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Biomimetic oxidation of N-nitrosodibenzylamine with molecular oxygen catalysed by chemical cytochrome P-450 in AOT reverse micelles

S M S CHAUHAN* and S SATAPATHY
Department of Chemistry, University of Delhi, Delhi 110007, India

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Abstract. Moderate yields of benzaldehyde, benzyl alcohol and benzylamine are obtained by the biomimetic oxidation of N-nitrosodibenzylamine with molecular oxygen catalysed by water soluble anionic manganese(III) 5,10,15,20-tetraphenylporphyrin acetate/sodium dithionite/methylene blue in aerosol-OT (AOT) reverse micelles, under phase transfer conditions with AOT concentration higher than 10^{-3} M. The formation of α-hydroxy-N-nitrosodibenzylamine and its decomposition products, benzaldehyde and benzyl alcohol in reverse micellar systems are governed by the ratio of water and AOT, pH and other changes in the microenvironment.

Keywords. Chemical cytochrome P-450; aerosol-OT (AOT) reverse micelles; biomimetic oxidation; reductive activation of oxygen.

1. Introduction

Cytochromes P-450 are membrane-bound heme enzymes which catalyse the regioselective, stereoselective hydroxylation, epoxidation and oxidation of different organic substrates by utilisation of NADPH and molecular oxygen (Hall 1985; Nebert and Gonzalez 1987). Iron(III) protoporphyrin IX in cytochrome P-450 is reduced to iron(II) by fast electron transfer from NADPH and cytochrome P-450 reductase either in mitochondria or microsomal systems. Molecular oxygen binds to iron(II) protoporphyrin leading to iron(III) peroxy radical which is further reduced by second slow electron transfer for NADPH and cytochrome P-450 reductase to form iron(III) peroxy ions. In the presence of a proton source, iron(III) peroxy ions are protonated and are transformed to high valent iron oxo radical cations and related species which are responsible for mono-oxygenase reaction of substrates (Coulson *et al* 1984; Guengrich and McDonald 1984; Nagalsu *et al* 1990). The high valent oxo iron intermediates of cytochrome P-450 are separated from the corresponding reducing components by biomembranes which are responsible for the high catalytic cycles of cytochrome P-450 (Hall 1985, 1986). Cytochrome P-450, cytochrome P-450 reductase and NADPH have been reconstituted in model membranes. In particular, these reconstituted systems in liposomes have been used for the oxidation of organic substrates (Ingelman-Sundberg *et al* 1981; Kunz *et al* 1985; Taniguchi *et al* 1987).

Different chemical models for short catalytic cycles of cytochrome P-450 in homogeneous organic solvents have been developed by reactions of different

* For correspondence

monooxygen donors with iron(III) and manganese(III) porphyrins (Mansuy 1987; Holm 1987; Mansuy *et al* 1989; Chauhan *et al* 1990; White 1990). Chemical models for long catalytic cycles of cytochrome P-450 utilise molecular oxygen and iron(III) or manganese(III) tetraarylporphyrins in presence of reducing agents in organic solvents as well as in biphase for biomimetic oxidation of organic substrates (Tabushi and Kodera 1986; Battioni *et al* 1987; Tabushi 1988). Excess of reducing agents deactivate the oxoiron or oxomanganese porphyrins and are responsible for the low catalytic turnover number of the models and the low yield of oxidation products.

Reverse micelles are simple, convenient and dynamic models for biological membranes which are formed by the solubilisation of sodium *bis*(2-ethylhexyl) sulpho succinate (aerosol-OT or AOT) and/or related surfactant molecules in *iso*-octane and related hydrocarbons (Luisi 1985; Luisi and Magid 1986; Martineck *et al* 1986; Fendler 1987). They provide a unique and variable micro-environment, depending on the water to surfactant ratio, to study different kinds of reactions (Ranganathan *et al* 1989; Briffraud *et al* 1990; Singh *et al* 1990). They are used for the solubilisation of proteins and other biomolecules in the aqueous interface and the organic phase depending on their nature (Helenius and Simons 1975; Luisi and Magid 1986; Ringsdorf *et al* 1988). Enzymes entrapped in reverse micelles have altered reaction rates and products due to the change in their conformations as compared to those in aqueous solutions (Erjomin and Metelitza 1983; Pshezhetskii *et al* 1987; Walde *et al* 1988). Certain heme enzymes and proteins also change their spin states, as compared to their states in aqueous solutions (Luisi 1985). Recently the biomimetic oxidation of alkanes following the short catalytic cycles of cytochrome P-450 using iron salts and H_2O_2 in reverse micelles has been reported (Briffaud *et al* 1990), but so far no study has been carried out using metalloporphyrins following the long catalytic cycle of cytochrome P-450 in reverse micelles. This is the first report about the biomimetic oxidation of an organic substrate, N-nitrosodibenzylamine, with molecular oxygen catalysed by chemical model for long catalytic cycles of cytochrome P-450 in AOT reverse micelles.

2. Materials and methods

2.1 *Starting materials*

Water soluble manganese(III)-5,10,15,20-tetra(4′-sulphonatophenyl)porphyrin TPPS–Mn(III) acetate (1a) and zinc(II)-5,10,15,20-tetra(4′-sulphonatophenyl) porphyrin TPPS–Zn(II) (1b) were prepared by modification of known procedures (Fleischer *et al* 1971; Srivastava and Tsutsui 1973). N-Nitrosodibenzylamine (7a) was prepared by the nitrosation of dibenzylamine (7b) with $NaNO_2$/HCl by modification of literature procedure (Looney *et al* 1957). Commercially available sodium *bis*(2-ethylhexyl) sulphosuccinate (AOT) was purified before use (Luisi and Magid 1986; Magid *et al* 1988).

2.2 *Formation of AOT reverse micelles with different water:surfactant ratios* (W_o)

Phosphate buffer (pH 7·0, 5·4, μl, 0·2 M) was injected into a solution of AOT (3 × 0·044 g, 0·3 mM) in *iso*-octane (3·0 ml). After shaking thoroughly it was allowed to stand for 3 min to obtain a transparent solution of AOT reverse micelles with the water to

surfactant ratio (W_0) = 1. Further, 5·4 μl of phosphate buffer, pH 7·0, was injected in to the above solution to get reverse micelles of $W_0 = 2$. Similarly reverse micelles of different W_0 values were prepared. The formation of reverse micelles were monitored by UV absorption at 210 nm (Luisi and Magid 1986).

2.3 *Incorporation of metalloporphyrins in AOT reverse micelles*

A stock solution of 1a (5·4μl, 3·6 mM) or 1b (5·4 μl, 6·5 mM) in phosphate buffer (pH 7·2, 0·2 M) was injected into a solution of AOT (0·1 M, 3 ml) in *iso*-octane. The solution was thoroughly shaken and was allowed to stand for 10 min to get a transparent solution of 1a or 1b in AOT reverse micelles. For each increase in W_0, 5·5 μl of additional buffer was added to the AOT solution. The incorporation of metalloporphyrin 1a or 1b in reverse micelle at different W_0 was confirmed by UV-visible spectroscopy (figures 1 and 2).

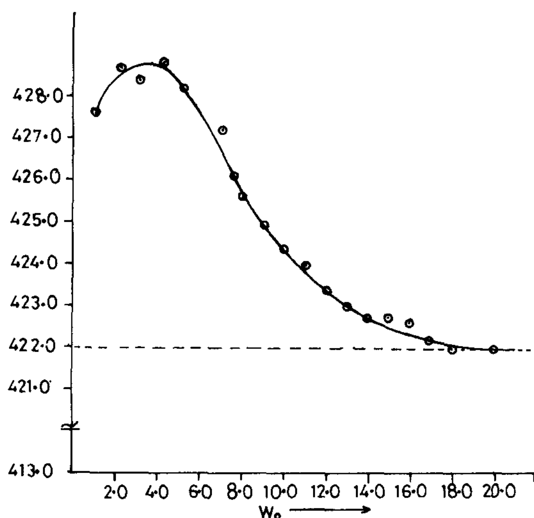


Figure 1. TPPS–Zn(II) at pH = 7 and at different values of W_0 in 0·1 M AOT/*iso*-octane.

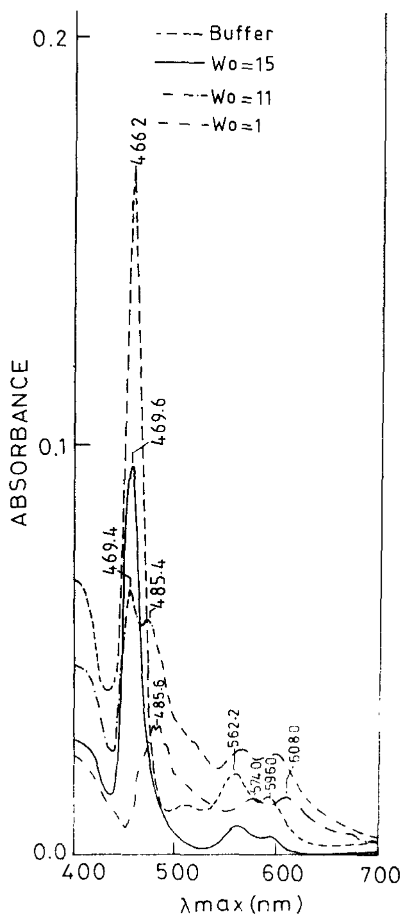


Figure 2. TPPS$_4$–Mn(II) OAc at pH = 7 and different values of W_0 in 0·1 M AOT/*iso*-octane.

Table 1. Volatile decomposition products of dibenzylnitrosoamine, water soluble manganese porphyrins, reducing agent sodium dithionite, methylene blue and molecular oxygen at pH 7 in biphase and reverse micellar system of 0·1 M AOT/*iso*-octane at different W_0.

W_0	System	Total amount of product (10^{-5} M)	Turnover number	% Yield* Benzal-dehyde **RT 1·5	% Yield* Benzyl alcohol RT 2·0	% Yield* Benzyl-amine RT 1·8
—	1a/$Na_2S_2O_4$/methylene blue/ tetrabutylammonium bromide/biphase/O_2	2·30	26·09	8·8	3·7	12·35
—	1a/$Na_2S_2O_4$/methylene blue/AOT(10^{-4} M)/ biphase/O_2	—	—	—	—	—
—	1a/$Na_2S_2O_4$/methylene blue/AOT(10^{-3} M)/ biphase/O_2	6·7	74·5	27·0	10·0	33·76
7	1a/$Na_2S_2O_4$/methylene blue/O_2/reverse micelle	2·95	129·6	121·0	47·8	—
10	1a/$Na_2S_2O_4$/methylene blue/O_2/reverse micelle	2·64	71·7	47·2	25·0	—
15	1a/$Na_2S_2O_4$/methylene blue/O_2/reverse micelle	3·20	141·7	38·3	23·0	—
15	1a/$Na_2S_2O_4$/O_2/reverse micelle	1·48	65·4	11·4	18·0	—
20	1a/$Na_2S_2O_4$/methylene blue/O_2/reverse micelle	1·75	47·5	29·1	19·8	—

* Yield is calculated with respect to reducing agent; ** RT – retention time

2.4 *Oxidation of N-nitrosodibenzylamine (7a) in AOT reverse micelles*

7a (0·2 m mol) was added to the above AOT reverse micellar solution containing 1a. Methylene blue (10 μl, 0·2 M) and sodium dithionite (1·74 mg, 7·1 μl mol) were added to the above well-stirred reverse micellar solution and reduction was monitored by UV spectroscopy. Oxygen gas was bubbled through the above reaction mixture for 12 h. The reaction mixture was concentrated and the residue was subjected to GLC analysis to monitor the volatile products. The yields of different volatile products with GC retention times under different reaction conditions are given in table 1.

3. Results

The water to surfactant (AOT) ratio (W_0) determines the structural and physical properties of AOT reverse micelles. At lower W_0 most of the water molecules are bound to the AOT molecules and above $W_0 = 6$–8 some free water exists in the water pool. The entrapment of 1b leads to a blue shift in soret, at $W_0 = 1$, pH 7, to 428 nm.

There is a gradual increase in λ_{max} with increase of W_0 and it reaches the same value as that in the buffer (422 nm) at $W_0 = 18$ (figure 1). The entrapment of 1a shows similar behaviour to that of 1b with blue shifts to 485, 574 and 608·0 nm at $W_0 = 1$, pH 7. It remains virtually constant till $W_0 = 10$. At $W_0 = 11$, there is a splitting of the 485 nm peak to 485 nm and 469 nm. Above $W_0 = 11$, only the absorptions at 466, 562 and 596 nm resemble the absorption of buffer (figure 2). The blue shifts in UV absorption can be accounted for by the formation of aggregates at lower W_0 and localisation of porphyrin at the interface.

3.1 *Effects of AOT concentration*

The reaction of N-nitrosodibenzylamine (7a) with molecular oxygen catalysed by 1a in benzene/phosphate buffer in the presence of AOT (at 10^{-5} M and 10^{-4} M concentrations) did not give any product, but at an AOT concentration of 10^{-3} M, benzaldehyde (11) and benzyl alcohol (14) are obtained in 27·0 and 10·0% yields respectively. The lack of reverse micelle formation may be responsible for the absence of decomposition products at AOT concentration less than 10^{-3} M. But above the cyclic micellar concentration (CMC) of AOT (10^{-3} M), decomposition products of α-hydroxy N-nitrosodibenzylamine (10) were obtained suggesting the formation of reverse micelles and microemulsions.

3.2 *Effect of water/surfactant ratio* (W_0)

The reaction of N-nitrosodibenzylamine (7a) with molecular oxygen in presence of 1a and $Na_2S_2O_4$/methylene blue in AOT reverse micelles at $W_0 = 7$ gave benzaldehyde and benzyl alcohol in 121 and 47·8% yields respectively. Increase of W_0 decreases the yield of both the products (table 1). Absence of methylene blue decreases the yield of the decomposition products (table 1), whereas omission of any of the above components does not give the decomposition products in biomimetic oxidation.

3.3 *Effect of imidazole*

The reaction of 7a with $Na_2S_2O_4$/N-methylimidazole/methylene blue/O_2 in reverse micelle at $W_0 = 15$, pH 7 gives benzaldehyde and benzyl alcohol in 41·4 and 28·8% yield respectively. When imidazole is replaced by N-methylimidazole, benzyl alcohol is obtained in 36·05% yield.

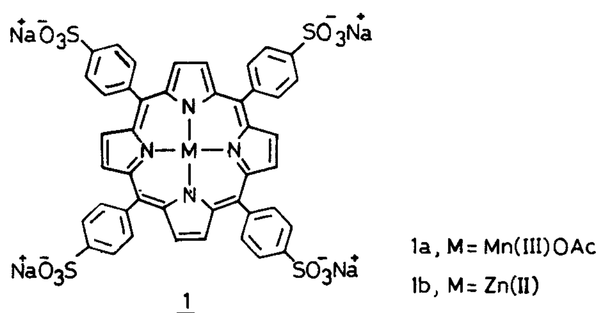
3.4 *Effect of pH*

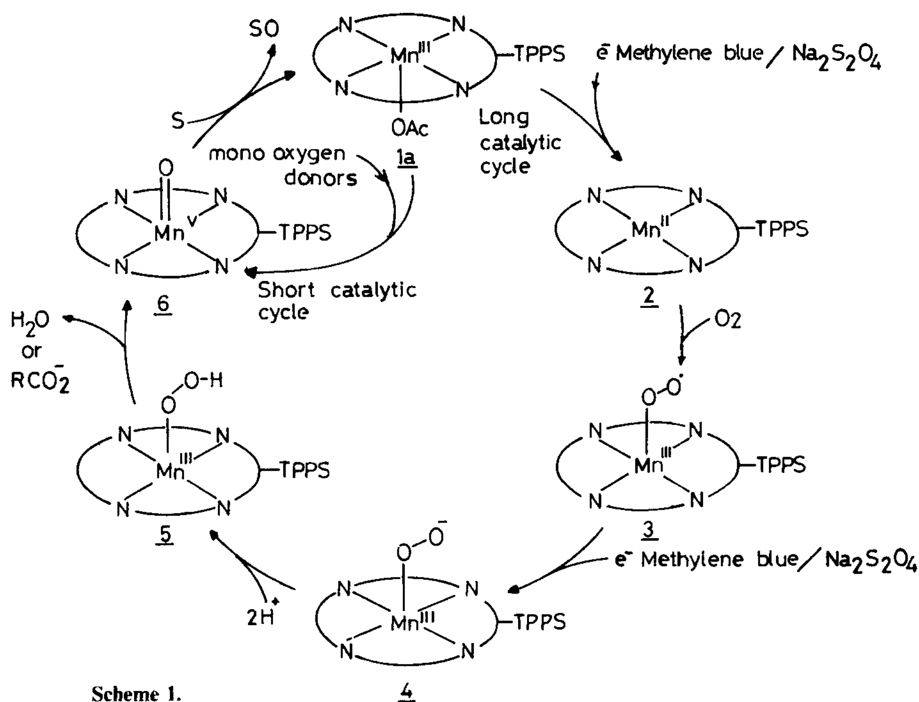
The reaction of 7a with 1a/$Na_2S_2O_4$/methylene blue/O_2 in reverse micelle at $W_0 = 15$, pH = 5·7, 7·0, 9·0 and 13·0, have been studied. The yields of the different decomposition products are given in table 2.

4. Discussion

The water soluble MnTPPS and molecular oxygen in presence of reducing agent, dihydropyridine, and electron transfering agent, flavin mononucleotide, are able to

epoxidise nerol and hydroxylates hydrocarbons (Tabushi and Kodera 1986). The reducing agent in the above example does not compete with the substrate for the reaction with high valent oxomanganese porphyrins (Tabushi 1988). Sodium dithionite is a powerful reducing agent in aqueous alkaline solution and it reduces the monomers as well as dimers of manganese(III) porphyrins to manganese(II) porphyrins (Harriman and Porter 1979; Duncan *et al* 1980). The TPPSMn(III)OAc (1a) is reduced to TPPSMn(II) by sodium dithionite dissolved in the aqueous pool of AOT reverse micelle and this reduction is facilitated by the presence of electron transferring agent methylene blue (Kurihara and Fendler 1983; Esch *et al* 1986). Manganese(II) porphyrin 2 reacts with molecular oxygen to form a complex which by inner electron transfer mechanism changes to TPPSMn(III)–O–Ȯ (3). The single



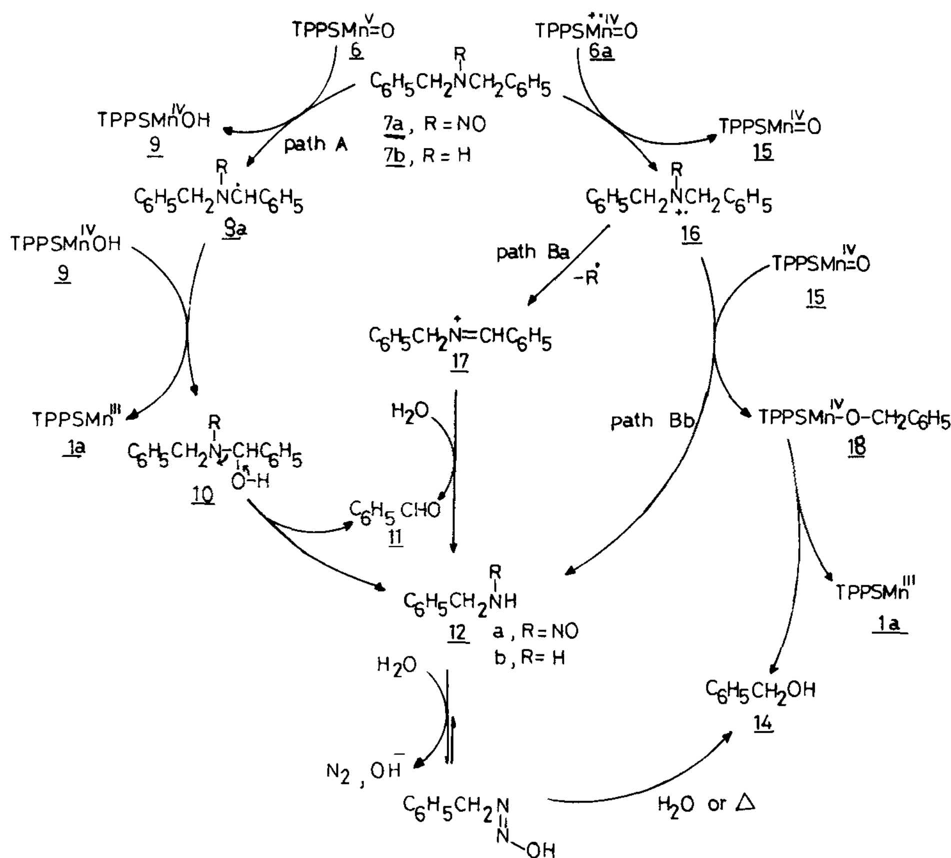


Scheme 1.

electron reduction of 3 gives TPPSMN–O–O$^-$ (4) which on protonation at the interface gives TPPSMn–O–OH (5). The heterolytic cleavage of 5 at interface gives oxomanganese porphyrin (6) and the corresponding radical cation, responsible for biomimetic reactions (scheme 1) (Lindsey-Smith and Mortimer 1986; Banfi *et al* 1990).

The high valent oxomanganese porphyrin (6) abstracts hydrogen radical *syn* to nitroso group (Fraser 1976) of N-nitroso dibenzylamine (7a) to form the radical species (8a) and hydroxy manganese porphyrin (9), both of which further recombine in the solvent cage to give α-hydroxy N-nitrosodibenzylamine (10). The spontaneous decomposition of 10 by fragmentation gives benzaldehyde) (11) and primary nitrosoamine 12a which isomerise to give diazohydroxide 13.

The nucleophilic attack of H_2O on 13 or its thermal decomposition gives benzyl alcohol (14) (scheme 2, path A) (Lindsey-Smith *et al* 1984). The high valent oxomanganese radical cationic species 6a may abstract radical from dibenzyl nitrosoamine (7a) leading to the formation of radical cation 16a which might eliminate HNȮ to give intermediate 17. The reaction of H_2O with 17 gives benzylamine (12b) and benzaldehyde (11) (scheme 2, path Ba). The reaction of radical cation 16 with high valent oxomanganese complex 15 may give 12 and unstable intermediate 18 which spontaneously decomposes to give benzyl alcohol (14) and manganese(III) porphyrin. The formation of alcohol by the oxidation of



Scheme 2.

Table 2. Volatile decomposition products of dibenzylnitrosoamine/dibenzylamine with water soluble tetraphenylporphyrinato manganese porphyrin, sodium dithionite, methylene blue and oxygen at different pH and $W_0 = 15$ in reverse micelle of 0·1 M AOT/*iso*-octane.

pH	System	Total amount of product (10^{-5} M)	Turn-over number	% Yield*	
				Benzaldehyde	Benzyl alcohol
5·7	Dibenzylnitrosoamine/1a/ $Na_2S_2O_4$/methylene blue/O_2	1·7	74·8	—	47·4
7·0	Dibenzylnitrosoamine/1a/ $Na_2S_2O_4$/methylene blue/O_2	3·2	141·8	106·9	25·0
7·0	Dibenzylamine/1a/$Na_2S_2O_4$/ methylene blue/O_2	0·51	56·6	4·5	68·0
9·0	Dibenzylnitrosoamine/1a/ $Na_2S_2O_4$/methylene blue/O_2	2·4	38·3	23·5	23·8
13·0	Dibenzylnitrosoamine/1a/ $Na_2S_2O_4$/methylene blue/O_2	4·8	212·3	108·5	28·6

*Yield is calculated with respect to reducing agent; turnover number = moles of product/moles of porphyrin; ratio of porphyrin: reducing agent: electron transfering agent: substrate 1:10:10:1000.

electron rich compounds by high valent oxometalloporphyrins have been reported recently (Baciocchi 1990). This mechanism may be operative in the formation of benzyl alcohol (14) from dibenzyl amine (7b) by the above model system in AOT reverse micelles (path Bb) (table 2).

The biomimetic oxidation of 7a gives high yield of decomposition products, benzaldehyde and benzyl alcohol, in reverse micelles as compared to biphase system with low concentration of AOT (10^{-3} M concentration or less). Thus reverse micelles offer unique and variable microenvironment in compartmentalising and catalysing the model reactions and may be used for selective transformation of both lipid soluble and aqueous soluble compounds in enzymatic and nonenzymatic reactions.

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