Free-radical chain oxidation of 2-nitropropane initiated and propagated by superoxide

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The superoxide radical $O_2^{\cdot-}$, whether produced by the xanthine/xanthine oxidase reaction or infused as KO_2 , solubilized by a crown ether in dry dimethyl sulphoxide, initiated a free-radical chain oxidation of anionic 2-nitropropane. Superoxide dismutase, but not catalase, inhibited oxidation of the nitroalkane. Xanthine oxidase suffered a syncatalytic inactivation, during the co-oxidation of 2-nitropropane, which was reversed by dialysis. Cyanide exacerbated this syncatalytic inactivation and rendered it irreversible. The frequently observed oxidations of nitroalkanes by flavoenzymes now need to be re-examined to clarify the extent to which $O_2^{\cdot-}$ -initiated free-radical chain oxidation contributed to the overall nitroalkane oxidation.

INTRODUCTION

The oxidation of nitroalkanes in biological systems has been under study for approximately 35 years. Thus Little (1951) reported that extracts of *Neurospora crassa* caused the oxidation of nitroethane; extracts of pea plants had a similar effect on 2-nitropropane (NP^-) (Little, 1957). Porter *et al.* (1972, 1973) studied the oxidation of nitroalkanes by D-amino acid oxidase and adduced evidence for a flavin–substrate adduct, which could be trapped by addition of cyanide, leading to a syncatalytic inactivation of the enzyme. Kido *et al.* (1976a,b) grew the yeast *Hansenula mrakii* on a medium in which a nitroalkane was the sole source of nitrogen and then isolated a flavoenzyme from a cell extract. This flavoenzyme catalysed the oxidation of nitroethane, 1-nitropropane or 2-nitropropane, with incorporation of oxygen, yielding nitrite plus a carbonyl compound, but not H_2O_2 . They named it 2-nitropropane dioxygenase and noted that catechols, which scavenge $O_2^{\cdot-}$, inhibited nitroalkane oxidation.

The possible role of $O_2^{\cdot-}$ in nitroalkane oxidation by 2-nitropropane dioxygenase was explored further. Thus Kido *et al.* (1978a) found that the oxidation of NP^- by this enzyme was inhibited by SOD and by a variety of compounds capable of scavenging $O_2^{\cdot-}$, such as ferricytochrome *c*, adrenaline (epinephrine) and NADH. Moreover, they demonstrated $O_2^{\cdot-}$ production during the enzymic oxidation of NP^- in terms of an SOD-inhibitable reduction of Nitro Blue Tetrazolium and by e.p.r. spectroscopy (Kido *et al.*, 1978b). Horseradish peroxidase catalyses the oxidation of NP^- , and in this case catalase inhibits completely and SOD inhibits partially (DeRycker & Halliwell, 1978). These authors proposed that slow autoxidation of NP^- -produced H_2O_2 and that the peroxidase plus H_2O_2 caused the univalent oxidation of NP^- to a radical that could then reduce O_2 to $O_2^{\cdot-}$. They further proposed that $O_2^{\cdot-}$ or $HO^{\cdot}$, derived therefrom, could propagate the process by the univalent oxidation of NP^- . Kido & Soda (1984) have demonstrated that the oxidation of NP^- , whether catalysed by glucose oxidase, D-amino acid oxidase or 2-nitropropane dioxygenase, was inhibited by SOD. They concluded that $O_2^{\cdot-}$ was a

catalytically essential intermediate in all enzymic oxidations of NP^- .

We considered the possibility that $O_2^{\cdot-}$ is able both to initiate and to propagate the free-radical chain oxidation of NP^- . In that case the oxidations of anionic nitroalkanes, catalysed by a variety of flavoenzymes, would need to be re-evaluated. We chose the xanthine oxidase reaction as a convenient source of $O_2^{\cdot-}$ (McCord & Fridovich, 1968; Fridovich, 1970; Nagano & Fridovich, 1985), and found that $O_2^{\cdot-}$ could initiate a free-radical chain oxidation of NP^- . We then used a mechanical infusion of KO_2 in dry dimethyl sulphoxide to verify that $O_2^{\cdot-}$ as such was the active species. We now describe these and related experimental results.

MATERIALS AND METHODS

Xanthine oxidase, prepared from unpasteurized bovine cream (Waud *et al.*, 1975), was kindly supplied by Dr. K. V. Rajagopalan. It was dissolved to $11.7 \mu M$ in 50 mM-potassium phosphate/0.1 mM-EDTA buffer, pH 7.8, and was stored, in small portions, at $-70^\circ C$ until used. The specific activity of this enzyme preparation, in terms of the activity/flavin ratio, was measured as ΔA_{295} divided by A_{450} and was found to be 53. Cu,Zn-SOD as generously supplied by Dr. L. Flohe and Dr. F. Otting of Grunenthal, Stolberg, West Germany. It was assayed as previously described (McCord & Fridovich, 1969) and was found to contain 3400 units of activity/mg of protein. 2-Nitropropane was purchased from Aldrich Chemical Co., and xanthine and bovine liver catalase were from Sigma Chemical Co. This catalase was assayed for SOD and was found to contain not more than 1.0 unit of SOD per 8.9×10^4 units of catalase, an amount too low to interfere with any of our measurements that were done with catalase present.

18-Crown-6 was purchased from Eastman Co. and was used without further purification. The NP^- was prepared by mixing 2-nitropropane compound with equimolar aqueous NaOH before use. The oxidation of NP^- was monitored in terms of O_2 consumption, measured with a Yellow Springs Instrument Co. no. 4004 Clark Oxygen

Fig. 1. Co-oxidation of NP^- during the xanthine oxidase reaction

Reaction mixtures contained 0.1 mM-xanthine, 0.1 mM-EDTA and 50 mM-potassium phosphate at pH 7.8 in a volume of 1.86 at 25 °C. At the times indicated by arrows 25 μl of 2.34 μM -xanthine oxidase and 100 μl of 180 mM- NP^- were added.

Fig. 2. Inhibition by SOD of the co-oxidation of NP^-

Reaction mixtures contained 0.1 mM-xanthine, 0.1 mM-EDTA, 50 mM-potassium phosphate, 18.7 mM- NP^- , 29 nM-xanthine oxidase and the indicated amounts of SOD at pH 7.8 at 25 °C. Rates of O_2 consumption are here presented as a function of the concentration of SOD.

Monitor. Standard reaction mixtures contained 29 nM-xanthine oxidase, 90 μM -xanthine and the indicated concentration of NP^- in 50 mM-potassium phosphate/0.1 mM-EDTA buffer, pH 7.8. The total reaction volume was 2.0 ml and the temperature was 25 °C. Xanthine oxidase activity was measured under these conditions, but in the absence of nitropropane, in terms of the rate of conversion of xanthine into urate, monitored at 295 nm. The rate of production of $\text{O}_2^{\cdot-}$ by the xanthine oxidase reaction was measured under these conditions in terms of the rate of reduction of ferricytochrome *c*, monitored at 550 nm. When this was done, the concentration of cytochrome *c* was 10 μM .

KO_2 from Alfa Products (Danvers, MA, U.S.A.) was dissolved to 50 mM in dimethyl sulphoxide that had been dried overnight over molecular sieve and that contained 0.1 M-18-crown-6. This solution was pumped into reaction mixtures, and the actual amount of $\text{O}_2^{\cdot-}$ being infused was measured in terms of the SOD-inhibitable reduction of cytochrome *c*. The pumping rate was

Fig. 3. Effect of varying the concentration of NP^- on the xanthine oxidase reaction

Reaction mixtures contained 0.1 mM-xanthine, 0.1 mM-EDTA, 50 mM-potassium phosphate, 29 nM-xanthine oxidase and the indicated concentrations of NP^- at pH 7.8 in a total volume of 2.0 ml at 25 °C.

adjusted so that the rate of infusion of $\text{O}_2^{\cdot-}$ was equal to that produced *in situ* by the xanthine oxidase reaction. Oxidation of NP^- was then measured in terms of NO_2^- production, by applying the colorimetric assay method of Schneider & Yearby (1973).

RESULTS

Co-oxidation of NP^- by xanthine oxidase

Addition of NP^- to the xanthine/xanthine oxidase reaction augmented the rate of O_2 consumption, as shown in Fig. 1. Addition of NP^- to xanthine oxidase in the absence of xanthine, or to xanthine in the absence of xanthine oxidase, caused no uptake of O_2 . SOD eliminated the extra O_2 consumption caused by NP^- , without affecting that O_2 uptake due only to the xanthine/xanthine oxidase reaction. This inhibitory effect of SOD is shown in Fig. 2. SOD was equally effective whether added before or after initiation of the reaction with xanthine oxidase.

Increasing the concentration of NP^- increased the extra O_2 consumption occasioned by its presence, as shown in Fig. 3. The rate of $\text{O}_2^{\cdot-}$ production during the xanthine oxidase reaction can be measured in terms of the SOD-inhibitable reduction of cytochrome *c* (McCord & Fridovich, 1968, 1969). This method is valid provided

Fig. 4. Chain length of the xanthine oxidase reaction as a function of the concentration of NP^-

Reaction mixtures were prepared as described in the legend to Fig. 3. The rate of O_2 consumption at each concentration of NP^- was corrected for the rate of O_2 consumption by the xanthine/xanthine oxidase reaction in the absence of NP^- , and NP^- oxidation was equated to this corrected O_2 consumption. The rate of $\text{O}_2^{\cdot-}$ production by the xanthine oxidase reaction was measured in terms of the rate of the SOD-inhibitable reduction of ferricytochrome *c*. Number of molecules of NP^- oxidized per molecule of $\text{O}_2^{\cdot-}$ produced is here presented as a function of the concentration of NP^- .

Fig. 5. Effect of catalase on the xanthine oxidase reaction

Reaction mixtures were prepared as described in the legend to Fig. 1. At the arrows $25\ \mu\text{l}$ of $2.3\ \mu\text{M}$ -xanthine oxidase and $50\ \mu\text{l}$ of catalase (1000 units/ml) were added. NP^- was absent from reaction *A* but was present at $9.0\ \text{mM}$ in reaction *B*.

that a saturating concentration of cytochrome *c* is used, which ensures that no significant amount of $\text{O}_2^{\cdot-}$ will be lost to the dismutation reaction. Under the conditions used here, $10\ \mu\text{M}$ -ferricytochrome *c* was saturating, in that higher concentrations did not result in greater rates of reduction, monitored at $550\ \text{nm}$. The number of molecules of O_2 consumed, due to the presence of NP^- , per molecule of $\text{O}_2^{\cdot-}$ generated, could then be calculated. As shown in Fig. 4, this number increased from 2.4 to 6.8 as the concentration of NP^- was raised from $9.4\ \text{mM}$ to $37.5\ \text{mM}$. It is clear that $\text{O}_2^{\cdot-}$ initiates a free-radical chain oxidation of NP^- .

Xanthine oxidase reduces O_2 univalently and divalently to yield both $\text{O}_2^{\cdot-}$ and H_2O_2 (Fridovich, 1970). Since the $\text{O}_2^{\cdot-}$ dismutates, the only stable product of O_2 reduction that accumulates is H_2O_2 . It follows that addition of catalase to a xanthine/xanthine oxidase reaction mixture should halve the rate of O_2 consumption and should cause a transient evolution of O_2 equal to half of the total O_2 consumed to that point. Trace *A* in Fig. 5 demonstrates that these expectations are met. Thus the rate of O_2 uptake was $5.6\ \mu\text{M}/\text{min}$ before the addition of catalase and was $2.6\ \mu\text{M}/\text{min}$ after catalase was added. Trace *B* in Fig. 5 shows that the same effects of catalase were evident, when the net O_2 consumption was increased by the co-oxidation of NP^- . Thus the rate of O_2 uptake during NP^- co-oxidation was $14.7\ \mu\text{M}/\text{min}$ without catalase and

Fig. 6. Inactivation of xanthine oxidase during co-oxidation of NP^-

Reaction mixtures contained $0.1\ \text{mM}$ -xanthine, $0.1\ \text{mM}$ -EDTA, $47\ \text{nM}$ -xanthine oxidase, $50\ \text{mM}$ -potassium phosphate and as indicated below NP^- , catalase and/or CN^- , all at $\text{pH}\ 7.8$ in a total volume of $2.5\ \text{ml}$ at $25\ ^\circ\text{C}$. Curve *a*, no additions; curve *g*, plus $9.5\ \text{mM}$ - NP^- ; curve *h*, plus $9.5\ \text{mM}$ - NP^- and $10.0\ \text{mM}$ - CN^- ; curves *b*-*f*, plus $9.5\ \text{mM}$ - NP^- with catalase at 3100, 1550, 155, 31 and 3.1 units/ml respectively. The catalase used was tested for SOD activity and contained only 1.1×10^{-5} units of SOD per unit of catalase.

Fig. 7. SOD protects xanthine oxidase against inactivation by NP^-

Reaction mixtures contained 0.1 mM-xanthine, 0.1 mM-EDTA, 28 nM-xanthine oxidase, 50 mM-potassium phosphate and as indicated below NP^- and SOD. Curve a, no additions; curve d, plus 9.5 mM- NP^- ; curve c, plus 9.5 mM- NP^- and 0.04 μg of SOD/ml; curve b, 9.5 mM- NP^- and 0.08 μg of SOD/ml; curve e, 9.5 mM- NP^- and 0.8 or 8.0 μg of SOD/ml.

Table 1. Effect of CN^- on the reversibility of the inhibition of xanthine oxidase by NP^-

Reaction mixtures contained 0.1 mM-xanthine, 0.1 mM-EDTA, 70 nM-xanthine oxidase, 50 mM-potassium phosphate and the indicated additions at pH 7.8 in a volume of 2.5 ml at 25 °C. After 25 min of reaction, residual activity was measured. Duplicate samples were dialysed for 6 h at 4 °C against 4 litres of 0.4 mM-xanthine in the phosphate/EDTA buffer and then for 10 h against 4 litres of the cold phosphate/EDTA buffer without xanthine. The dialysed samples were then assayed for residual activity.

Additions	Residual activity (normalized)	
	Before dialysis	After dialysis
None	100	100
10 mM- CN^-	102 ± 2	68 ± 18
9.5 mM- NP^-	3 ± 2.5	87 ± 1
10 mM- CN^- + 9.5 mM- NP^-	3 ± 2.5	34 ± 9

was 6.8 $\mu\text{M}/\text{min}$ in the presence of catalase. It follows that H_2O_2 was a product of NP^- oxidation.

Inhibition of xanthine oxidase by NP^-

Were NP^- to inhibit xanthine oxidase, chain lengths, calculated as molecules of NP^- oxidized per molecule of $\text{O}_2^{\cdot-}$, such as those shown in Fig. 4 would be underestimated. Since co-oxidation of NP^- during the xanthine oxidase reaction contributes to O_2 consumption, such inhibition could be masked in assays performed on

Table 2. Oxidation of NP^- by an infusion of KO_2

Reaction mixtures contained 9.5 mM- NP^- , 0.1 mM-EDTA, the indicated amounts of SOD or catalase and 50 mM-potassium phosphate at pH 7.8 at 25 °C. KO_2 dissolved in dry dimethyl sulphoxide containing 0.1 M-18-crown-6 was infused as described in the Materials and methods section, and NO_2^- accumulation was assayed after 0, 10 and 20 min of infusion. The precision in the determination of NO_2^- was estimated to be ± 5% on the basis of pilot assays with standards.

Time (min)	SOD ($\mu\text{g}/\text{ml}$)	Catalase (units/ml)	NO_2^- (μM)
0	0	0	0
0	200	0	0
0	0	3000	0
10	0	0	210
10	200	0	0
10	0	3000	220
20	0	0	580
20	200	0	0
20	0	3000	550

the basis of O_2 consumption. We therefore turned to spectrophotometric monitoring of the xanthine oxidase reaction at 295 nm. Fortunately, neither NP^- nor acetone absorbs significantly at this wavelength. As shown in Fig. 6 (curves a and g), NP^- caused a progressive inhibition of xanthine oxidase, which was exacerbated by 10 mM- CN^- (curve h). It should be noted that 10 mM- CN^- did not inhibit xanthine oxidase in the absence of NP^- (curve a). Since the co-oxidation of NP^- during the xanthine oxidase reaction leads to increased H_2O_2 production and since H_2O_2 is known to inactivate xanthine oxidase (Kellogg & Fridovich, 1977), it appeared possible that some of the inactivation seen in the presence of NP^- was actually due to H_2O_2 . That this was the case is shown by the partial protection against the NP^- inactivation by catalase (Fig. 6, curves b–g).

Acetone is also a product of the oxidation of NP^- , and acetone can form an adduct with H_2O_2 (Sauer & Edwards, 1972). This raised the possibility that the formation of an acetone- H_2O_2 adduct might fully account for the effect of NP^- on xanthine oxidase. Our results (not shown) demonstrate that 5.0 mM-acetone had no effect on the activity of xanthine oxidase and did not exacerbate the progressive inactivation caused by 5.0 mM- H_2O_2 . NO_2^- is also a product of the oxidation of NP^- , but 10.0 mM- NO_2^- had no effect on the xanthine oxidase reaction, in the absence or in the presence of 10 mM-acetone (results not shown).

SOD, which prevented the co-oxidation of NP^- during the xanthine oxidase reaction, fully prevented the progressive inactivation of xanthine oxidase by NP^- . This is shown in Fig. 7. It follows that some intermediate of the free-radical chain oxidation of NP^- initiated by $\text{O}_2^{\cdot-}$ was responsible for that part of inactivation of xanthine oxidase which was not preventable by catalase.

The results in Table 1 demonstrate that most of the activity of xanthine oxidase, which was lost during the co-oxidation of NP^- , was restored by dialysis. CN^- , which did not inhibit in the absence of NP^- , caused half of the inhibition seen in the presence of NP^- to become irreversible.

Initiation of NP^- oxidation by KO_2

Xanthine oxidase did not cause detectable oxidation of NP^- in the absence of xanthine. This, and the inhibition by SOD, suggested that $O_2^{\cdot-}$, rather than some interaction with the flavin, was the cause of NP^- oxidation. It nevertheless seemed important to demonstrate the oxidation of NP^- in the absence of any flavoenzyme. This was achieved by a mechanical infusion of KO_2 , dissolved with the aid of a crown ether in dry dimethyl sulphoxide. The pumping rate was adjusted such that the rate of $O_2^{\cdot-}$ infusion equalled that of $O_2^{\cdot-}$ generation *in situ* during the xanthine/xanthine oxidase co-oxidation of NP^- , and NP^- oxidation was monitored in terms of the rate of accumulation of NO_2^- .

As shown in Table 2, infusion of KO_2 caused the oxidation of NP^- to NO_2^- over a period of 20 min. This oxidation was completely suppressed by SOD, but was unaffected by catalase. The rate of accumulation of NO_2^- , i.e. $21 \mu M/min$, approximated the increase in the rate of O_2 uptake by the xanthine/xanthine oxidase reaction caused by the presence of NP^- , i.e. $14.5 \mu M/min$. Since both the rates of introduction of $O_2^{\cdot-}$ and the concentration of NP^- were the same in both reaction systems, this result indicates that $O_2^{\cdot-}$ was the agent responsible for the oxidation of NP^- . This conclusion is buttressed by the inhibitory effect of SOD and the lack of effect by catalase.

DISCUSSION

The oxidation of 2-nitropropane by enzymic or chemical sources of $O_2^{\cdot-}$ can be explained by the following sequence of reactions:

This scheme accounts for initiation by univalent oxidation of NP^- by $O_2^{\cdot-}$, for propagation by reaction of the hydroxyisopropyl radical with O_2 and for termination by the dismutation of $O_2^{\cdot-}$ or by any other radical/radical reaction. These reactions are in accord with the formation of H_2O_2 , which was shown by the effect of catalase (Fig. 3). They also explain why more

than 1 molecule of NP^- was oxidized per molecule of $O_2^{\cdot-}$ introduced into the reaction mixture, and why the chain length increased with increasing concentration of NP^- . The latter effect depends upon a competition between reactions (b) and (e) for $O_2^{\cdot-}$. Thus increasing the concentration of NP^- favours initiation (reaction b) over termination (reaction e) and so increases chain length.

Since a number of flavoprotein dehydrogenases have been shown capable of producing $O_2^{\cdot-}$ (Massey *et al.*, 1969), the $O_2^{\cdot-}$ -initiated mode of NP^- oxidation could contribute to the oxidation of NP^- by virtually any flavoenzyme. The 2-nitropropane dioxygenase of *H. mrakii* is inhibited by scavengers of $O_2^{\cdot-}$, such as tiron (Kido *et al.*, 1976a), cytochrome *c* or SOD (Kido *et al.*, 1978a,b). The oxidations of NP^- by glucose oxidase and by D-amino acid oxidase were similarly inhibited by SOD (Kido & Soda, 1984). These results indicate that $O_2^{\cdot-}$ is an essential intermediate in the oxidation of NP^- by these flavoenzymes and suggest that the free-radical chain reaction pathway (reactions b, c and d) was probably involved. This pathway requires that H_2O_2 be among the products; yet Kido *et al.* (1976b) observed that catalase had no effect on the rate of the reaction catalysed by the *H. mrakii* enzyme. One possible explanation for this seeming contradiction would be the presence of a 2-nitropropane peroxidase activity in the *H. mrakii* enzyme.

The syncatalytic inactivation of xanthine oxidase in the presence of NP^- suggests that some intermediate of the free-radical pathway of NP^- oxidation was the responsible agent. This view is reinforced by the observation that SOD, which blocks the co-oxidation of NP^- , prevents this

syncatalytic inactivation. The gradual syncatalytic inactivation of D-amino acid oxidase during catalysis of the oxidation of nitromethane has been noted (Porter *et al.*, 1972, 1973). These workers reported that CN^- irreversibly inhibited this enzyme only during its action on nitroalkanes. They proposed an adduct between the

nitroalkane, or a derivative thereof, and the enzyme-bound FAD to which CN^- could add. This was demonstrated by the finding that the inactive FAD adduct could be decomposed to FAD, acetaldehyde and CN^- by heating to 70 °C, at pH above 8.0. The inactivation of xanthine oxidase during the co-oxidation of NP^- , described above, was similarly exacerbated and rendered irreversible by CN^- . We conclude that an intermediate of the free-radical chain oxidation of NP^- , probably the hydroxyisopropyl radical, can add on to the flavin and that CN^- can in turn add on to and stabilize the flavin-isopropane adduct.

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