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Superoxide Radical as an Intermediate in the Oxidation of Hydroxylamines by Mixed Function Amine Oxidase

ELMER J. RAUCKMAN,[1] GERALD M. ROSEN,[2] AND BARBARA B. KITCHELL

Departments of Surgery and Pharmacology, Duke University Medical Center, Durham, North Carolina 27710

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SUMMARY

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Previous observations have shown that mixed function amine oxidase (MFAO), a flavoprotein, is responsible for the oxidation of hydroxylamines to nitroxides. We had assumed that the hydroperoxyflavin directly attacked the hydroxylamine giving the hydroxylamine oxide as an intermediate which rapidly decomposed to yield a nitroxide and a hydroxyl radical. A kinetic investigation showed that MFAO does not directly attack the hydroxylamine but suggested that MFAO releases an oxidizing agent that is responsible for hydroxylamine oxidation. The Haber-Weiss reaction, known to produce hydroxyl radicals, was shown not to be involved in the formation of nitroxide free radicals. It was found that superoxide is solely responsible for MFAO mediated nitroxide formation.

INTRODUCTION

Nitroxide radicals have been postulated as intermediates in the hepatic microsomal oxidation of primary and secondary amines (1) as shown in figure 1. Unfortunately, the instability of these radicals has hampered efforts to study this oxidative pathway. However, it has recently been found that certain secondary amines form stable or relatively stable nitroxides which may be studied using epr[3] techniques. For example, Floyd *et al.* (2) have demonstrated that a nitroxide is an intermediate in the microsomal oxidation of N-acetyl-N-hydroxy-2-aminofluorene (figure 2) and have suggested that this radical, rather than its nitrosoamine product, is the active carcinogen.

Mixed function amine oxidase (MFAO) is a flavoprotein which is responsible for the hepatic microsomal oxidation of various sulphur and nitrogen containing compounds (3, 4). Specifically, this enzyme oxidizes unhindered secondary amines and hydroxylamines while primary alkylamines and unsubstituted hydroxylamines are not true substrates for MFAO (5). We have previously shown that MFAO will oxidize certain hindered secondary hydroxylamines to stable nitroxides (6, 7) which may

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[3] The abbreviations used are: epr, electron paramagnetic resonance; MFAO, mixed function amine oxidase; TEMPO, 2,2,6,6-tetramethylpiperidinoxyl; DTBNO, di-t-butylnitroxide; OXANO, 2-ethyl-2,4,4-trimethyl-3-oxazolidinyloxy; PROLO, 2-ethyl-2,5,5-trimethylpyrrolidinoxyl.

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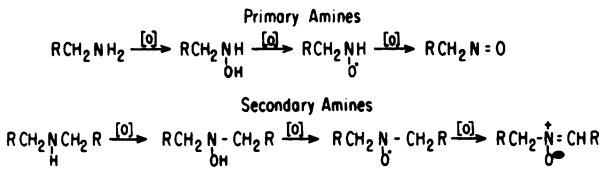


FIGURE 1

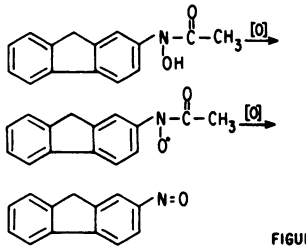


FIGURE 2

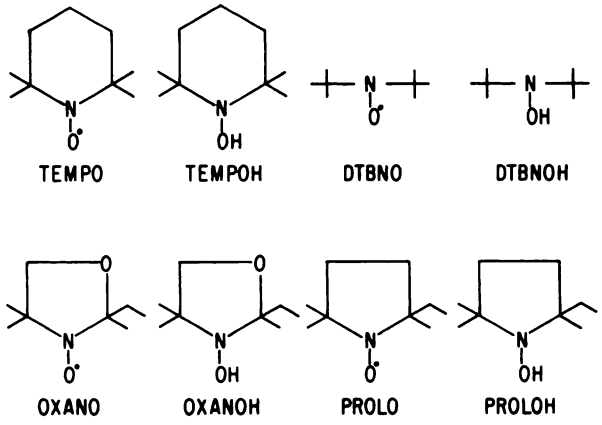
be quantitated by epr. This report describes experiments demonstrating that this oxidation proceeds by way of a single electron transfer process that is mediated by superoxide radical.

MATERIALS AND METHODS

Purified mixed function amine oxidase from pig liver microsomes was a gift from Dr. Dan Ziegler. The enzyme was stored at −20° as a lyophilized powder from 0.1 M phosphate buffer, pH 8.3 and dissolved with water immediately before use. Xanthine and xanthine oxidase (grade I) were obtained from Sigma Chemical Co. NADPH was purchased from Boehringer Mannheim Biochemicals. Superoxide dismutase, bovine erythrocyte, was a gift from Dr. Irwin Fridovich and was assayed for activity by the inhibition of the xanthine/xanthine oxidase reduction of cytochrome c.

The spin labeled probes, OXANO, TEMPO and PROLO were prepared according to the methods of Keana *et al.* (8, 9) and Rauckman *et al.* (10). DTBNO was obtained from Polysciences, Inc. Reduction of OXANO, TEMPO, DTBNO and PROLO to their corresponding hydroxylamines, OXANOH, TEMPOH, DTBNOH and PROLOH, was carried out by hydrogenation at 1 atm. using platinum oxide as catalyst. The purity of the hydroxylamines was demonstrated by means of thin layer chromatography. A chloroform extract of the aqueous reduced nitroxides (using Eastman silica gel thin layer plates eluted with benzene:ethyl acetate - 3:1) gave one spot identical with the parent nitroxide. It was found that hydroxylamines rapidly air oxidize to their corresponding nitroxides.

The rate of hydroxylamine oxidation was determined by measuring the initial rate of



nitroxide formation using a Varian Associates model E-9 spectrometer equipped with a thermostated cell holder. In a typical experiment, the reaction mixture contained 10 mM hydroxylamine, 0.2 mM NADPH, 0.1 mg/ml MFAO in 0.1 M phosphate buffer at pH 8.3 or 0.25 mM xanthine and 1 μg/ml xanthine oxidase in 0.1 M phosphate buffer at pH 7.8.

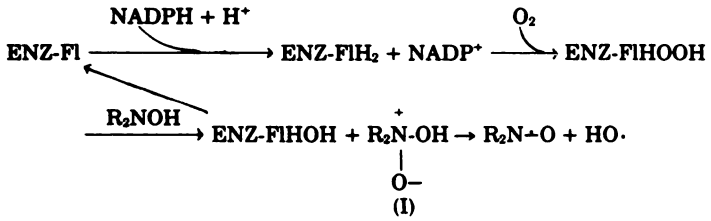
Superoxide production was measured by following the rate of cytochrome c reduction at 550 nm using a Varian/Cary 118 spectrophotometer equipped with a thermostated cell holder. In a typical experiment, the reaction mixture contained 10 μM cytochrome c (Sigma Chemical Co.), 0.2 mM NADPH and 0.1 mg/ml MFAO in 0.1 M phosphate buffer at pH 8.3 or 0.25 mM xanthine and 1 μg/ml xanthine oxidase in 0.1 M phosphate buffer at pH 7.8.

Control experiments were undertaken to demonstrate catalase cannot act as a peroxidase and in doing so, oxidize OXANOH to its corresponding nitroxide. Catalase (0.01 μg/ml) and hydrogen peroxide (10 mM) were mixed with OXANOH (1 mM) in phosphate buffer pH 7.8. In a different experiment, glucose (20 mg/ml), glucose oxidase (0.2 units/ml; Sigma Chemical Co.), catalase (50 μg/ml) and OXANOH (1 mM) were mixed in phosphate buffer at pH 6.8. In both cases, the rate of nitroxide formation was not greater than the rates obtained in the absence of catalase and hydrogen peroxide in the first experiment and glucose oxidase and catalase in the latter experiment.

RESULTS AND DISCUSSION

The oxidation of hydroxylamines to nitroxides is overall a one electron transfer process that is carried out by MFAO, a flavoprotein. Since this enzyme had not previously been implicated in one electron transfer reactions, we were prompted to investigate this oxidative reaction in some detail.

Our initial supposition was that the hydroxylamine was directly attacked by an oxygenated form of the reduced enzyme, represented here as the hydroperoxyflavin for simplicity, to give the hydroxylamine oxide (I). This species, being unstable, would undergo homolytic cleavage to a stable nitroxide and hydroxyl radical as shown:



(Equation 1)

This mechanism appeared reasonable in light of chemical studies by Hoffmann *et al.* (11) who suggested that (I) is an intermediate in the reductive conversion of 2-methyl-2-nitropropane to di-t-butylnitroxide. It was suggested that this intermediate decomposed to the nitroxide and hydroxyl radical. If in fact the hydroxyl radical were also produced during MFAO mediated hydroxylamine oxidation as shown in equation 1, hydroxyl radical could react with a number of species present in the reaction mixture to form radical products; however the two most likely chain termination reactions are shown by equations 2 and 3. The rates of both reactions should be diffusion limited, owing to the high reactivity of the hydroxyl radical. Since the concentration of the hydroxylamine is high compared to the hydroxyl radical, the pathway shown by equation 2 is likely to predominate. On this basis, one would anticipate that two moles of nitroxide would be produced for every mole of NADPH oxidized.

$$HO\cdot + R_2NOH \rightarrow R_2N\text{-}O + H_2O \quad \text{(Equation 2)}$$

$$HO\cdot + HO\cdot \rightarrow H_2O_2 \quad \text{(Equation 3)}$$

Upon addition of OXANOH to a mixture of NADPH and MFAO, the following observations were made: 1) NADPH oxidation rate did not increase over that of control (without OXANOH) and 2) the rate of

TABLE 1

Enzyme system	Rate NADPH oxidation	Cytochrome c reduction	Rate N → O formation
Mixed function amine oxidase			
1) pH 7.4	1.7 μM/min	0.063 μM O_2^-/min	
2) pH 8.3	5.6 μM/min	0.200 μM O_2^-/min	
Mixed function amine oxidase[a]			
1) OXANOH	5.6 μM/min		0.20 μM/min
2) TEMPOH	5.6 μM/min		0.08 μM/min
3) DTBNOH	5.6 μM/min		0.04 μM/min
4) PROLOH	5.6 μM/min		0.08 μM/min
5) OXANOH or TEMPOH or DTBNOH, or PROLOH + SOD[b] (0.5 μg/ml)	5.6 μM/min		0
Xanthine-xanthine oxidase[c]			
1) control		2.10 μM O_2^-/min	
2) OXANOH			1.36 μM/min
3) TEMPOH			0.48 μM/min
4) DTBNOH			0.56 μM/min
5) OXANOH or TEMPOH or DTBNOH + SOD[b] (0.5 μg/ml)			0

[a] pH 8.3
[b] Superoxide dismutase
[c] pH 7.8

OXANO formation, as measured by epr spectroscopy, significantly increased over that of control (without NADPH or without MFAO). If the mechanism depicted in equation 1 were correct, one would anticipate an increase in NADPH oxidation upon addition of a hydroxylamine. Since this was not observed, the mechanism shown in equation 1 is unlikely. A mechanism more consistent with these observations is one in which MFAO releases an oxidizing agent in the presence of NADPH. The two most likely agents are superoxide and hydrogen peroxide which are both known to be products of the xanthine/xanthine oxidase reduction of oxygen (12). However, Rozantsev (13) has demonstrated that hydrogen peroxide will not oxidize hydroxylamines without an appropriate catalyst (e.g., sodium tungstate). Furthermore, the kinetics of nitroxide formation are not consistent with the participation of hydrogen peroxide as the oxidizing agent.[4]

[4] If hydrogen peroxide were the oxidizing agent, the rate of nitroxide formation would be proportional to the concentration of hydrogen peroxide generated giving exponential kinetics. However, the rate of nitroxide formation in the presence of MFAO or xanthine/xanthine oxidase decreases slightly with time.

The second candidate, superoxide, had not previously been identified as a product of MFAO reduction of oxygen.[5] However, we observed that a mixture of MFAO and NADPH reduces cytochrome c and that this reduction was inhibited by superoxide dismutase (Table 1), suggesting that superoxide is produced by MFAO in the presence of NADPH. In addition, superoxide dismutase inhibited the NADPH dependent MFAO mediated oxidation of OXANOH (Table 1). Furthermore, the xanthine/ xanthine oxidase system, a known producer of superoxide, was also capable of oxidizing OXANOH to its nitroxide. This oxidation was inhibited by superoxide dismutase (Table 1).

The close correlation between superoxide flux, as measured by cytochrome c reduction, and nitroxide formation, as measured by epr spectroscopy, is in accord with the hypothesis that superoxide is the oxidizing agent. However, hydrogen peroxide is also generated by MFAO in the presence of NADPH and oxygen (14) and thus we had to consider the possibility that hydroxy radical, formed by the Haber-Weiss reaction (15) (equation 4), could play a role in the

[5] D. M. Ziegler, personal communication.

oxidation of hydroxylamines to nitroxides (equation 5), since superoxide dismutase inhibitory experiments do not discount the Haber-Weiss reaction but merely show that superoxide is an active participant in the oxidation of hydroxylamines to nitroxides.

$$H_2O_2\cdot + O_2^{-} \rightarrow HO\cdot + O_2 \quad \text{(Equation 4)}$$

$$HO\cdot + R_2NOH \rightarrow H_2O + R_2N\text{-}O \quad \text{(Equation 5)}$$

Accordingly, an experiment was undertaken to determine whether the Haber-Weiss reaction is involved in the superoxide dependent oxidation of OXANOH to OXANO. Catalase (50 μg/ml) was added to a xanthine/xanthine oxidase OXANOH mixture with the idea that it should inhibit the Haber-Weiss reaction by virtue of its rapid elimination of hydrogen peroxide. In the presence of catalase, the rate of nitroxide formation was not measurably reduced over the control rate, suggesting that the Haber-Weiss reaction does not participate in this oxidation. Two control experiments were performed and are described in the experimental section. Since these experiments were found to be negative, we concluded that superoxide alone is responsible for MFAO mediated nitroxide formation.

The role of superoxide in the oxidation of hydroxylamine to nitrite has received some attention. For example, Bors *et al.* (16) have studied the pulse radiolytic oxidation of hydroxylamine to nitrite. They reported that under their conditions, only hydroxyl radicals efficiently attacked hydroxylamine while superoxide radical is necessary for the subsequent formation of nitrite. Elstner and Heupel (17) had previously reported that superoxide formed by the xanthine/xanthine oxidase system oxidized hydroxylamine to nitrite. In the experiments described by Elstner, it is possible that hydroxyl radicals could be produced from either a Haber-Weiss reaction or a Fenton type reaction. Another possibility is that superoxide produced in low concentrations may directly attack hydroxylamine. However, with more sterically hindered hydroxylamines like OXANOH, superoxide can easily form nitroxides via a one electron oxidation. Kono (18) has reported that the autoxidation of hydroxylamines produces superoxide radicals. In this system, the participation of superoxide in the further oxidation of hydroxylamine is minimal since superoxide dismutase was found to inhibit only 12% of the nitrite formed. The mechanism he suggested involves initial attack of hydroxylamine by a metal cation to give a radical species which then reacts with oxygen to produce nitrite.

OXANOH was oxidized more rapidly than either TEMPOH or DTBNOH (see Table 1) with either MFAO or xanthine/xanthine oxidase as a source of superoxide. Although the reason for this phenomenon is not known, we have examined several possibilities and are proposing a hypothesis that is consistent with our observations. Differences in electrochemical potential may be eliminated since we previously found that all three substrates have identical redox potentials of 300 mV (19). We also determined that neither reduced MFAO nor superoxide, produced by either MFAO or xanthine/xanthine oxidase, reduced TEMPO or DTBNO. Thus, neither superoxide nor MFAO acts as a reducing agent for these nitroxides. It has been reported that the E_0 for O_2^{-}/O_2 is in the range of −280 to −330 mV (20). Since these nitroxides have an E_0 of 300 mv, it is likely that the E_0 for O_2^{-}/O_2 is less than −300 mV. Furthermore, since superoxide is small, a simple steric effect where superoxide cannot readily attack either TEMPOH or DTBNOH specifically is unlikely.

A hypothesis which is consistent with our observations is that the ether oxygen in OXANOH enhances the free radical oxidation of this hydroxylamine to its corresponding nitroxide. This theory was tested by measuring the rate of oxidation of PROLOH, a carbon analogue of OXANOH, by MFAO. This rate was similar to that observed for both TEMPOH or DTBNOH suggesting that the ether oxygen is, in fact, responsible for the increased oxidative rate of OXANOH. This phenomenon is not restricted to pig liver MFAO since we recently reported that in rat liver microsomes, cytochrome P-450 selectively reduces certain nitroxides (19). It is noteworthy that OXANO is not included in this group.

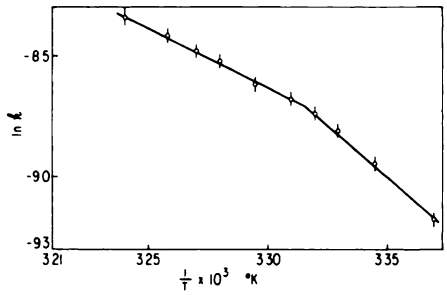


FIG. 3. *An Arrhenius plot for cytochrome c reduction by mixed function amine oxidase generated superoxide.*

This plot shows a definitive break at 29° where the calculated energy of activation, E_a, changes from 17.1 kcal/mole to 9.9 kcal/mole at temperatures above the transition point. The corresponding entropy of activation, ΔS^*, changes from 21.1 cal/deg mole to −3.24 cal/deg mole. Experimental conditions are described in the MATERIALS AND METHODS section. Error bars represent estimated maximal experimental error.

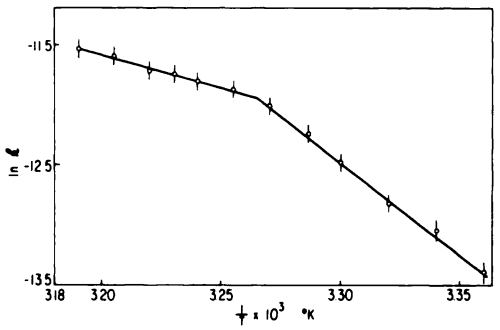


FIG. 4. *An Arrhenius plot for the oxidation of OXANOH by mixed function amine oxidase generated superoxide*

This plot shows a definitive break near 33° where the calculated energy of activation, E_a, changes from 31.1 kcal/mole to 11.2 kcal/mole at temperatures above the transition point. The corresponding entropy of activation, ΔS^*, changes from 37.8 cal/deg mole to −27.8 cal/deg mole. Experimental conditions are described in the MATERIALS AND METHODS section. Error bars represent estimated maximal experimental error.

In a previous report, we showed that MFAO undergoes a transition near 33° which affects both the physical properties and reactivity of the enzyme toward true substrates (21). It was found that Arrhenius plots of superoxide produced by MFAO, as measured by cytochrome c (Fig. 3) or OXANOH oxidation (Fig. 4), also show this transition. A control Arrhenius plot using xanthine/xanthine oxidase gave a straight

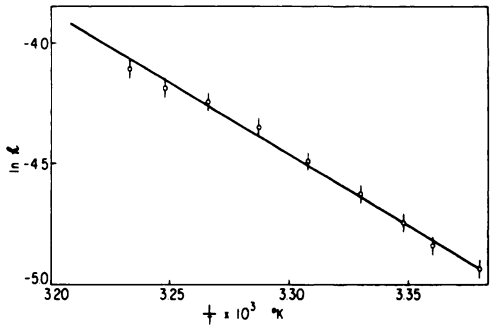


FIG. 5. *An Arrhenius plot for cytochrome c reduction by xanthine/xanthine oxidase generated superoxide*

This plot, unlike fig. 3, shows no transition over the range of temperatures examined where the calculated energy of activation, E_a, is 11.8 kcal/mole. The corresponding entropy of activation, ΔS^*, was calculated to be 0.15 cal/deg mole. Experimental conditions are described in the MATERIALS AND METHODS section. Error bars represent estimated maximal experimental error.

line which verified that MFAO alone is responsible for this transition (Fig. 5). The similarity in kinetic behavior of MFAO for production of superoxide and for a true substrate oxidation (e.g., N,N-dimethylaniline) suggests that a common rate limiting intermediate is involved in both processes. This will be the subject of a future paper.

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