Superoxide dismutase-inhibitable reduction of cytochrome *c* by the alloxan radical

Implications for alloxan cytotoxicity

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Cytochrome *c* was reduced when superoxide was generated from xanthine oxidase in the presence of alloxan, and by the reaction of alloxan and with reduced glutathione. In each case, most of the reduction was inhibited by superoxide dismutase, but considerably more enzyme was required than with superoxide alone. This indicates that the superoxide dismutase-inhibitable cytochrome *c* reduction was mainly due to a direct reaction with the alloxan radical, and implies that other reactions that are inhibited by superoxide dismutase could be due to either alloxan radicals or superoxide.

Superoxide dismutase has been shown to protect against the destruction of pancreatic islet cells by the diabetogenic drug alloxan (Grankvist *et al.*, 1979a), and against the manifestation of diabetes in alloxan-fed rats (Grankvist *et al.*, 1981). Reduction of alloxan yields dialuric acid, which rapidly autooxidizes in air to give superoxide ($O_2^{\bullet-}$) and H_2O_2 (Cohen & Heikkila, 1974). It is thought that these activated oxygen species are involved in its cytotoxicity, possibly by reacting to produce hydroxyl radicals via the iron-catalysed Haber–Weiss reaction, and that superoxide dismutase prevents this reaction (Cohen & Heikkila, 1974; Grankvist *et al.*, 1979a,b; Fischer & Hamburger, 1980a,b; Grankvist, 1981).

Houée and co-workers (Houée-Levin *et al.*, 1979; Houée *et al.*, 1981) have shown that the alloxan radical is an intermediate in the reduction of alloxan and oxidation of dialuric acid, and suggest that it can exist in equilibrium with $O_2^{\bullet-}$. In other situations where there is equilibrium between semiquinone radicals and $O_2^{\bullet-}$, superoxide dismutase indirectly inhibits reactions of the organic radicals (Winterbourn *et al.*, 1978; Winterbourn, 1980, 1981; Sutton & Sangster, 1982). In particular, the superoxide dismutase-inhibitable cytochrome *c* reduction that occurs when semiquinone radicals are produced in the presence of O_2 is in many cases primarily due to the semiquinone rather than to $O_2^{\bullet-}$. I have therefore investigated whether superoxide dismutase can also inhibit reactions of the alloxan radical.

Abbreviation used: GSH, reduced glutathione.

Methods

Cytochrome *c* (30 μ M unless otherwise stated) in 50 mM-sodium phosphate buffer, pH 7.4, was made to react either with alloxan (0.1 or 0.2 mM) and GSH (0.1 or 0.075 mM) or with xanthine (80 μ M), xanthine oxidase [3.6×10^{-3} units (μ mol/min)/ml] and alloxan (0.2 or 1 mM). The alloxan was dissolved in water and added to the buffer immediately before the start of the reaction. Rates of cytochrome *c* reduction were determined, in the presence of catalase to prevent re-oxidation by H_2O_2 , by continuously monitoring A_{550} ($\epsilon = 21.1 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ for ΔA_{550}). The kinetics of inhibition were determined by conducting the reaction in the presence of 0–16.6 μ g of superoxide dismutase/ml. Values of I_{max} and K_i were compared with those for reduction of cytochrome *c* (30 μ M) by $O_2^{\bullet-}$ produced from xanthine and xanthine oxidase alone. All biochemicals were obtained from the Sigma Chemical Co., St. Louis, MO, U.S.A., except superoxide dismutase, which was from Diagnostic Reagents, Thame, Oxon, U.K.

Results

As shown in Table 1, reduction of cytochrome *c* by xanthine plus xanthine oxidase in air occurred at the same rate in the presence or in the absence of alloxan. There was very little cytochrome *c* reduction or xanthine oxidation (increase in A_{295} in the absence of cytochrome *c*) in N_2 in either case, and therefore no direct electron transfer from the enzyme to alloxan. Hence if the alloxan radical ($HA^{\bullet}$) is

formed from xanthine oxidase in air, it should be via the forward reaction of equilibrium (1):

There was no increase in reaction rate on doubling the concentration of cytochrome *c* (Table 1), indicating efficient trapping of all the radicals produced. In each case most of the cytochrome *c* reduction was inhibited by superoxide dismutase. However, a much higher concentration of enzyme was required to inhibit reduction in the presence than in the absence of alloxan (Figs. 1*a* and 1*b*). K_i

increased with increasing alloxan concentration (Table 1). The effect of alloxan depended on the O_2 concentration. When this was decreased 4-fold, the percentage inhibition by superoxide dismutase in the presence of a given alloxan concentration decreased (Table 2). Decreasing the O_2 concentration made little difference in the absence of alloxan. In air 1 mM-alloxan had approximately the same effect as 0.25 mM-alloxan in air/ N_2 (1:3).

The possibility that the superoxide dismutase was inactivated or irreversibly inhibited was excluded by pretreating the enzyme with alloxan. With no

Fig. 1. Reciprocal plot of percentage inhibition of cytochrome *c* reduction versus superoxide dismutase concentration (a) Xanthine + xanthine oxidase (●); (b) xanthine + xanthine oxidase plus 0.2 mM-alloxan (○) or 1 mM-alloxan (■); (c) 0.1 mM-GSH plus 0.1 mM-alloxan (□) and 0.075 mM-GSH plus 0.2 mM-alloxan (▲). Reaction conditions were the same as for Table 1. Lines were drawn by the least-squares method.

Table 1. Rates of cytochrome *c* reduction and kinetic constants for inhibition by superoxide dismutase

Assays were performed as described in the Methods section. Unless otherwise stated, reactions were conducted in air with 30 μM -cytochrome *c*. Each rate was determined from the linear increase in A_{550} over the first few minutes of the reaction, and is expressed as the mean ($\pm$ S.D.) for three assays. Kinetic constants were calculated from the slopes and intercepts of the lines shown in Fig. 1.

Reaction conditions	Concn. of alloxan (mM)	Rate of cytochrome <i>c</i> reduction (nmol/min per ml)	I_{max} (%)	K_i ($\mu\text{g/ml}$)
Xanthine + xanthine oxidase	0	2.24 ± 0.10	93	0.18
With 60 μM -cytochrome <i>c</i>	0	2.34	—	—
In N_2	0	0.10	—	—
Xanthine + xanthine oxidase	1	2.34 ± 0.24	84	1.50
With 60 μM -cytochrome <i>c</i>	1	2.24	—	—
In N_2	1	0.14	—	—
Xanthine + xanthine oxidase	0.2	2.30	96	0.84
0.1 mM-GSH	0	0.16	—	—
0.075 mM-GSH	0.2	3.57 ± 0.36	76	1.18
0.1 mM-GSH	0.1	3.68 ± 0.39	62	0.87

Table 2. Effect of varying the O_2 concentration on inhibition of cytochrome *c* reduction by superoxide dismutase
For reaction conditions see the legend to Table 1.

Concn. of alloxan (mM)	Percentage inhibition by superoxide dismutase at 0.6 μ g/ml	
	Air	Air/ N_2 (1:3)
0	74.0 $\pm$ 1.8	68.6 $\pm$ 3.6
0.25	44.4 $\pm$ 3.1	11.5 $\pm$ 3.2
1	12.5 $\pm$ 2.8	0 $\pm$ 4

alloxan, or a concentration of 0.02 mM, cytochrome *c* reduction was inhibited by 70.9 $\pm$ 2.5% (mean $\pm$ S.D. for three assays) by superoxide dismutase at 0.6 μ g/ml. Pretreatment of the enzyme with 1 mM-alloxan for 30 min, then dilution to give a final alloxan concentration of 0.02 mM, did not alter this inhibition (72.9 $\pm$ 1.7%), whereas in the presence of 1 mM-alloxan the inhibition was decreased to 12.5 $\pm$ 2.8%.

Similar results were obtained for cytochrome *c* reduction by alloxan and GSH. In this case, only about 70% of the reaction was inhibitable by superoxide dismutase. Approximately 5% of the remainder was due to direct reduction by GSH (Table 1), and the rest was probably due to the reaction of dialuric acid with cytochrome *c* (Fee *et al.*, 1975). For the superoxide dismutase-inhibitable reaction, the K_i values were higher than those measured with $O_2^{\cdot-}$ alone, and were comparable with those measured with similar alloxan concentrations in the presence of xanthine and xanthine oxidase (Table 1 and Fig. 1c). Under N_2 cytochrome *c* was also reduced by alloxan and GSH, but there was no inhibition by superoxide dismutase at 8 μ g/ml.

Discussion

The present study has shown that inhibition of cytochrome *c* reduction by an $O_2^{\cdot-}$ -generating system required less superoxide dismutase than did a similar system in the presence of alloxan, or when alloxan radicals were generated by reducing alloxan with GSH. If cytochrome *c* reduction were due solely to $O_2^{\cdot-}$, under the conditions employed where all the radicals were trapped, the concentration of superoxide dismutase required to inhibit the reaction should depend only on the cytochrome *c* concentration and be independent of the source of $O_2^{\cdot-}$. The finding that much higher superoxide dismutase concentrations were required in the presence of alloxan therefore indicates either that the superoxide dismutase was not fully active or that cytochrome *c* reduction was not primarily due to $O_2^{\cdot-}$.

Crouch *et al.* (1981) have proposed that alloxan inhibits superoxide dismutase, but this is an unlikely explanation for my results. Firstly, there was no irreversible inhibition or inactivation of the enzyme, since prior treatment with alloxan did not alter its inhibitory capacity. Reversible inhibition is also unlikely. This type of inhibition would not be expected to vary with O_2 concentration, but the effect of alloxan was greater at lower O_2 concentrations.

The results are explicable, however, if superoxide dismutase was preventing cytochrome *c* reduction by the alloxan radical ($HA^{\cdot}$). This would be formed either directly by the reaction of alloxan with GSH, or from $O_2^{\cdot-}$ via reaction (1). As shown by the reaction with alloxan and GSH in N_2 , it is able to reduce cytochrome *c*. Two routes for cytochrome *c* reduction are therefore possible:

Reaction (3) was not inhibited by superoxide dismutase in N_2 , discounting its reaction directly with the alloxan radical. However, superoxide dismutase can inhibit reaction (3) in the presence of O_2 by removing $O_2^{\cdot-}$ and displacing equilibrium (1) to the left. This would either prevent the formation of the alloxan radical or accelerate its removal, thus inhibiting its reaction with cytochrome *c*. This mechanism is similar to that proposed to account for the apparent inhibition of superoxide dismutase by pamoic acid (Hassan *et al.*, 1980), and could also explain some of the findings obtained by Crouch *et al.* (1981) with alloxan and other diabetogenic drugs. It requires that reaction (1) is reversible, which is in accordance with the proposal of Houée *et al.* (1981), and with the behaviour of other quinones (Winterbourn, 1981; Sutton & Sangster, 1982). The requirement for more enzyme to inhibit the reaction with alloxan than with $O_2^{\cdot-}$ alone implies that reaction (3) is faster than reaction (2) and predominates. This is also the case with other semiquinone radicals whose reactions are inhibited by superoxide dismutase (Winterbourn, 1981), and for which Sutton & Sangster (1982) have measured k_{+3} values 10–1000 times greater than k_{+2} .

It is therefore concluded that superoxide dismutase indirectly inhibits cytochrome *c* reduction by the alloxan radical. An implication from these findings is that where superoxide dismutase has been observed to protect biological systems against alloxan (Grankvist *et al.*, 1979a, 1981; Fischer & Hamburger, 1980a; Yamamoto *et al.*, 1981) it could be a result of its removing either $O_2^{\cdot-}$ or the alloxan radical. Fee *et al.* (1975) have suggested that the alloxan radical may be involved in erythrocyte haemolysis, but otherwise no distinction has been

made between the two. Heikkilä *et al.* (1976), Grankvist *et al.* (1979a,b) and Fischer & Hamburger (1980b) have shown that hydroxyl-radical scavengers protect against the diabetogenic effects of alloxan, suggesting involvement of the hydroxyl radical, but it does not appear to be involved in alloxan-induced erythrocyte lysis (Fee *et al.*, 1975). I have found no evidence for a direct reaction between the alloxan radical and H_2O_2 (C. C. Winterbourn, unpublished work), and most other studies have implicated the iron-catalysed Haber-Weiss reaction as the source of hydroxyl radicals (Grankvist *et al.*, 1979a,b; Fischer & Hamburger, 1980b). Whether the alloxan radical can form hydroxyl radicals by another mechanism, or participate in other biologically undesirable reactions, is not known. But, if this is the case, such reactions are likely to be indirectly inhibited by superoxide dismutase, and should not be overlooked as a possible explanation for superoxide dismutase-inhibitable effects of alloxan.

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