in s^{-1} while the remaining rate constants are expressed in M^{-1} s^{-1}. [b] Calculated from K and k_f. [c] The pH-independent K adjusted for pH. [d] Taken from ref 18. [e] K_{27} was calculated by combining K_{25} and K_{26} with the global equilibrium constant for $FlH_2 + O_2 \rightleftharpoons Fl + H_2O_2$. The latter was calculated from the redox potentials in refs 20 and 28. [f] This work.

Energetics of 1,5-Dihydroflavin Autoxidation. Table II reveals that the k_{H1} values vary little between 5-alkyl and 5-H flavin 4a-hydroperoxides. This suggests that K_{14} and K_{24} are close to each other and thus we assume $K_{24} \simeq 10^{-4}$.

$$5\text{HFl}^+ + H_2O_2 \rightleftharpoons \text{FlH-4a-OOH} + H^+ \qquad (24)$$

Combining K_{24} with the above discussed microscopic $pK_a \simeq -4$ of 5H-Fl^+, we obtain the important equilibrium constant $K_{25} \simeq 10^8$ M.

$$\text{FlH-4a-OOH} \rightleftharpoons \text{Fl} + H_2O_2 \qquad (25)$$

This value is probably accurate within 2 orders of magnitude, implying 10^7 M $< K_{25} < 10^9$ M.

Utilizing K_{25} together with other known equilibria one can resolve the complete autoxidation of normal 1,5-dihydroflavin into individual steps. The relevant equilibria and rates are compiled in Table III while the process is represented pictorially in Figure 4. With the help of the cartoon, several issues can be addressed. It is seen that $K_{28} = K_{26}K_{27}$ is very large, implying that any equilibrium between FlH_2, O_2, and FlH-4a-OOH should be impossible to observe in water.

$$\text{FlH}_2 + O_2 \rightleftharpoons \text{FlH}^\bullet + HO_2^\bullet \qquad (26)$$

$$\text{FlH}^\bullet + HO_2^\bullet \rightleftharpoons \text{FlHO}_2\text{H} \qquad (27)$$

$$\text{FlH}_2 + O_2 \rightleftharpoons \text{FlHO}_2\text{H} \qquad (28)$$

Furthermore, since these equilibria refer to neutral species they should be fairly invariant with the solvent, changing perhaps by 2 orders of magnitude when the species is transferred to an aprotic environment. In addition, since the pK_a values of FlH_2 and FlH-4a-OOH differ by only ca. 4 units (see above), K_{28} should not diminish by more than a factor of 10^4 upon increasing the basicity of the solutions. It follows that, even with the uncertainties indicated, equilibrium 28 should be shifted completely to the right in all chemically realistic situations. Thus, in our opinion, the reported observations[25,40] attesting to the rapid attainment of eq 28 in either water or DMF are only apparent.

Inspection of Table II reveals that the ratio between the rates of heterolysis and homolysis of the C(4a)-O bond in water is ca. 10^8. As the polarity and proticity of the solvent are diminished, the heterolysis rate should decrease while the homolysis should remain virtually unaffected. We conclude, therefore, that by simply varying the solvent the stability of the 4a-OOH species can be increased by up to ~8 orders of magnitude with respect

(31) Merényi, G.; Lind, J. In *Tenth International Symposium of Flavins and Flavoproteins*; Como, Italy, in press.
(32) Everett, A. J.; Minkoff, G. J. *Trans. Faraday Soc.* **1953**, *49*, 410.
(33) Müller, F. In *Topics in Current Chemistry 108*; Boschke, F. L., Ed.; Springer: Berlin, 1983; pp 71–108.
(34) Eberlein, G.; Bruice, C. T. *J. Am. Chem. Soc.* **1983**, *105*, 6679.
(35) Eigen, M. *Angew. Chem., Int. Ed. Engl.* **1965**, *3*, 1.
(36) Eberlein, G.; Bruice, C. T. *J. Am. Chem. Soc.* **1983**, *105*, 6685, and references therein.
(37) Venkataram, U. V.; Bruice, T. C. *J. Am. Chem. Soc.* **1984**, *106*, 5703.
(38) Bell, R. P. *Adv. Phys. Org. Chem.* **1966**, *4*, 1.
(39) Ghisla, S.; Macheroux, P.; Müller, F.; Rüterjans, H. In *Tenth International Symposium of Flavins and Flavoproteins*; Como, Italy, in press.
(40) Massey, V.; Palmer, G.; Ballou, D. In *Oxidases and Related Redox Systems*; King, T. E., et al., Eds.; University Park Press: Baltimore, MD, 1973; Vol. 1, pp 25–49.

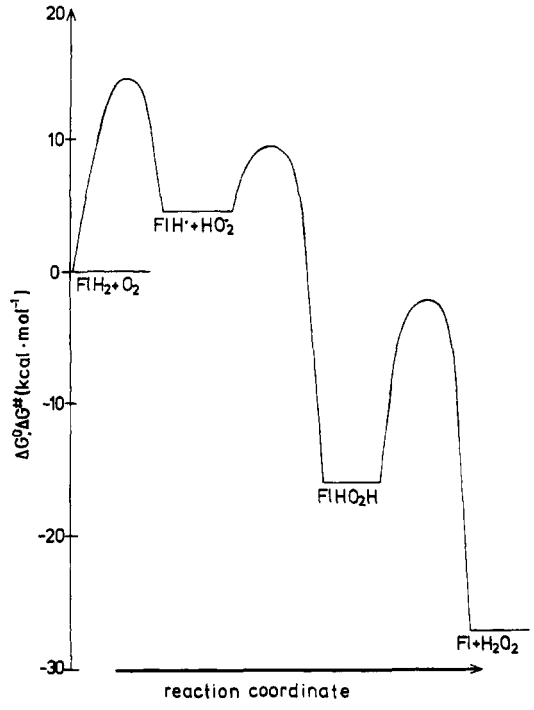


Figure 4. Reaction cartoon over the various steps constituting the overall conversion of 1,5-dihydroflavin and oxygen into fully oxidized flavin and hydrogen peroxide.

to their breakdown into oxidized flavin and H_2O_2.

Now, for most hydroperoxides, ROOH, the pK_a values[32] and O–H bond dissociation enthalpies[41] are close to that of H_2O_2. Therefore, the equilibrium constants K_{27} and K_{25} as well as the corresponding rate constants k_{-27} and k_{25} should not change much if H is substituted for R. This is in keeping with the finding that k_{25} for 4a-OOH, 4a-OOCH(OH)CH_3, and 4a-OOCH_2C(OH)$(CH_3)_2$ are equal within a factor of ca. 3. In conclusion, the above considerations for the 4-OOH species should also apply to other 4a-peroxides.

Homolysis of the Peroxidic O–O Bond in Relation to Monooxygenase Activity. In the foregoing discussion, the energetics of heterolysis and homolysis of the C(4a)–O bond in the flavin peroxides has been elucidated in some detail. However, the most fascinating reactions in the flavin enzymes involve the rupture of the O–O bond. For some reactions, this rupture was shown by Bruice[12] to be a heterolysis, and the good monooxygenating activity of FlH-4a-O_2H has been rationalized in terms of the low pK_a (9.2) of the pseudobase, FlHOH, as compared to that of ordinary alcohols.

There is, however, an increasing number of observations in the literature that appear to implicate a homolytic cleavage of the peroxydic O–O bond as the rate-determining step in the monooxygenating process. Bacterioluminescence[7] and the search[42] for the identity of intermediate II in phenolhydroxylases are two major cases in point.

In order to establish the feasibility of O–O cleavage in successful competition with the rather facile C(4a)–O rupture, we would need to estimate the O–O bond strength in flavin peroxides. Until recently no sufficient data were extant for such a calculation. The breakthrough is provided by a work of Mager et al.,[43] in which the one-electron oxidation potential of 5Et-LUMF-4a-OH was determined in acetonitrile.

$$\mathrm{FlOH^{\bullet+}} + e^- \rightarrow \mathrm{FlOH} \qquad (29)$$

(41) Cremer, D. In *The Chemistry of Peroxides*; Patai, S., Ed.; John Wiley: Chichester, 1983; p 43.
(42) Anderson, R. F. In *Oxidases and Related Redox Systems*; King, T. E., Mason, H. S., Morrison, M. M., Eds.; Alan Liss: New York, 1988; pp 167–178.
(43) Mager, H. I. X.; Sazou, D.; Liu, Y. H.; Tu, S.-C.; Kadish, K. M. *J. Am. Chem. Soc.* **1988**, *110*, 3759.

Table IV. O–O Bond Dissociation Enthalpies,[a] ΔH^0_{BDE}, of Peroxides (kcal/mol)

compound	ΔH^0_{BDE}	compound	ΔH^0_{BDE}
HO–OH	51	$CH_3C(O)O$–$OC(O)CH_3$	30
CH_3O–OH	45	FlO–OH	<26
CH_3O–OCH_3	38		

[a] Except for FlO–OH, the data were taken from ref 41.

This potential was found surprisingly low, with E^0_{29} = 1.29 V vs NHE. Working with pulse radiolysis[44] in water by utilizing the $N_3^\bullet$ radical as oxidant (E^0 ($N_3^\bullet/N_3^-$) = 1.33 V), we found E^0_{29} to be ≤1.12 V at pH 7. Furthermore, $\mathrm{FlOH^{\bullet+}}$ appears to be a rather strong acid,[45] deprotonating well below pH 5.5. The resulting neutral radical, $\mathrm{FlO^\bullet}$, is probably a zwitterion, with the unpaired electron mainly on N(5). These results imply that E^0_{30} ≤ 1.53 V in water.

$$\mathrm{FlO^\bullet} + H^+ e^- \rightarrow \mathrm{FlOH} \qquad (30)$$

In order to calculate ΔG^0_{31} for the O–O homolysis (reaction 31)

$$\mathrm{FlO\text{-}OH} \rightarrow \mathrm{FlO^\bullet} + \mathrm{OH^\bullet} \qquad (31)$$

we need the following thermodynamic cycle (all species in water):

$$\mathrm{FlO_2H} + H_2O \rightleftharpoons \mathrm{FlOH} + H_2O_2 \qquad (32)$$

$$H_2O_2 \rightarrow 2\ \mathrm{OH^\bullet} \qquad (33)$$

$$\mathrm{OH^\bullet} + H^+ + e^- \rightarrow H_2O \qquad (34)$$

$$\mathrm{FlO^\bullet} + H^+ + e^- \rightarrow \mathrm{FlOH} \qquad (30)$$

Then, $\Delta G^0_{31} = \Delta G^0_{32} + \Delta G^0_{33} - 23.06(E^0_{34} - E^0_{30})$. The essential identity of K_{12} and K_{14} (see Results) implies that $\Delta G^0_{32} \approx 0$. From the gaseous O–O bond dissociation enthalpy of H_2O_2, $\Delta H^0_{33}(g)$ = 51 kcal/mol,[46] $T_{298}\Delta S^0_{33}(g)$ = 9.6 kcal/mol,[47] and the ΔG^0 values of hydration of H_2O_2 (−6.8 kcal/mol)[48] and of $\mathrm{OH^\bullet}$ (−2.4 kcal/mol),[49] ΔG^0_{33} = 43.4 kcal/mol is calculated in water. The value of E^0_{34} is 2.72 V[49] and $E^0_{30} \leq 1.53$ V. Thus, $\Delta G^0_{31} \leq 0 + 43.4 - 23.06(2.72 - 1.53)$ = 16 kcal/mol. Allowing for ca. 10 kcal/mol to account for the $T\Delta S^0$ effect and transfer to the gas phase (note that for reaction 33 the correction is no more than 8 kcal/mol), the gaseous bond dissociation enthalpy, $\Delta H^0_{31}(g)$, turns out to be lower than 26 kcal/mol. Now, this is a staggeringly low figure, showing that the O–O bond in FlO–OH is not only weaker than in normal alkyl hydroperoxides, but is even below that of diacyl peroxides. The bond strengths are compiled in Table IV. The very weak O–O bond in flavin peroxides hinges on the low redox potential of $\mathrm{FlO^\bullet}$ as compared to ordinary alkoxyl radicals. Indeed, the flavin pseudobase resembles more a phenol than an aliphatic alcohol, both in terms of its acidity and its electrochemical properties.

The free energy in water of O–O bond fission of flavin 4a-O_2H, $\Delta G^0_{31} < 16$ kcal/mol, can be compared to $-\Delta G^0_{27} \approx 21$ kcal/mol, its free energy of C(4a)–O bond homolysis (calculated from Table III). In the preceding section it has been argued that, as the solvent becomes less and less polar and protic, the heterolysis of the C(4a)–O bond is slowed down and that the transition state more and more approaches that of the C(4a)–O homolysis. Given the finding that $\Delta G^0_{31} < -\Delta G^0_{27}$ one can imagine that, in sufficiently apolar enzyme pockets, homolysis of the O–O bond may become more favorable than other processes. In fact, we propose that the uniqueness of flavin 4a-peroxides may reside in the fact that the C(4a)–O and O–O bonds are of comparable strength. This allows

(44) Unpublished results.
(45) Mager, H. I. X.; Tu, S.-C.; Merényi, G.; Lind, J., to be published.
(46) *J.A.N.A.F. Thermochemical Tables*; NSRDS-NBS 37; 1971, National Standard Reference Data Series; U.S. National Bureau of Standards: Washington, DC, 1971.
(47) Benson, S. W.; Shaw, R. In *Organic Peroxides*; Swern, D., Ed.; Wiley: New York, 1970; pp 105–139.
(48) Wagman, D. W.; Evans, W. H.; Parker, V. B.; Schumm, R. H.; Halow, I.; Bailey, S. M.; Churney, K. L.; Nuttall, R. L. The NBS Tables of Thermodynamic Properties. Selected Values for Inorganic and C1 and C2 Organic Substances in SI Units. *J. Phys. Chem. Ref. Data* **1981**, *11* (Suppl. No. 2).
(49) Schwarz, H. A.; Dodson, R. W. *J. Phys. Chem.* **1984**, *88*, 3643.

the enzyme to achieve selectivity by subtle manipulations of the environment. For example, as the C(4a)–O and O–O bonds are adjacent, the enzyme could slightly stretch one of them while compressing the other. Although intermediate II in phenol hydroxylases is yet to be positively identified, the suggestion that its formation may involve a radical pair[42] appears entirely plausible in view of the facile O–O bond cleavage. The proposed capture of the OH• radical by the phenolic substrate in concert with O–O homolysis would certainly lower the energy of the transition state even further.

The model for bioluminescence as proposed in refs 6, 50, and 51 is even more appealing. First, in analogy with alkyl peroxides (see Table IV), the O–O bond in the peroxyhemiacetal should be weaker than in the hydroperoxide (perhaps by as much as 7 kcal/mol). The invocation in these models of an intramolecular electron transfer (CIEEL) is not necessary, as the facile O–O homolysis is directly connected with the low redox potential of the pseudobase radical. Obviously, when electron-donating substituents are introduced into the flavin moiety, this redox potential will further diminish, thus explaining the substituent effect on the bioluminescent reaction rate.[51] The uniqueness of the flavin peroxyhemiacetal to produce chemiluminescence is readily understood from the homolysis model. Clearly, from the point of view of energy content, the low reduction potential of the pseudobase radical proves a disadvantage in the back electron transfer from any complementary radical, R•, to FlO•. Indeed, with ca. 410 nm as the shortest wavelength emitted in the bioluminescence, the total exothermicity in this process has to exceed 3.0 eV. This demands R• to be a very strong reductant with an E^0 value below −1.5 V. Few radicals qualify, but the α-dihydroxyalkyl radical, $RC^{\bullet}(OH)_2$ (which is proposed[6,50,51] to form in a rapid rearrangement of the alkoxy radical, the primary product of the hemiacetal homolysis), is an ideal candidate. The one-electron redox potentials of α-hydroxy radicals in water are known to be in the vicinity of −1.3 V.[52] The reducing properties of α-dihydroxyalkyl radicals should be better, and the $CH_3C^{\bullet}(OH)_2$ radical was shown[53] to be a rapid one-electron reductant. From the data in a recent work[54] we estimate the gaseous bond dissociation enthalpy for reaction 35 to be 92 ± 2 kcal/mol. Assuming

$$CH_3CH(OH)_2 \rightarrow CH_3C^{\bullet}(OH)_2 + H^{\bullet} \qquad (35)$$

similar hydration free energies for the hydroxylic species in reaction 35 and utilizing literature values for the free energies of formation in water of acetic acid, acetaldehyde, and water as well as the hydration equilibrium of acetaldehyde, we calculate $E^0_{36} = -1.9 \pm 0.2$ V.

$$CH_3COOH + H^+ + e^- \rightarrow CH_3C^{\bullet}(OH)_2 \qquad (36)$$

E^0_{36} is sufficiently negative to satisfy the above-mentioned exothermicity criterion. Of course, the long-standing problem of the fluorescent[7,55] (or rather nonfluorescent) properties of the flavin pseudobase as a function of solvent or enzyme still awaits complete clarification.

In conclusion, while not giving details, the exceptionally weak O–O bond in flavin 4a-peroxides provides a powerful thermodynamic rationale for radical-mediated monooxygenation processes.

Experimental Section

Pulse radiolysis was carried out at room temperature with a 7-MeV microtron accelerator. Details of the setup[56] and the computerized optical detection system[57] have been described elsewhere. The length of the applied pulses was between 1×10^6 and 4×10^{-6} s, corresponding to doses of 130–500 Gy. The concentration of radicals generated in such pulses was in the range $(0.7–2.8) \times 10^{-4}$ mol/dm^3. Dosimetry was performed by means of aerated aqueous solutions containing 10 mM $Fe(CN)_6^{4-}$ employing $Ge = 2.8 \times 10^{-5}$ J/m^2. A halogen lamp equipped with a 460-nm interference filter was used as the analyzing light.

Pulsed conductivity was performed in balanced cells. The measured signals were calibrated against a dosimeter to yield directly the amount of protons produced. The dosimeter was an argon-flushed aqueous solution saturated with CCl_4. During irradiation of the dosimeter HCl is formed according to the reaction sequence

$$H_2O \rightarrow H^+, e^-_{aq}, OH^{\bullet}, H^{\bullet}$$

$$e^-_{aq} + CCl_4 \rightarrow CCl_3^{\bullet} + Cl^-$$

The yield of HCl is 3.1×10^{-7} mol/J.

The specific conductivities of H^+, Cl^-, and HCO_2^- were taken from the literature.

For chemiluminescence detection an ethanol solution containing 10^{-4} M TARF, 5×10^{-4} M O_2, and 0.1 M acetaldehyde was pumped through an irradiation chamber into a LKB luminometer. Calibration of the luminometer was done by a persulfate/luminol/H_2O_2 system.[58] The transit time between irradiation and detection was ca. 3 s.

Stopped-flow experiments were performed on a High Tech SF-3L unit with a mixing time of 2 ms.

Tetraacetylriboflavin (TARF) was synthesized by heating a suspension of riboflavin in a 1/1 mixture of AcOH/pyridine in the presence of benzenesulfonyl chloride. TARF was methylated to afford 3-methyl-TARF (3Me-TARF) by following ref 59. 3-Methyl-5-ethyl-TARF$^+$-ClO_4^- (5TARF$^+ClO_4^-$) was produced from 3Me-TARF according to ref 26. 5-Ethyl-FMN$^+ClO_4^-$ (5FMN$^+ClO_4^-$) was prepared from FMN according to prescriptions in ref 11. Riboflavin, flavin mononucleotide, ethanol, acetaldehyde, methanol, sodium formate, and the various buffers were of the highest commercial quality available. D_2O (97%) was purchased from Norsk Hydro. Water was triple distilled in quartz.

Acknowledgment. Thanks are due to the Swedish Natural Science Research Council for financial support.

(50) Mager, H. I. X.; Addink, R. In *Flavins and Flavoproteins*; Bray, R. C., Engel, P. C., Mayhew, S. G., Eds.; de Gruyter: Berlin, 1984; p 37.
(51) Macheroux, P.; Eckstein, J.; Ghisla, S. In *Flavins and Flavoproteins*; Edmondson, D. E., McCormick, D. B., Eds.; de Gruyter: Berlin, 1987; pp 613–619.
(52) Schwarz, H. A.; Dodson, R. W. *J. Phys. Chem.* **1989**, *93*, 409.
(53) Schuchmann, M. N.; von Sonntag, C. *J. Am. Chem. Soc.* **1988**, *110*, 5698.
(54) Lind, J.; Merényi, G. *The Seventh Tihany Symposium on Radiation Chemistry*; Akademiai Kiado: Budapest, in press.
(55) Kurfürst, M.; Macheroux, P.; Ghisla, S.; Hastings, J. W. *Biochim. Biophys. Acta* **1987**, *924*, 104.
(56) Rosander, S. Thesis, The Royal Institute of Technology, Stockholm, 1974; TRITAEEP-74-16, p 28.
(57) Eriksen, T. E.; Lind, J.; Reitberger, T. *Chem. Scr.* **1976**, *10*, 5.
(58) Rauhut, M. M.; Semsel, A. M.; Roberts, B. G. *J. Org. Chem.* **1966**, *31*, 2431.
(59) Hemmerich, P. *Helv. Chim. Acta* **1964**, *47*, 464.