J. Biosci., Vol.5, Number 1, March 1983, pp. 7 – 19 © Printed in India.

Naphthalene oxygenase from *Corynebacterium renale:* Characterisation and mechanism of oxygenation

PALURI S. RAO« and MEERA SRINIVASAN
Department of Chemistry, Indian Institute of Technology, New Delhi 110 016

MS received 19 October 1982; revised 9 February 1983

Abstract. The formation of *cis*-l,2,-dihydroxy-l,2,-dihydronaphthalene from naphthalene by naphthalene oxygenase, purified from *Corynebacterium renale* ATCC 15075, was demonstrated to involve oxidation of a mol NADH and consumption of one mol oxygen. The enzyme contains one g-atom Fe^{2+} and one FAD. Catalase inhibited product formation and H_2O_2 could substitute for NADH in the reaction. Superoxide dismutase inhibited enzyme activity when either NADH or H_2O_2 was present; the generation of superoxide anion on addition of NADH to the enzyme, in the absence of naphthalene, was detected by the nitro blue tetrazolium reduction method. Hydroxyl radical scavengers, ethanol, mannitol and sodium benzoate, inhibited product formation when either NADH or H_2O_2 was present. Electron spin resonance studies, under aerobic conditions, indicated that iron of the enzyme underwent valence changes during the course of the reaction.

Keywords. Iron; FAD; hydrogen peroxide; superoxide anion; hydroxyl radical; electron spin resonance.

Introduction

It has been demonstrated that the initial step in the metabolism of aromatic hydrocarbons by bacteria is catalysed by iron-containing NAD(P)H-dependent dioxygenases and the products formed are *cis*-dihydrodiols (Gibson *et al.,* 1968 a, b; Axcell and Geary, 1975). Catterall *et al.* (1971) reported that naphthalene dioxygenase from *Pseudomonas* sp. contained tightly bound Fe^{2+} and was NADPH-dependent. Jeffrey *et al.* (1975) demonstrated the formation of (+) *cis*- l,2-dihydroxy-l,2,-dihydronaphthalene from naphthalene using cells and extracts of *Pseudomonas putida* and established that the enzyme is a dioxygenase. Laborde and Gibson (1979) reported that naphthalene dioxygenase from *Pseudomonas* NCIB 9816 is a multicomponent enzyme system consisting of NADH-cytochrome *c* reductase, iron-sulphur protein and a small protein.

At present, the mechanism by which the stereospecific *cis*-l,2-dihydrodiols are formed from many aromatic hydrocarbons by bacterial dioxygenases is not clearly understood. The formation of strained dioxetanes (cyclic peroxides) as likely intermediates which on reduction could lead to the dihydrodiols has been postulated by many workers (Kobayashi *et al.,* 1964; Milne *et al.,* 1968; Reiner and

« To whom reprint requests should be addressed.

Hegeman, 1971). Jeffrey, *et al.* (1975) speculated the mechanisms, for naphthalene dioxygenase, involving either the stereospecific introduction of elements of hydrogen peroxide across the C_1 -C_2 double bond of naphthalene or the formation of 1,2-dioxetane as an intermediate.

In the present communication, role for NADH, evidence for the generation of superoxide anion and the formation of hydroxyl radical by the mechanism of Haber and Weiss (1934) during the course of naphthalene oxygenase (EC 1:13:99) catalysed reaction is presented. A probable mechanism for the formation of *cis*-l,2-dihydroxyl-l,2,-dihydronapthalene by the enzyme from *C. renale* is proposed.

Materials and methods

The following materials were obtained from the sources specified: Superoxide dismutase (EC 1:15:1:1), catalase (EC 1:11:1:6), peroxidase (EC 1:11:1:7), bovin serum albumin, NADH, FAD, FMN and GSH from Sigma Chemical Co., St. Louis, Missouri, USA; 2,6-dichloroquinone-4-chloroimide, Kieselguhr G, Silica gel G and 1,10-phenanthroline from E. Merck, Darmstadt, Germany; Sephadex G-75 from Pharmacia Fine Chemicals, Uppsala, Sweden; DEAE-cellulose and nitro blue tetrazolium from V.P. Chest Institute, New Delhi. All other chemicals used were of analytical grade.

Enzyme purification

Naphthalene oxygenase was purified to homogeneity from the crude extracts of *Corynebacterium renale* ATCC 15075 as described by Dua and Meera (1981).

Enzyme assay

Naphthalene oxygenase activity was assayed either by measuring oxidation of NADH at 340 nm using Pye-Unicam SP 500 Spectrophotometer or by determining the product formed colorimetrically with 2,6-dichloroquinone-4-chloroimide using-1-naphthol as standard as described by Booth and Boyland (1958).

Protein determination

Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Determination of iron

Following the method of Brumby and Massey (1967) the iron present in the enzyme (5 mg protein) was extracted into trichloroacetic acid, and then determined colorimetrically with 1,10-phenanthroline using ferrous ammonium sulphate as standard. The essentiality of iron for the enzyme activity was determined by the reconstitution of the apoenzyme, prepared by 1,10-phenanthroline treatment, with Fe^{2+} as described by Nakazawa *et al.* (1969).

Identification and determination of flavin

The flavin moiety of naphthalene oxygenase was liberated by trypsin digestion process as described by Kondo *et al.* (1960) and was used for both its identification

and determination. It was identified by TLC on 0.25 mm thick Kieselguhr G coated plate using 5% (w/v) Na_2HPO_4, $12H_2O$ as developing solvent (Fazekas and Kokai, 1971).

The FAD content of the enzyme was determined spectrophotometrically by recording the spectrum of the trypsin digest using Pye-Unicam SP 700 Recording Spectrophotometer and measuring its absorption at 450 nm.

Identification of product

Standard *cis*-l,2-dihydroxy-l,2-dihydronaphthalene was prepared according to the method of Jeffrey *et al.* (1974) and the product of the enzyme reaction was purified and identified by TLC using butan-1-ol/ethanol/water (7: 1: 2, by vol) as developing solvent (Meera, 1980).

Electron spin resonance studies

ESR studies were conducted using Varian Model E-12 X-band Spectrometer at 93°K.

Detection of superoxide anion (O^-_2) and hydroxyl radicals

The generation of superoxide anion in the enzyme reaction was detected by the method of Beauchamp and Fridovich (1971) which involves inhibition of reduction of nitro blue tetrazolium to formazan by superoxide dismutase. Hydroxyl radical (OH) formation was detected using scavengers-ethanol, mannitol and sodium benzoate as described by Beauchamp and Fridovich (1970).

Results

Determination of iron

Spectroscopic analysis of the enzyme showed the presence of only iron. Using a molecular weight of 99,000 for the enzyme (as determined by Sephadex G-100 filtration method), the mol ratio of Fe:enzyme was found to be 1:1.05. Preincubation of the enzyme with 1,10-phenanthroline and subsequent exhaustive dialysis resulted in 80% loss in activity and the original activity was regained by the addition of Fe^{2+} only (figure 1) which suggested that the enzyme contained Fe^{2+}.

Identification and determination of flavin

The TLC of trypsin digest of naphthalene oxygenase showed the presence of only FAD (table 1). Using a molar extinction coefficient of 11.3×10^3, at 450 nm and molecular weight 99,000 for the enzyme, the mol ratio FAD: enzyme was calculated to be 1:1.02 (figure 2) .

Effect of catalase

Catalase, although enhanced NADH oxidation, inhibited the product formation by 85%; also, in the absence of naphthalene, catalase caused a small amount of NADH oxidation (table 2).

Effect of hydrogen peroxide

The enzyme catalysed the formation of 1,2-dihydrodiol when NADH was replaced by H_2O_2 in the reaction mixture (table 3). When naphthalene oxygenase was replaced by peroxidase, no product was detected.

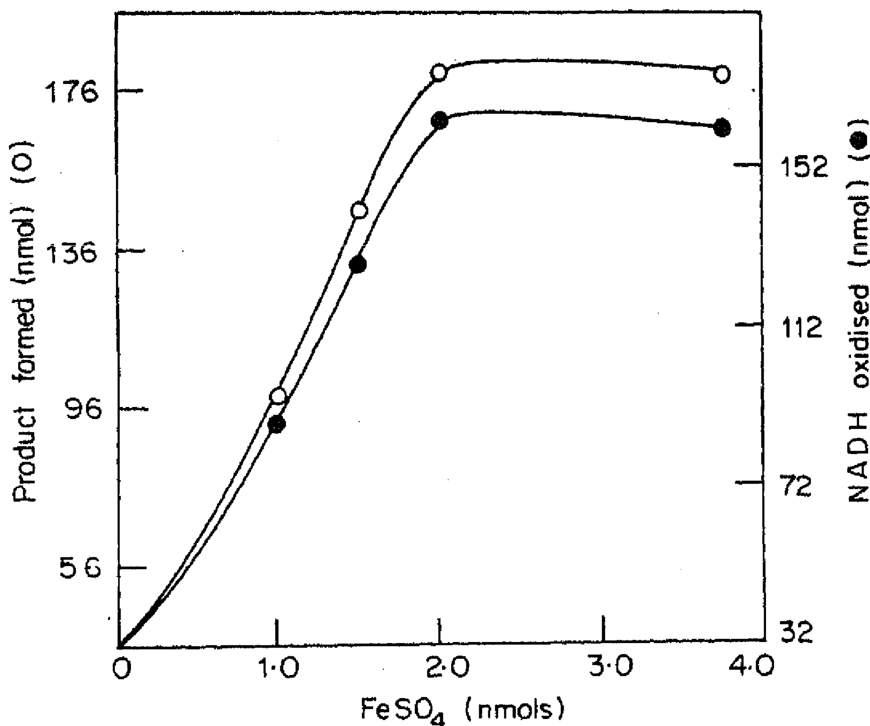


Figure 1. Reconstitution of apoenzyme with iron.

Naphthalene oxygenase (1 mg protein) in 0.05 M phosphate buffer (pH 6.5) was incubated with 2 µmol 1,10-phenanthroline in a total volume of 2.5 ml for 30 min at 40°C and dialysed against 500 ml phosphate buffer (0.05 M; pH 7.0) with three changes for 60 h at 4°C. Aliquots of the dialysed protein were preincubated for 5-10 min at 30°C with different amounts of $FeSO_4$ and assayed for oxygenase activity. The final reaction mixture in each case contained: apoenzyme (0.2 mg protein), 0.8 µmol NADH, 0.2 µmol naphthalene in 0.02 ml 2-rnethoxyethanol, 0.05 M phosphate buffer (pH 6.5) and different amounts of $FeSO_4$, as indicated, in a total volume of 2.2 ml. The reaction was conducted at 30°C till the oxidation of NADH was complete.

(O) Product formed; (●) NADH oxidized

Table 1. Identification of flavin.

Compound	R_f
FAD	0.426
FMN	0.298
Riboflavin	0.142
Trypsin digest	0.433
Trypsin digest + FAD	0.433
Trypsin digest + FMN	0.433; 0.30

Purified naphthalene oxygenase (2 mg protein in 2.2 ml 0.05 M phosphate buffer, pH 6.5), after heat denaturation at 80°-100°C for 10 min was digested with 0.3 mg trypsin at pH 8.0 for 5 h at 37°C and treated further as described by Kondo *et al.* (1960). The TLC of the trypsin digest was performed on 0.25 mm thick Kieselguhr G plate using 5% (w/.v) $Na_2HPO_4.12H_2O$ as developing solvent. The spots were located under u.v. light.

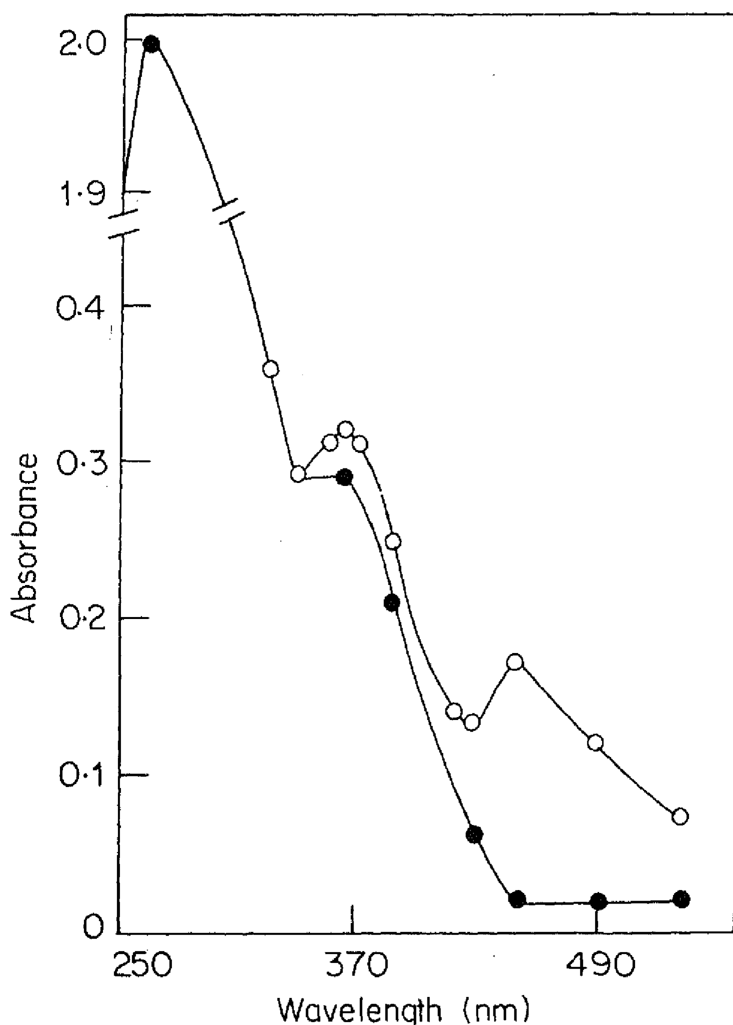


Figure 2. Determination of FAD.
The experimental details were as given in table 1, with the exception that 3 mg oxygenase was used. After digestion, trypsin was destroyed by heating at 80°C for 15 min, the mixture cooled and centrifuged at 2000 *g* for 10 min. The absorption spectrum of the clear supernatant was recorded.
(O) Spectrum of trypsin digest; (●) Spectrum of trypsin digest after addition of 2 mg $Na_2S_2O_4$.

Table 2. Effect of catalase on naphthalene oxygenase catalysed reaction.

Omissions	NADH Oxidised (nmols)	Activity (%)	Product formed (nmols)	Activity (%)
Catalase	190	100	197	100
Nil	226	119	27	14
Naphthalene	49	26	0	0
Naphthalene + Catalase	0	0	0	0

The complete reaction mixture contained: enzyme (0.2 mg protein), 0.8 µmol NADH, 0.2 µmol naphthalene in 0.02 ml 2-methoxyethanol, 100 µg catalase and 0.05 M phosphate buffer (pH 6.5) in a total volume of 2.2 ml. The reaction was conducted for 30 min at 30°C.

Table 3. Effect of substitution of hydrogen peroxide for NADH on product formation

Addition to control	Amount (μmol)	Product formed (nmol)	Activity (%)
NADH	1	268	100
H_2O_2	1	267	100
	4	282	105.5
	9	307	114
	12	321	120
	18	305	114
	36	250	93.5

Control contained: enzyme (0.2 mg protein), 0.5 μmol naphthalene in 0.05 ml 2-methoxyethanol and 0.05 M phosphate buffer (pH 6.5) in a total volume of 2.2 ml. NADH or H_2O_2 was added as indicated and the reaction was conducted for 15 min at 30°C in a water bath shaker.

Identification of product

The product formed in a reaction mixture containing naphthalene, NADH or H_2O_2, naphthalene oxygenase and phosphate buffer (0.05 M; pH 6.5) had the same R_f(single spot in each case R_f= 0.87) as that of standard *cis*-1,2-dihydroxyl-1,2-dihydronaphthalene when subjected to TLC on Silica gel G using butanol/ethanol/water (7:1:2 by vol) as developing solvent.

Effect of superoxide dismutase

Superoxide dismutase, at 2.73 μM, inhibited the enzyme reaction in terms of both NADH oxidation and product formation by 45%; however, at 6.8 μM, the inhibition was nearly 90% (table 4a). Superoxide dismutase inhibited the reaction even when H_2O_2 was substituted for NADH (table 4b).

Table 4a. Effect of superoxide dismutase on NADH oxidation and product formation.

Additions	NADH oxidised (nmol)	Activity left (%)	Product formed (nmol)	Activity left (%)
Nil	190.0	100.0	197	100.0
Superoxide dismutase (6 nmol)	86.5	45.5	86	43.5
Superoxide dismutase (15 nmol)	18.0	9.5	15	7.5

The reaction mixture contained: enzyme (0.2 mg protein), 0.8 μmol NADH, 0.25 μmol naphthalene in 0.025 ml 2-methoxyethanol and 0.05 M phosphate buffer (pH 6.5) in a total volume of 2.2 ml; superoxide dismutase was added as indicated. The reaction was conducted for 30 min at 30°C.

Table 4b. Effect of superoxide dismutase on naphthalene oxygenase catalysed reaction in the presence of hydrogen peroxide

Additions	Product formed (nmol)	Activity left (%)
Nil	268	100
Superoxide dismutase (15 nmol)	31	14

The reaction mixture contained: enzyme (0.2 mg protein), 1 μmol H_2O_2, 0.5 μmol naphthalene in 0.05 ml 2-methoxyethanol and 0.05 M phosphate buffer (pH 6.5) in a total volume of 2.2 ml; superoxide dismutase was added as indicated. The reaction was conducted for 15 min at 30°C.

Detection of superoxide anion

The enzyme alone did not reduce nitro blue tetrazolium (NBT), but on the addition of NADH, in the absence of naphthalene, reduced NBT which was inhibited by superoxide dismutase by 85% (figure 3). Although, addition of GSH to the enzyme caused some reduction of NBT, no product formation from

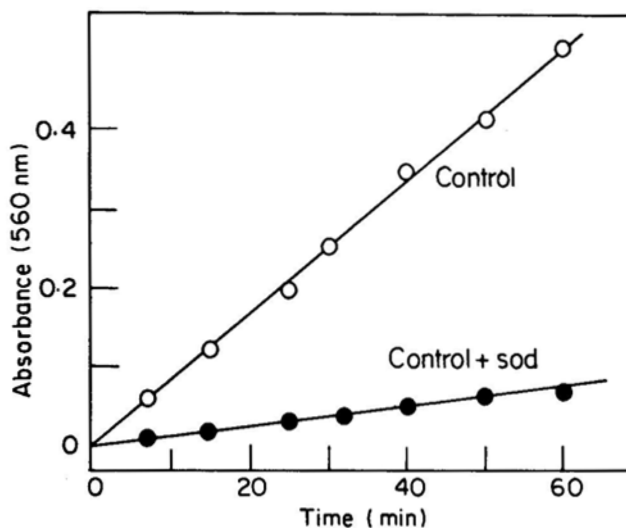


Figure 3. Rate of reduction of NBT by naphthalene oxygenase in the presence of NADH.

The rate of reduction of NBT was measured as change in optical density units at 560 nm. Control contained: enzyme (0.3 mg protein), 1 μmol NADH, 1 μmol NBT and 0.05 M phosphate buffer (pH 6.5) in a total volume of 3.11 ml. Reaction conditions were as given in table 5.

(O) Control; (●) Control plus.6 nmol superoxide dismutase.

naphthalene, in the absence of NADH or H_2O_2 was detected. Addition of naphthalene, H_2O_2, mannitol or sodium benzoate did not cause reduction of NBT. These observations indicated the possibility that NADH could be modifying the

naphthalene oxygenase in an allosteric fashion to cause the generation of superoxide anion (table 5).

Table 5. Detection of superoxide anion by nitro blue tetrazolium reduction method.

Additions to control	Reduction of NBT (%)	Reduction of NBT in presence of superoxide dismutase (%)
Nil	0	0
NADH	100	13
GSH	50	4
Naphthalene	0	0
H_2O_2	0	0
NADH plus mannitol or benzoate	100	12
Mannitol or benzoate	0	0

Control contained: naphthalene oxygenase (0.3 mg protein), 1 µmol nitro blue tetrazolium (NBT), 0.05 M phosphate buffer (pH 6.5) in a final volume of 3.11 ml. The amounts used for added compounds were: 1 µmol NADH; 4 µmol GSH; 1 µmol naphthalene in 0.1 ml 2-methoxyethanol; 1 µmol H_2O_2; 12 µmol mannitol or benzoate. The reaction mixture was incubated for 60 min at 30°C in a water bath shaker and NBT reduction was measured as optical density units at 560 nm using Spectronic 20 absorptiometer in the presence and absence of 6 nmol superoxide dismutase.

Effect of hydroxyl radical scavengers

Ethanol, mannitol and sodium benzoate inhibited product formation when either NADH or H_2O_2 was present in the reaction mixture; benzoate had maximum inhibitory effect. Whereas, the inhibition was much greater in presence of H_2O_2 compared to that of NADH for the same concentrations of the scavengers, there was also simultaneous inhibition of NADH oxidation in the NADH system (table 6). These results suggested the formation of .OH radical in the enzyme reaction.

ESR spectral studies

The enzyme alone showed one sharp ESR signal at g=2.07 and on addition of NADH, under aerobic conditions, a new signal at g= 4.3 appeared which on subsequent incubation with naphthalene nearly disappeared while retaining the signal at g=2.07 (figure 4). These observations suggested that the iron of the enzyme underwent valence changes from Fe^{2+} to Fe^{3+} and back during the course of the enzyme reaction. ESR studies with initial addition of naphthalene to the enzyme could not be conducted as naphthalene was found to separate out on freezing.

Effect of ultraviolet irradiation

Irradiation of a mixture containing naphthalene, H_2O_2, naphthalene oxygenase and phosphate buffer (0.05 M; pH 6.5) at 240 nm showed that (a) the yield of 1,2-

Table 6. Effect of OH radical scavengers on the naphthalene oxygenase catalysed reaction.

		NADH System				H_2O_2 System	
Compound added	Amount (μmol)	NADH Oxidised (nmol)	NADH (Oxidation (per cent inhibition)	Product formed (nmol)	Product formation (per cent inhibition)	Product formed (nmol)	Product formation (per cent inhibition)
—	—	259	0	265	0	265	0
Sodium benzoate	1.0	200	23.0	183	31.0	85	68
	2.0	172	33.5	150	43.5	45	83
	4.0	147	43.0	113	57.5	0	100
	8.0	114	56.0	75	83.0	—	—
Mannitol	1.0	214	17.5	197	25.5	186	30
	2.0	193	25.5	168	36.5	122	54
	4.0	172	33.5	134	49.5	32	88
	8.0	155	40.0	115	56.5	—	—
Ethanol	2×10^2	245	5.5	250	5.5	240	9.5
	10.3×10^2	210	19.0	200	24.5	160	40
	41.5×10^2	180	30.5	160	40.0	80	70

The reaction mixture contained: enzyme (0.2 mg protein), 0.5 μmol naphthalene in 0.05 ml 2-methoxyethanol, 1 μmol either NADH or H_2O_2 and 0.05 M phosphate buffer (pH 6.5) in a total volume of 2.2 ml. The scavengers were added as indicated. The reaction was conducted in each case for 15 min at 30°C.

dihydrodiol was not affected, (b) but when superoxide dismutase was included, some unidentified compounds (which gave phenolic reaction on acid treatment and whose overall yield as 1-naphthol was only 50%) were formed and (c) absorbance at 240 nm in presence and absence of superoxide dismutase also simultaneously decreased. In contrast, in the absence of oxygenase, no change in absorbance at 240 nm was observed, whether superoxide dismutase was present or absent, although some unidentified compounds whose qualitative tests and yield were similar to that of oxygenase system in presence of superoxide dismutase were formed; besides, mannitol and benzoate completely inhibited the formation of these compounds (table 7).

Discussion

Dua and Meera (1981) reported that purified naphthalene oxygenase from *Corynebacterium renale* oxidised a mol NADH for the incorporation of one mol oxygen into naphthalene and that the product of the reaction was *cis*-1,2-dihydroxy-l,2,-dihydronaphthalene. The enzyme was found to contain one g-atom Fe^{2+} and one FAD as determined by colorimetric and absorptiometric methods respectively. The ESR signal at $g= 2.07$, reconstitution of apoenzyme with Fe 2^+ and lack of absorption in the Soret region by the enzyme alone or after its treatment with CO for 5 min in dark indicated the presence of non-heme Fe^{2+}.

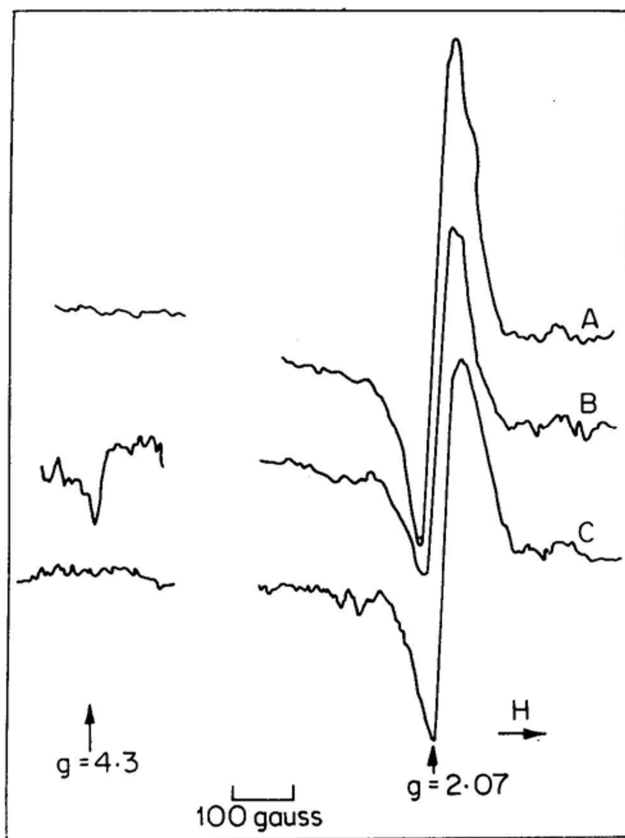


Figure 4. ESR spectrum of naphthalene oxygenase, in presence of air, under different conditions at 93°K; modulation amplitude 5 G.

Enzyme (1 mg protein) dissolved in 0.05 M phosphate buffer (pH 6.5) was frozen and the spectrum recorded. To this enzyme soln, 2 µmol NADH was added, incubated for 20 min at 30°C and the spectrum recorded. Finally, to the NADH preincubated enzyme, 2 µmol naphthalene was added, incubated again for 20 min at 30°C and the ESR spectrum recorded.

(A) Enzyme alone; (B) Enzyme+NADH; (C) Enzyme+NADH+napthalene.

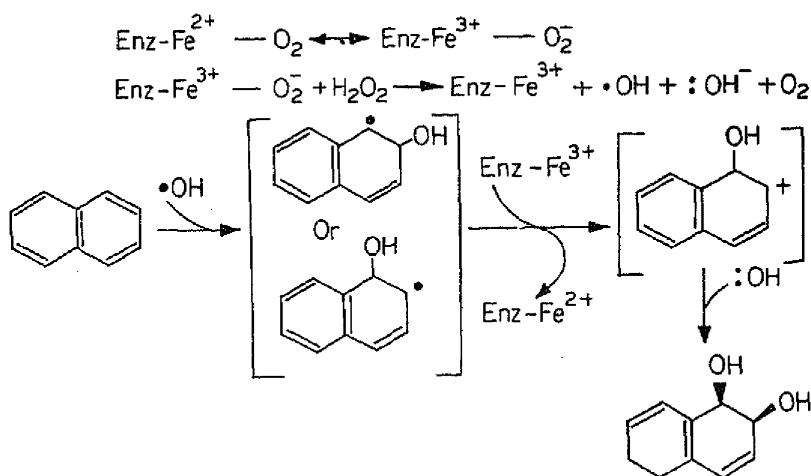
Experiments with catalase, which inhibited product formation significantly but enhanced NADH oxidation suggested that the binding of naphthalene to the enzyme initiated the generation of H_2O_2; subsequent studies confirmed that H_2O_2 itself could substitute for NADH in the enzyme reaction. The enzymatic reaction in presence of either NADH or H_2O_2 was inhibited by superoxide dismutase; although, high amount of superoxide dismutase was required to cause significant inhibition, analysis of the reaction showed that the addition of NADH alone to the enzyme caused the generation of O_2^- as tested by the NBT reduction method. The results suggested that (a) NADH binds to the enzyme initially so as to expose its Fe^{2+} to O_2 and (b) the second function of NADH is to produce H_2O_2 by oxidation *via* FAD of the enzyme only when naphthalene was present. The hydroxyl radical scavengers inhibited product formation when either NADH or H_2O_2 was present; however, there was also simultaneous inhibition of NADH oxidation. At present,

Table 7. Effect of U.V. irradiation on system containing naphthalene and hydrogen peroxide in the presence and absence of naphthalene oxygenase.

Additions to control	ΔA_{240}	Products as 1-naphthol (nmol)
Enzyme	0.17	495
Enzyme plus superoxide dismutase	0.17	250
Nil	0	253
Superoxide dismutase	0	246
H_2O_2	0	250
Mannitol	0	0
Sodium benzoate	0	0

Control contained: 0.5 μmol naphthalene in 0.05 ml 2-methoxyethanol, 1 μmol H_2O_2 and 0.05 M phosphate buffer (pH 6.5) in a total volume of 2.32 ml. The amounts used for added compounds were: 0.2 mg naphthalene oxygenase; 15 nmol superoxide dismutase; 4 μmol H_2O_2; 4 μmol mannitol or sodium benzoate. The reaction was terminated in each case by addition 0.23ml conc.HCl after irradiating for 30 min at 30°C at 240 nm in a 1 cm cuvette. The products were determined as 1-naphthol after heating for 15 min at 100°C by the method of Booth and Boyland (1958).

we do not know the reason for this unexpected phenomenon but it is likely that the rate of generation of O_2^- was also indirectly affected as the scavengers are not known to react with either O_2^- or H_2O_2 (Fridovich, 1976). The appearance of a new ESR signal at *g*= 4.3, under aerobic conditions, on the addition of NADH to the enzyme, and its near disappearance on subsequent incubation with naphthalene suggested that during the course of the reaction the enz-Fe^{2+} underwent valence changes to Fe^{3+} and back (Meera and Rao, 1979). Also, when NADH was replaced by H_2O_2, there is evidence for the involvement of O_2^- in the enzyme reaction; *viz.* inhibition by superoxide dismutase and decrease in absorbance at 240 nm (ε_{240} for O_2^- =1980 $M^{-1}cm^{-1}$; Behar *et al.,* 1970) during the course of the reaction in presence or absence of superoxide dismutase; here, the results suggested that O_2^- generation took place only on the addition of naphthalene to the mixture previously containing H_2O_2 and enzyme. The hydroxyl radical scavengers inhibited this system more than the NADH system. The formation of unidentified compounds on irradiation of the mixture containing naphthalene in 2-methoxyethanol, H_2O_2 and phosphate buffer at 240 nm is likely to be due to .OH radicals, generated by the dismutation of H_2O_2 (Hochanadel, 1962), reacting with naphthalene. The above results suggested (a) operation of regulatory mechanisms in the production of H_2O_2 and O_2^- by the enzyme, (b) that O_2^- is generated by the reaction, Fe^{2+} ---- O_2 ---- Fe^{3+} ---- O_2^- and (c) that .OH radical is produced by the Haber-Weiss (1934) mechanism. We propose the following sequence which involves incorporation of .OH radical into naphthalene in the formation of *cis*-1;2-dihydroxy-l,2-dihydronaphthalene by naphthalene oxygenase from *C. renale.*



The mechanism proposed is consistent with the stoichiometry of the reaction. The slow rate of Haber-Weiss reaction (k = 0.1 to 10 $M^{-1}s^{-1}$) and the subsequent fast addition of O H radical to C_1-C_2 double bond of naphthalene (k=(9.5 ± 1.5)$\times10^9$ $M^{-1}s^{-1}$; Michael and Hart, 1970) ensure that hydroxyl radical does not accumulate in the system. Based on the presumption that naphthalene oxygenase from *C. renale* is a dioxygenase, we postulate that both the hydroxyl groups of *cis*-l,2-dihydrodiol arise from O^-_2. Experiments confirming the validity of the above scheme using $H_2{}^{18}O_2$, $H_2{}^{18}O$ and $^{18}O_2$are in progress. The hydroxyl radical has been implicated in other systems: hydroxylation of '*p*-cresol and *p*-nitrophenol (Goscin and Fridovich, 1972) production of ethylene from methional (Beauchamp and Fridovich, 1970); peroxidation of lysosomal membranes (Fong *et al.,* 1973) and deploymerization of hyaluronic acid (McCord, 1974).

Acknowledgement

The authors are grateful to Council of Scientific and Industrial Research, New Delhi for financial assistance.

References

Axcell, B. C. and Geary, P.J. (1975) *Bioch em. J.* **146,** 173.
Beauchamp, C. and Fridovich, I. (1970) *J. Biol. Chem.,* **245,**4641.
Beauchamp, C. and Fridovich, I. (1971) *Anal. Biochem.* **44,** 276.
Behar, D., Czapski, G., Rabani, J., Dorfman, L.M. and Schwarz,H. A. (1970) *J. Phys. Chem.* **74,** 3209.
Booth, J. and Boyland, E. (1958) *Biochem. J.,* **70,** 681.
Brumby, P. E. and Massey, V. (1967) *Methods Enzymol.,* **10,** 463.
Catterall, F. A., Murray, K. and Williams, P. A. (1971) *Biochim. Biophys. Acta,* **237,** 361.
Dua, R. D. and Meera, S. (1981) *Eur. J. Biochem.,* **120,** 461.
Fazekas, A. G. and Kokai, K. (1971) *Methods Enzymol.,* **B18,** 390.
Fong, K. L, McCay, P. B., Poyer, J. L., Keele, B.B. and Misra, H. (1973) *J. Biol Chem.* **248,** 7792.
Fridovich, I. (1976) in *Free Radicals in Biology,* ed. W. A. Pryor (New York: Academic Press) vol 1, p. 247.
Gibson, D. T., Koch, J. R. and Kallio, R. E. (1968a) *Biochemistry,* **7,** 2653.
Gibson, D. T., Koch, J. R., Schuld, C. L. and Kallio, R. E. (1968b) *Biochemistry,* **7,** 3795.
Goscin, S. A. and Fridovich, I. (1972) *Arch. Biochem. Biophys.,* **153,** 778.
Haber, F. and Weiss, J. (1934) *Proc. Roy. Soc. (London) Ser. A.,* **147,** 332.
Hochanadel, C.J. (1962) *Radiation Res.,* **17,** 286.

Jeffrey, A. M., Yeh, H.J. C. and Jerina, D. M. (1974) *J. Org. Chem.* **39,** 1405.

Jeffrey, A. M., Yeh, H. J. C., Jerina, D. M., Patel, T. R., Davey, J. F. and Gibson, D. T. (1975) *Biochemistry,* **14,** 575.

Kobayashi, S., Kuno, S., Itada, N., Hayaishi, O., Kozuka, S. and Oae, S. (1964) *Biochem.Biophys.Res. Commun.,* **16,** 556.

Kondo, H., Friedmann, H. C. and Vennesland, B. (1960) *J. Biol. Chem.,* **235,** 1533.

Laborde, A. L. and Gibson, D. T. (1979) *Abstr. Annu. Meet. Am. Soc. Microbiol.,* p. 148.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.,* **193,** 265.

McCord, J. M. (1974) *Science,* **185,** 529.

Meera, S. (1980) *Purification, characterisation and studies on the mechanism of action of naphthalene oxygenase from Corynebacterium renale,* Ph.D. Thesis, Indian Institute of Technology, New Delhi.

Meera, S. and Rao, P. S. (1979) *Indian J. Biochem. Biophys.,* **16,** 81.

Michael, B. D. and Hart, E. J. (1970) *J. Phys. Chem.,* **74,** 2878.

Milne, G. W. A., Goldman, P. and Holtzman, J. L. (1968) *J. Biol. Chem.,* **243,** 5374.

Nakazawa, T., Nozaki, M., Hayaishi, O. and Yamano, T. (1969) *J. Biol. Chem.,* **244,** 119.

Reiner, A. M. and Hegeman, G. D. (1971) *Biochemistry,* **10,** 2530.