EVIDENCE FOR FORMATION OF OXYGENATED FLAVINS

Masaki Yamasaki and Toshio Yamano

Department of Biochemistry, Medical School,
Osaka University, Osaka, Japan

Received February 16,1973

SUMMARY: Oxygenated flavins have been detected in the reactions of fully reduced flavins and flavoproteins with molecular singlet oxygen ($^1\Delta_g$). The structure of the oxygenated flavins was derived on the basis of the absorption spectra and the computer-aided high-resolution mass spectra. The absorption bands, especially in the 500-680 nm region and a broad peak centered at 375 nm, were found to be characteristic of the oxygenated form. A mechanism is presented for the oxygenation reaction of the flavin enzymes with molecular oxygen.

One of the major problems in flavoprotein catalysis is to understand the reactivity of reduced flavins and flavoproteins with molecular oxygen. Recently, Massey et al. proposed the occurrence of an oxygenated form of p-hydroxybenzoate hydroxylase,[1] while a spectral species ascribed to oxygenated flavin was reported by Hayaishi et al.,[2] but the reaction mechanisms have remained unclear in detail. Reaction of oxygen at the 1a position of the flavin ring system has been implicated by Mager and Berends,[3] while Hemmerich et al.[4] implicate the 4a position. Massey et al. have also suggested the 1a position on the basis of recent work of Müller.[5] No direct chemical evidence supported these hypotheses. Some model reactions proposed by Hamilton et al.[6] suggest that substrates of alcohol and amine dehydrogenases form a covalent bond with the 4a position of the flavin ring system.

The excited state of oxygen is currently receiving considerable attention in photochemistry. Molecular oxygen in its first excited state ($^1\Delta_g$), especially, should be capable of concerted two electron reactions. Recent studies have shown that photooxygenation of monoolefins with singlet oxygen usually leads stereospecifically to the formation of perhydroxyl products.[7]

In this communication, we report the successful detection of oxygenated

flavins in the reaction of reduced flavins and flavoproteins with singlet oxygen.

MATERIALS AND METHODS

3-Methyllumiflavin was synthesized by a slight modification of the procedure of Hemmerich et al.[8] [ir (KBr) 1699 and 1667 cm^{-1}; nmr ($CDCl_3$, TMS) δ 2.44, 2.55, 3.50, 4.10 (each 3H, s), 7.40, and 8.03 (each 1H, s); mass m/e 270 (M^+)] Riboflavin, FMN, and FAD were commercial products.

D-Amino acid oxidase was prepared according to the method of Kubo et al.[9] and further purified by treatment with hydroxylapatite following the method of Massey et al.[10] Benzoate bound to the enzyme was removed by passing the enzyme through a Sephadex G-100 column immediately after addition of a small amount of 1 M DL-alanine solution to the column. The concentration of the enzyme was expressed in terms of bound FAD (ε_{455} = 11300 $M^{-1}cm^{-1}$).[11] The enzymatic reactions were carried out in 0.1 M sodium pyrophosphate buffer, pH 8.3 unless otherwise specified. Enzymic activity was assayed by using a galvanic electrode (Kyusui Kagaku Institute, Tokyo). Other chemicals used were of analytical grade.

The absorption spectra were recorded with a Cary model 14 recording spectrophotometer and a Hitachi model 124 spectrophotometer. High-resolution mass spectra were taken on a JEOL model JMS-01-SG spectrometer.

A special anaerobic spectrophotometric cuvette was designed for the experiments that required anaerobic additions or a record of the absolute spectrum of the anaerobic contents.

RESULTS AND DISCUSSION

No indication of the formation of oxygenated flavin was observed in the reactions of normal reduced flavins (2, R"=H) prepared by EDTA-light irradiation under anaerobic conditions [12] with singlet oxygen ($^1\Delta_g$). It seems likely that normal reduced flavins react rather poorly with singlet oxygen; conversely, they react rapidly with ground state oxygen. However, stable fully reduced flavins in which the benzyl group was fixed to the N-5 position of the

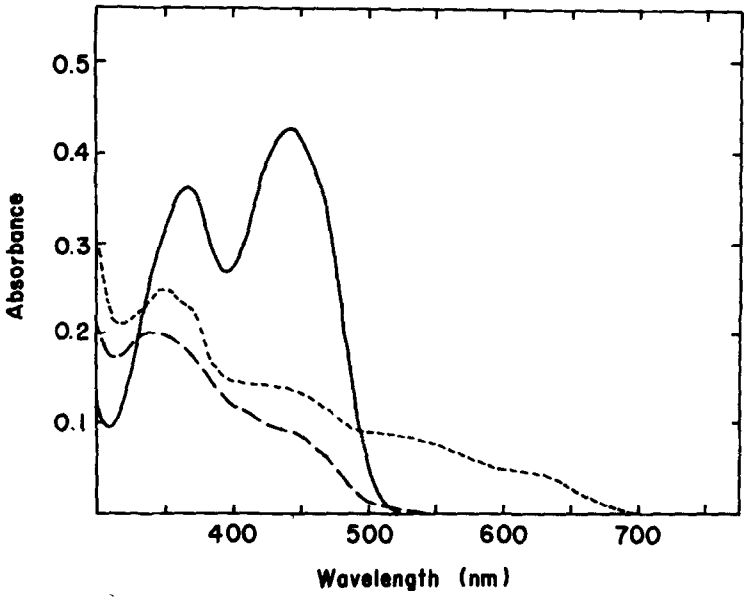
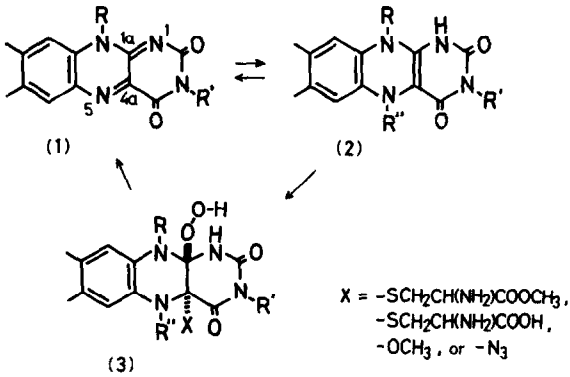


Fig. 1 The absorption spectrum of the oxygenated form observed in the reaction of 3-methyllumiflavin with molecular singlet oxygen. 3-Methyllumiflavin, 1.2×10^{-4} M in 3.0 ml aqueous solution (pH 6.3-6.4) was treated with a twofold excess of phenyl acetic acid by visible light under anaerobic conditions at 0° to give the stable fully reduced flavin (2, R=R'=CH_3, R''=Bz), which was further reacted with singlet oxygen in the presence of rose bengal (8.3×10^{-6} M) under cooling after the anaerobic addition of sodium azide (5.1×10^{-4} M) to afford the oxygenated flavin (3, R=R'=CH_3, R''=Bz, X=N_3). The absorbancy was measured in a cell of 1.0 cm path length. Solid line, absorption spectrum of oxidized form before the reaction; broken line, stable fully reduced form; dotted line, oxygenated form obtained with molecular oxygen.

Scheme 1



flavin ring system (2, R''=Bz) were found to react rapidly only with singlet oxygen in the presence of trapping reagents to afford oxygenated flavins in which molecular oxygen was directly incorporated into the flavin ring.

It is convenient to study first, the simplest example, the reaction of 3-methyllumiflavin (1, R=R'=CH_3) with singlet oxygen, but as a preliminary some basic chemical results must be examined. Under anaerobic conditions, the reaction of 3-methyllumiflavin in visible light with phenyl acetate in aqueous reaction mixture at pH 6.4 gave the expected 5-benzyl-3-methyllumiflavin (2, R=R'=CH_3, R''=Bz),[13] which was further reacted with singlet oxygen in the presence of rose bengal as photosensitizer and sodium azide to afford the corresponding oxygenated flavin (3, R=R'=CH_3, R''=Bz, X=N_3). The structure of this oxygenated flavin was mainly confirmed on the basis of its absorption spectra and the results of computer-aided high-resolution mass spectra.

The characteristic absorption due to the oxygenated form was observed in the 500-680 nm region and in a broad peak centered at 375 nm (Figure 1).

The high-resolution mass spectral data of this oxygenated flavin was treated with a computer-aided method, which could calculate all of the possible elemental compositions within a few millimass units of an experimentally found mass, search the magnetic tapes for all masses fitting within the experimental error, and then print out the found elemental compositions.

The essentially different patterns between the fully reduced form and the oxygenated form were obtained in both high-resolution and low-resolution mass spectra. An element map of stable fully reduced flavin (2, R=R'=CH_3, R''=Bz) obtained with the computer-aided techniques is given in Table 1. The molecular ion peak at m/e 362.1723 (47%) or 362.1716 (48%), which has a composition of $C_{21}H_{22}N_4O_2$ was observed. Debenzylation from the N-5 position results in the peak at m/e 271.1195 or 271.1204 (each 81%) corresponding to an elemental composition of $C_{14}H_{15}N_4O_2$. Under these conditions, there is no peak at m/e 361 corresponding to that observed in the following case of oxygenated flavin, which was probably formed as a result of removing the hydrogen atom from the N-1 position on the flavin ring.

It will be noticed (Table 2) that a very particular peak at mass 361.1664, 361.1663, or 361.1658 (each above 82%), which is due to $C_{21}H_{21}N_4O_2$ was observed in the calculation result from the computer-aided high-resolution mass spectral

TABLE 1

CALCULATION RESULT OF HIGH RESOLUTION MASS SPECTRAL DATA

OF 5-BENZYL-3-METHYLLUMIFLAVIN

LEVEL	20									
START	381									
CONST.	5	INT	OBSD.	CALCD.	ERROR	C12	C13	H	N	O
INT.	30	47	362.1723	.1756	- 3.2	23	0	24	1	3
RANGE	350			.1742	- 1.8	21	0	22	4	2
	365			.1720	+ 2.1	16	0	22	6	4
C13 =	0									
N =	6	INT	OBSD.	CALCD.	ERROR	C12	C13	H	N	O
O =	4	48	362.1716	.1670	+ 4.6	27	0	22	0	1
X =	0			.1756	- 3.9	23	0	24	1	3
S =	0			.1742	- 2.5	21	0	22	4	2
P =	0			.1702	+ 1.4	16	0	22	6	4
ERROR	5									

LEVEL	20									
START	319									
CONST.	5	INT	OBSD.	CALCD.	ERROR	C12	C13	H	N	O
INT.	40	81	271.1195	.1208	- 1.3	16	0	17	1	3
RANGE	270			.1235	- 4.0	19	0	15	2	0
	300			.1195	+ 0.0	14	0	15	4	2
C13 =	0			.1154	+ 4.0	9	0	15	6	4
N =	6									
O =	4	INT	OBSD.	CALCD.	ERROR	C12	C13	H	N	O
X =	0	81	271.1204	.1208	- 0.3	16	0	17	1	3
S =	0			.1235	- 3.0	19	0	15	2	0
P =	0			.1195	+ 0.9	14	0	15	4	2
ERROR	5									

TABLE 2

CALCULATION RESULT OF HIGH RESOLUTION MASS SPECTRAL DATA

OF OXYGENATED FLAVIN (3, R=R'=CH_3, R''=Bz, X=N_3)

LEVEL	20									
START	381									
CONST.	5	INT	OBSD.	CALCD.	ERROR	C12	C13	H	N	O
INT.	50	82	361.1664	.1677	- 1.3	23	0	23	1	3
RANGE	355			.1704	- 4.0	26	0	21	2	0
	365			.1664	+ 0.0	21	0	21	4	2
C13 =	1			.1624	+ 4.0	16	0	21	6	4
N =	7			.1651	+ 1.3	19	0	19	7	1
O =	4			.1633	+ 3.1	22	1	22	1	3
X =				.1660	+ 0.4	25	1	20	2	0
S =				.1619	+ 4.4	20	1	20	4	2
P =				.1705	- 4.0	16	1	22	5	4
ERROR	5									

INT	OBSD.	CALCD.	ERROR	C12	C13	H	N	O
83	361.1663	.1677	- 1.4	23	0	23	1	3
		.1704	- 4.1	26	0	21	2	0
		.1664	- 0.1	21	0	21	4	2
		.1624	+ 3.8	16	0	21	6	4
		.1651	+ 1.2	19	0	19	7	1
		.1633	+ 2.9	22	1	22	1	3
		.1660	+ 0.3	25	1	20	2	0
		.1619	+ 4.3	20	1	20	4	2
		.1705	- 4.2	16	1	22	5	4

INT	OBSD.	CALCD.	ERROR	C12	C13	H	N	O
82	361.1658	.1677	- 1.9	23	0	23	1	3
		.1704	- 4.6	26	0	21	2	0
		.1664	- 0.5	21	0	21	4	2
		.1624	+ 3.4	16	0	21	6	4
		.1651	+ 0.7	19	0	19	7	1
		.1633	+ 2.5	22	1	22	1	3

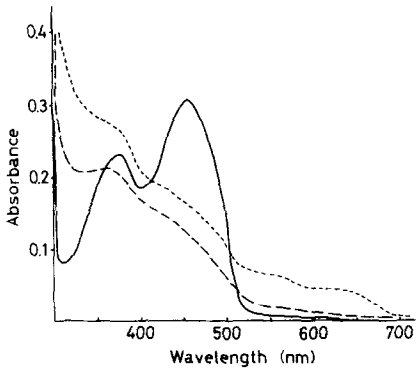


Fig. 2.

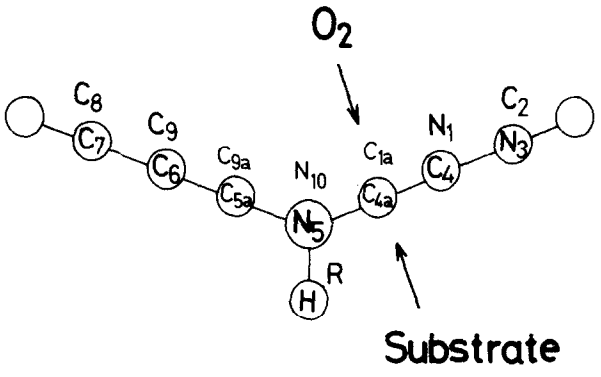


Fig. 3.

Fig. 2 The absorption spectrum of the oxygenated enzyme during the reaction of D-amino acid oxidase with molecular singlet oxygen. The conditions were similar to those specified in Fig. 1. The enzyme reaction was performed in 0.1 M phosphate buffer, pH 6.4 at 0° in a total volume of 3 ml. Solid line, absorption spectrum of oxidized enzyme (31.5 μM in terms of FAD) before the reaction; broken line, stable fully reduced enzyme; dotted line, oxygenated enzyme obtained by the reaction with molecular oxygen.

Fig. 3 The mechanism of the oxygenated reaction of the flavin enzymes with molecular oxygen.

---

data of oxygenated flavin (3, R=R'=CH_3, R''=Bz, X=N_3). This peak was attributed to the ion formed by cleavage of the N-H bond in the β position to the oxygen atom as generally expected, which was based on the direct incorporation of molecular oxygen into the flavin ring; no peak at m/e 362 could be found anywhere in the 355-365 mass range (Table 2).

These results together with the well known reaction mechanisms of C-C double bond with singlet oxygen, strongly indicated the existence of the oxygenated flavin, with addition of molecular oxygen occurring at C-1a of the flavin ring with N_3 as trapping reagent at C-4a.

Similar observations were made under the same conditions with FAD, FMN, and riboflavin. Furthermore fully reduced D-amino acid oxidase, benzylated at the N-5 position of the flavin ring, also reacted with singlet oxygen, strongly indicating that oxygenated enzyme was also found in phosphate buffer at pH 6.4 under these same conditions. The spectrum similar to the above-mentioned oxygenated flavins was observed in the oxygenated enzyme (Figure 2). The re-

oxidized spectrum was also obtained from this enzymic solution following the readmission of air and the enzymic activity of D-amino acid oxidase was recovered more than 85 %.

Spectral evidence for the oxygenated reactions of reduced flavins and flavoproteins could be obtained using the following nucleophilic trapping reagents: L-cysteine, L-cysteine methylester, methanol, and sodium azide.

Control experiments showed that benzylated stable reduced flavin did not react rapidly with ground state oxygen in the dark or upon irradiation in the absence of sensitizer. In addition, no spectral change due to oxygenated flavin was observed in the absence of trapping reagent under the usual conditions and the effect of adding the sensitizer, rose bengal, in this case is negligible. There was no evidence from EPR experiments for free-radical intermediates as neutral semiquinones during the oxygenation steps of flavoprotein or model flavins.

On the mechanisms of the oxygenation, the first step involves the electrophilic addition of the excited molecular oxygen ($^1\Delta_g$) to the C(1a)-(4a) double bond of fully reduced flavin. Molecular oxygen seems to be exclusively introduced on the side away from the two bulky groups. In the second step, nucleophilic attack towards the perepoxide or peroxirane intermediate proceeds from the other side as shown Figure 3.

We suggest that the oxygenation of the flavin enzymes by molecular oxygen in reactions involving natural substrates proceed by a similar mechanism. The mechanism of enzymic oxygen activation and the existence of molecular singlet oxygen ($^1\Delta_g$) in the enzymatic reactions have remained unclear, but will engage the attention as one of the most fascinating aspects of this subject.

REFERENCES

1. Spector, T. and Massey, V., J. Biol. Chem., 247, 5632 (1972).
2. Yamamoto, S., Hirata, F., Yamauchi, T., Nozaki, M., Hiromi, K., and Hayaishi, O., J. Biol. Chem., 246, 5540 (1971).
3. Mager, H.I.X. and Berends, W., Biochem. Biophys. Acta, 118, 440 (1966).
4. Walker, W.H., Hemmerich, P., and Massey, V., Helv. Chim. Acta, 50, 2269 (1967).
5. Müller, F., in Flavins and Flavoproteins, Third International Symposium (H. Kamin, ed) p363, University Park Press, Baltimore (1971).

6. Brown, L.E. and Hamilton, G.A., J. Amer. Chem. Soc., 92, 7225 (1970).
7. Kearns, D.R., Chem. rev., 71, 395 (1971).
8. Hemmerich, P., Fallab, S., and Erlenmeyer, H., Helv. Chim. Acta, 39, 1242 (1956).
9. Kubo, H., Yamano, T., Iwatsubo, M., Watari, H., Shiga, T., and Isomoto, A., Bull. Soc. Chim. Biol., 42, 569 (1960).
10. Massey, V., Palmer, G., and Bennet, R., Biochem. Biophys. Acta, 48, 1 (1961).
11. Whitby, L.G., Biochem. J., 54, 437 (1953).
12. Frisell, W.R., Chung, C.W., and Mackenzie, C.G., J. Biol. Chem., 234, 1297 (1959).
13. Hemmerich, P., Massey, V., and Weber, G., Nature, 213, 728 (1967).