O_2-AFFINITY OF FLAVIN RADICAL SPECIES AS STUDIED BY PULSE RADIOLYSIS

Moshe FARAGGI
Nuclear Research Center, Negev, Beer Sheva, Israel

and

Peter HEMMERICH
Fachbereich Biologie, Universität Konstanz, 775 Konstanz, West Germany

and

Israel PECHT
Weizmann Institute of Science, Rehovot, Israel

Received 3 December 1974

1. Introduction

The activation of molecular oxygen* by metal free biological systems is presently receiving much attention and investigation. For the case of flavins in *aqueous solution*, Massey et al. [1] postulated 'adduct formation' of O_2 and the activating species, reduced flavin ($Fl_{red}H_2$), (scheme 1, A) in obvious violation of spin conservation. This was based upon kinetic evidence obtained from stopped flow and irrespective of the type of bonding involved between Fl und O_2. Later on, studies of the same group [2,3] with protein bound flavin disclosed spectra of short-lived FlH_2-O_2 intermediates in the catalytic pathway of certain monooxygenases ($\lambda_{max} \approx 370$–$410$ nm).

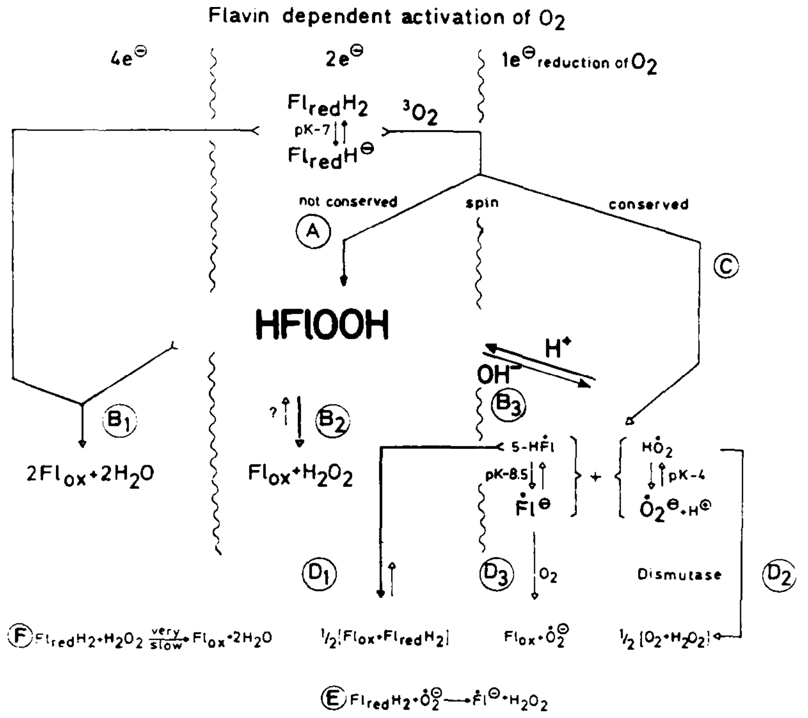
At an earlier stage, Mager and Berends [4,5,6] had provided evidence for an intermediate species in *aprotic* autoxidation of flavin derivatives, which circumvented H_2O_2 formation (scheme 1, B_1). They proposed for the active intermediate a peroxide structure HFl-OOH. From this it follows that HFl-OOH is an efficient autocatalyst of $Fl_{red}H_2$ autoxidation under aprotic conditions, while in aqueous solution it decays rapidly as discussed below.

Autocatalysis of $Fl_{red}H_2$ autoxidation had earlier been found to occur also in aqueous solution [7] and was then thought to be due to the intermediate formation of flavin radical (scheme 1, C). Later on, however, it became obvious from studies of Hemmerich et al. [8], that autocatalysis of $Fl_{red}H_2$ autoxidation by O_2 is also found under aprotic conditions and in suitably modified flavin systems where – in analogy to the biological case of flavoprotein oxidases – no intermediate radical formation is observed, in agreement with chemical structure.

As next best candidate for the autocatalytic species, superoxide anion $\dot{O}_2^-$ was demonstrated (step E) by Massey et al. [1] with the aid of superoxide dismutase (step D_2 favored). Stoichiometric amounts of O_2^-, however, could only be detected at pH > 8. The authors suggested this fact as being due to the well known acceleration of superoxide dismutation (pathway D_2). But it is equally obvious that pathway B_3 (scheme 1) is efficiently reversed at pH < 8, i.e. whenever protonation of the radical anion $\dot{Fl}^-$ ($pK_{HFl}^{H} \approx 8.4$ [9]) becomes

---

* The use of the term 'O_2-activation' instead of O_2-reduction in the biochemical literature is obviously meant to emphasize the initial polarization of O_2-symmetry and should not be misunderstood in the sense of formation of spectroscopically excited states.



Scheme 1: Pathways of flavin mediated O_2-reduction. Note that B_1 (unpolar medium) and B_3 (polar medium) are both autocatalytic, the catalysts being HFl-OOH for B_1 and $\dot{Fl}^-$ (D_3) as well as $\dot{O}_2^-$ (E) for B_3. The redox balance is $2\ Fl_{ox} + 2H_2O$ (= $4e^-$-transfer) for B_1, $Fl_{ox} + H_2O_2$ (= $2e^-$-transfer) for B_2 and $1/2\ Fl_{ox} + \dot{O}_2^-$ (= $1e^-$- transfer) for B_3, simulating oxygenase (B_1) and e^--transferase (B_3) action.

predominant. The presence of superoxide dismutase, on the other hand, might force this equilibrium towards the right even at lower pH, thus distorting the whole picture.

Fl_{ox} and H_2O_2 (step F) as final products of the autoxidation can be eliminated from the list of potential catalysts, since extraneous addition of either species did not induce catalysis of $Fl_{red}H_2$ autoxidation in most flavin systems.

Hence, one is tempted to see HFl-OOH as an autocatalytic intermediate by itself and source of 'active oxygen', which could account for three different modes of O_2-reduction (scheme 1, B_{1-3}), the aprotic medium reflecting flavoprotein oxygenases (B_1), aqueous acid conditions reflecting flavoprotein oxidases (B_2), and alkaline media electron-transferring flavoproteins (B_3). We even suspect a different HFl-OOH isomer being responsible for each of the three reactions [10].

In order to verify this scheme, one of the requirements is the direct measurement of O_2-affinities inherent in the two flavosemiquinone species that may exist under physiological conditions, i.e. the blue [11,12] $H\dot{Fl}$ and the red [9,12] $\dot{Fl}^-$. This problem has been tackled first by Vaish and Tollin [13] studying by flash photolysis the flavin–phenol system

$$Fl_{ox} + C_6H_5\text{-}OH \underset{\text{dark}}{\overset{h\nu}{\rightleftarrows}}$$

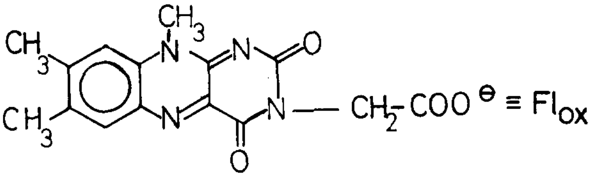
$$H\dot{Fl} + C_6H_5\text{-}\dot{O} \xrightarrow{O_2} \text{cf. scheme 1}$$

This method has the disadvantage, that the photoreduction of flavin is reversed in the dark and that the presence of phenol and phenoxyl redicals may give rise to disturbing side products and side effects. Hence, we have now used flavin radicals produced by reaction of Fl_{ox}

with hydrated electrons generated by pulse radiolysis [14]. These experiments should decide which of the radical pathways (by way of C or $A \longrightarrow B_3$ (scheme 1)), are essential in flavin dependant O_2-activation, and over the need to search for mechanisms of rapid intersystem crossing from triplet oxygen other than the 'trivial' but biologically dissatisfactory pathway C.

2. Materials and methods

Lumiflavin-3-acetate



prepared according to Hemmerich [15] was used throughout this study.

The experimental procedure of pulse radiolysis has been described in detail earlier [14,16]. All solutions contained 0.1 M *t*-butanol as scavenger for $\dot{\mathrm{O}}\mathrm{H}$ radicals. Saturation by argon, oxygen or air was achieved by the bubbling for ca. 20 min. Various concentrations of O_2 were made up by mixing the appropriate volumes of argon- and O_2-saturated solutions. The pH of the solution was maintained either by the lumiflavin acetate or by 10^{-2} M borate or phosphate buffers. Total Fl concentrations varied between $1 \cdot 10^{-5}$ to $5 \cdot 10^{-4}$ M, up to 1% of which were reduced by e^-_{aq} produced by a single pulse of 5 MeV electrons [16].

3. Results

The reaction of hydrated electrons (e^-_{aq}) with Fl_{ox} in anaerobic solution leads to the formation of the radical anion $\dot{\mathrm{Fl}}^-$ in a reaction, which is first order in each reactant, and at a rate of 3.10^{10} M^{-1} sec^{-1}. The spectrum of the species produced at 4<pH<8 is that of the neutral radical $\mathrm{H\dot{F}l}$ (fig.1). Since the p*K* of $\mathrm{H\dot{F}l}$ is 8.4 [9], production of $\dot{\mathrm{Fl}}^-$ is followed by immediate protonation at lower pH. The flavin radicals are found to decay anaerobically in a reaction, which is second order in the decaying species, i.e. by dismutation (fig.2). This reaction is slower for $\dot{\mathrm{Fl}}^-$ because of charge repulsion and reaches at pH 7 a specific rate of 2.10^8 M^{-1} sec^{-1}. Up to this point our data are essentially an extension of the observations of Land and Swallow [14], who obtained in the anaerobic study $k = 5.7 \times 10^8$ M^{-1} sec^{-1} for FMN at pH 5 for the $\mathrm{H\dot{F}l}$ ($\lambda_{max} = 570$ nm) dismutation (D_I, scheme 1). Our slightly lower values may be due to the fact that our Fl-model (lumiflavin-

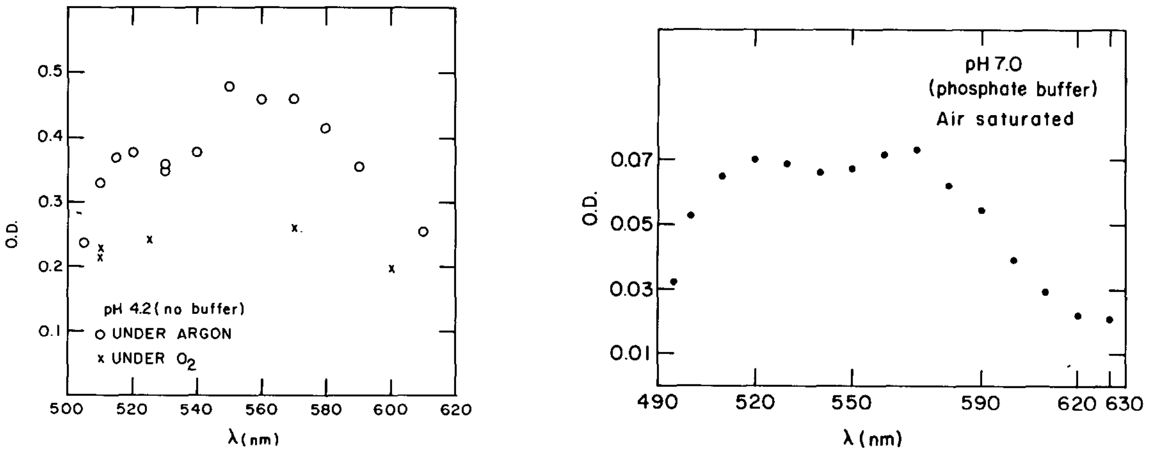


Fig.1. The transient absorption spectra produced by the reaction of e^- aq with lumiflavin-3-acetate under different conditions of pH and saturating gases.

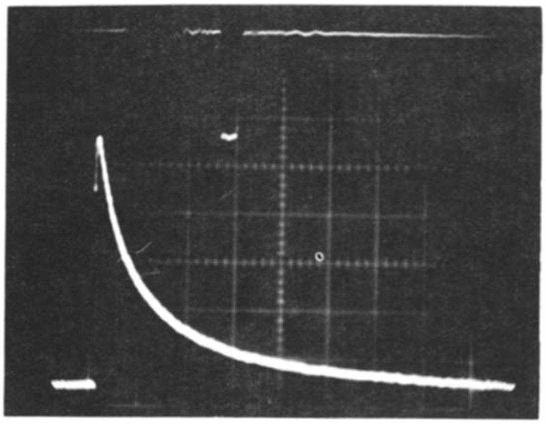


Fig.2. The second order decay of the transient absorption of $\mathrm{H\dot{F}l}$ in aqueous phosphate buffered solution pH 7.0 sweep rate: 500 μsec/scale unit, sensitivity 20 mv/scale unit followed at 570 nm.

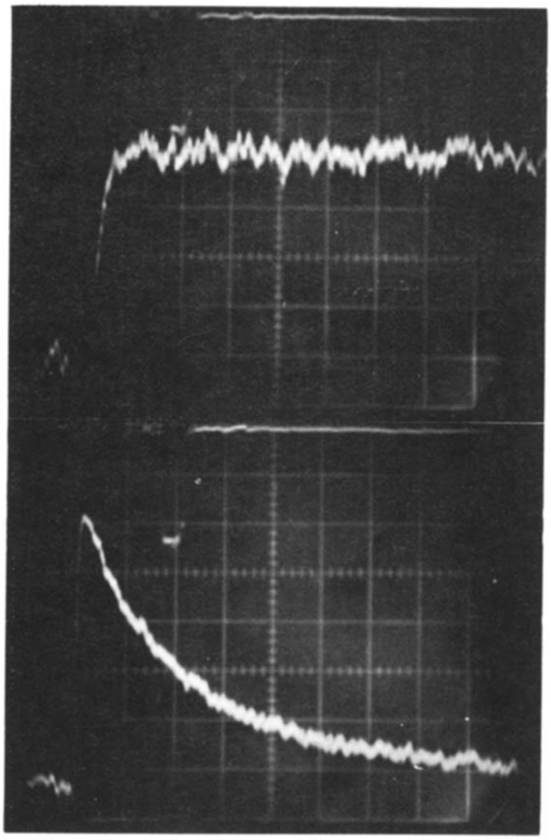


Fig.3. Formation (upper trace) and decay (lower trace) of the $\mathrm{H\dot{F}l}$ radical in air saturated neutral solution followed at 570 nm. Sweep rates: upper 10 μsec/scale unit. Sensitivity 5 mv/scale unit.

3-acetate) carries a negative charge closer to the redox-system, if compared with the phosphate charge of FMN.

In aerobic solution, however, the flavin radical decay becomes strongly pH-dependent: At pH 10, the formation of $\mathrm{\dot{F}l^-}$ ($\lambda_{max} \approx 480$ nm) may be observed directly, but only at $[O_2] \leqq 2.10^{-4}$. The radical ($[\mathrm{Fl^-}] \approx 5.10^{-7}$) is decaying under these conditions in a pseudo first order process at a rate of $2.5 \pm 0.3 \times 10^8$ $\mathrm{sec^{-1}\ M^{-1}}$, by reaction with O_2 (D_3, scheme 1). This is in very good agreement with the value of 3×10^8 $\mathrm{M^{-1}\ sec^{-1}}$ extrapolated for the same reaction by Vaish and Tollin [13] at alkaline pH in their flash photolysis study. At pH 7, however, we observe the formation of HFl in the presence of O_2-concentrations up to 10^{-3} M. This requires, however, total Fl-concentration to be increased towards 10^{-4} M in order to compete with the reaction of e_{aq}^- with O_2. The presence of O_2 does not alter the $\mathrm{H\dot{F}l}$-spectrum and the radical decay pattern remains under these conditions pseudo first order ($k = 2.5 \times 10^7$ $\mathrm{M^{-1}\ sec^{-1}}$). At O_2 lower than 10^{-4} M, however, the decay becomes first order in both, $\mathrm{H\dot{F}l}$ and O_2, at pH 7. The above mentioned anaerobic decay mode, i.e. dismutation, is at pH 7 still negligible, as compared to the autoxidation. If we assume, that the autoxidative decay of $\mathrm{H\dot{F}l}$ is occurring entirely via $\mathrm{\dot{F}l^-}$, we can calculate from the rate observed at pH 7 the specific rate for $\mathrm{\dot{F}l^-}$-autoxidation, using the expression $k_{spec} = k_{obs}$ $(1 + H^+/K)$ with $pK = 8.4$ [9], and we obtain $k_{spec} = 4.1 \times 10^8$ $\mathrm{M^{-1}\ sec^{-1}}$, in good agreement with the above mentioned value, we determined directly at pH 10. Hence, the neglect of $\mathrm{H\dot{F}l}$ as $\mathrm{O_2^{\cdot -}}$-reductant is justified. Consequently, the aerobic radical decay changes drastically in shape from pseudo first (i.e. autoxidation) to second order in HFl (i.e. dismutation) upon lowering the pH further. At pH 4.1 the aerobic and anaerobic decay of HFl become identical, $k = 2.10^8$ $\mathrm{M^{-1}\ sec^{-1}}$.

4. Discussion

It turns out that the two flavin radical species which may exist in the physiological range of pH, $\mathrm{H\dot{F}l}$ and $\mathrm{\dot{F}l^-}$, differ greatly in oxygen affinity, in agreement with the results of Vaish and Tollin [13]. With the one exception of glucose oxidase, flavoproteins are known to stabilize only one of the above mentioned radical species alternatively [10,12]: O_2-activating flavoproteins produce no superoxide while e^--transferring flavoproteins – when forced to react with the 'false' acceptor O_2 – produce stoichiometric amounts of it.

We can combine our results with previous knowledge in the following way: In O_2-activating flavoproteins the reduced state $\text{Fl}_{\text{red}}\text{H}_2$ has, by definiation, a high O_2-affinity. These flavoproteins stabilize red radicals $\dot{\text{Fl}}^-$, as mentioned above, and $\dot{\text{Fl}}^-$ has a high O_2-affinity, as we find. Hence, up to 2 moles of $\dot{O}_2^-$ were to be formed, if radicals were essential in the biocatalysis, but none is found.

In e^--transferring flavoproteins, on the other hand, O_2-affinity of $\text{Fl}_{\text{red}}\text{H}_2$ is low. These flavoproteins stabilize blue radicals $\text{H}\dot{\text{Fl}}$, whose O_2-affinity is even lower, as we find. Hence, one and only one mole of $\dot{O}_2^-$ must be found, if radicals were essential, and at the most one mole is indeed found. The low O_2-reactivity of protein bound $\text{H}\dot{\text{Fl}}$ groups is not due to its O_2-inaccessibility of the protein active site, as one might suspect. Hence, the oxidative decay of $\text{H}\dot{\text{Fl}}$ occurs, preferably, via disproportionation, at least in the free state. But this seems even to be valid for the protein bound $\text{H}\dot{\text{Fl}}$, where the intermolecular self contact is generally more difficult, but still not to be excluded. In many cases the shape of the apoprotein might even be such as to favour interflavin contact, be it inter- or, in the case of the many flavoproteins having two flavins per molecule, intramolecular contact [17].

Applied to the pathways of radical formation in scheme 1, the conclusions read as follows:

1) The pH-independent step C would be followed at $\text{pH} > 8$ by D_3, owing to the high O_2-affinity of $\dot{\text{Fl}}^-$, rather than by recombination with $\dot{O}_2^-$ (reversal of B_3), and would yield two moles of $\dot{O}_2^-$ per Fl_{ox} formed. The actual yield, however, is always less than 1 mole. This rules out pathway C in favour of $A \longrightarrow B_3$, where B_3 still competes with B_1 and B_2.

2) At $\text{pH} < 8$, B_3 is also suppressed, owing to the low O_2-affinity of $\text{H}\dot{\text{Fl}}$, thus leaving only B_1 (unpolar) and B_2 (polar) open for O_2-reduction.

Hence, mechanisms of intersystem crossing of $^3O_2$ other than $\dot{O}_2^-$ radical formation should be considered. A proposal has been made elsewhere about how $\text{Fl}_{\text{red}}\text{H}_2$ by itself might facilitate this process [18].

References

[1] Massey, V., Palmer, G. and Ballou, D. (1973) in: Oxidases and Related Redox Systems II (King, T. E., Mason, H. S. and Morrison, M. eds.) p. 25, University Park Press, Baltimore.

[2] Strickland, S. and Massey, V. (1973) J. Biol. Chem. 248, 2953.

[3] Entsch, B., Massey, V. and Ballou, D. (1974) Biochem. Biophys. Res. Comm. 57, 1018.

[4] Mager, H. I. X. and Berends, W. (1966) Biochim. Biophys. Acta 118, 440.

[5] Mager, H. I. X., Addink, R. and Berends, W. (1967) Rec. Trav. Chim. 86, 833.

[6] Mager, H. I. X. and Berends, W. (1972) Rec. Trav. Chim. 91, 611, 630, 1137.

[7] Gibson, Q. H. and Hastings, J. W. (1962) Biochem. J. 83, 368.

[8] Hemmerich, P., Bhaduri, A. P., Blankenhorn, G., Brüstlein, W., Haas, W. and Knappe, W.-R. (1973) in: Oxidases and Related Redox Systems II (King, T. E., Mason, H. S. and Morrison, M. eds.) p. 3, University Park Press, Baltimore.

[9] Ehrenberg, A., Müller, F. and Hemmerich, P. (1967) European J. Biochem. 2, 286.

[10] Massey, V. and Hemmercih, P. in: Enzymes, 3rd edition, (Boyer, P. ed.) Volume on Oxidoreduction, in press.

[11] Müller, F. Brüstlein, M., Hemmercih, P., Massey, V. and Walker, W. H. (1972) Eur. J. Biochem. 25, 573.

[12] Palmer, G. and Massey, V. (1968) in: Biological Oxidations, (Singer, T. P. ed.) p. 263 Interscience, New York.

[13] Vaish, S. P. and Tollin, G. (1971) Bioenergetics 2, 61.

[14] Land, E. J. and Swallow, A. J. (1969) Biochemistry 8, 2117.

[15] Hemmerich, P. (1964) Helv. Chim. Acta 47, 464.

[16] Pecht, I. and Faraggi, M. (1972) Proc. Nat. Acad. Sci., USA, 69, 902.

[17] Hemmerich, P., Nagelschneider, G., Veeger, C. (1970) FEBS Lett. 8, 69.

[18] Hemmerich, P. and Schuman-Jorns, M. (1973) in: Enzymes: Structure and Function, (Veeger, C., Drenth, J. and Oosterbaan, R. A. eds.) p. 95 North Holland, Amsterdam.