SUPEROXIDE DISMUTASE AS AN INHIBITOR OF REACTIONS OF SEMIQUINONE RADICALS

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Received 26 June 1978

1. Introduction

The only known reaction of superoxide dismutase is the catalysis of the reaction:

$$2\,O_2^- + 2\,H^+ \longrightarrow O_2 + H_2O_2 \qquad (1)$$

and the enzyme has been used extensively as an indicator of the involvement of superoxide in a variety of reactions [1,2]. It is generally assumed that superoxide dismutase prevents reactions either of superoxide directly, or of a product of a subsequent reaction of superoxide with another component of the reaction mixture [2–5]. This assumption is based on the premise that superoxide is produced irreversibly. However, evidence is presented here that the reaction of O_2 with the semiquinone of menadione produces superoxide reversibly, and that the effect of the dismutase in this instance is primarily to lower the concentration and thereby inhibit other reactions of the semiquinone. Many reactions that produce superoxide proceed via free radical intermediates similar to the menadione semiquinone. This may therefore be an example of a more general protective function of superoxide dismutase, that is, to lower the levels of such free radical intermediates, which may in fact be a greater threat to the cell than superoxide itself.

This study arose out of an investigation of the reaction of oxyhaemoglobin with menadione and a need to explain the marked acceleration of haemoglobin oxidation on adding superoxide dismutase.

2. Experimental

Semiquinone radicals were produced by irradiating N_2-bubbled solutions of menadione and benzoquinone at 365 nm, and by heating 1,4-naphthoquinone-2-sulphonate (NQS) solutions at 40°C under N_2. Reactions were carried out in 0.05 M sodium phosphate buffer (pH 7.4) with menadione solutions containing 5–10% isopropanol to increase its solubility. The radicals were detected by electron spin resonance (ESR) spectroscopy using a Varian E12 spectrometer with 10 kHz modulation. Methaemoglobin was prepared as in [6]. Menadione was obtained from Calbiochem, San Diego, CA, NQS from Eastman Kodak Co., Rochester, NY, benzoquinone from BDH Ltd., Poole and xanthine oxidase, catalase and superoxidase dismutase from the Sigma Chemical Co., St Louis, MO.

3. Results and discussion

Irradiation of menadione produced a radical with an ESR spectrum identified as that of the menadione semiquinone [7]. There was no obvious decrease in concentration over about 15 min under N_2, but the radical disappeared on bubbling O_2 through the solution. Addition of methaemoglobin (under N_2) destroyed the radical, and the absorption spectrum of the solution showed that the methaemoglobin had been reduced to deoxyhaemoglobin. The radical also reduced

methaemoglobin to oxyhaemoglobin in air, indicating that it reacts more rapidly with methaemoglobin than O_2. The fully reduced hydroquinone, which gradually accumulates in menadione solutions exposed to light, did not contribute significantly to the methaemoglobin reduction.

Superoxide produced by the xanthine oxidase-catalysed oxidation of xanthine, (with catalase present to remove H_2O_2), reacted with menadione to produce a species with the properties of the semiquinone radical. It could not be detected directly because by necessity O_2 was present in the solution, but it did reduce methaemoglobin to oxyhaemoglobin (fig.1). Although superoxide itself can reduce methaemoglobin [6,8], as can be seen in fig.1, this reaction is very much slower than that in the presence of menadione, and cannot bring about such complete reduction. The reaction was dependent on superoxide, since it was almost completely inhibited by superoxide dismutase. It occurred at the same rate in air or O_2. Much less oxyhaemoglobin was produced in the absence of xanthine oxidase. This reaction was also inhibited by superoxide dismutase.

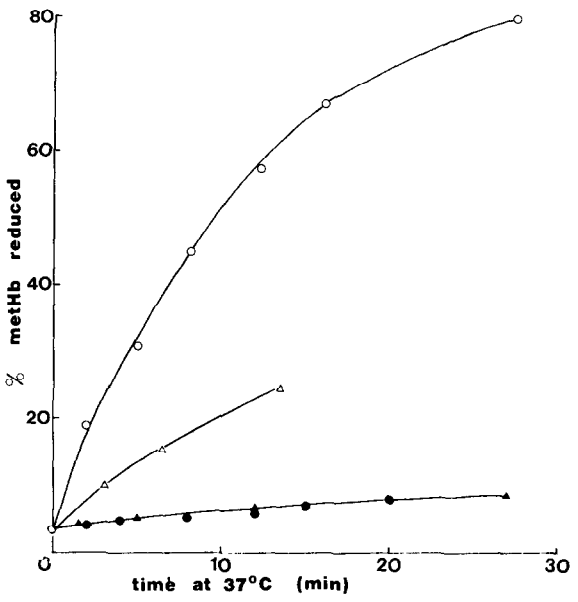

Fig.1. Reduction of methaemoglobin by superoxide and menadione. Solutions contained 25 μM methaemoglobin, 200 μM menadione, 1 mM xanthine, 20 mU/ml xanthine oxidase and 30 μg/ml catalase. (○) All components present; (●) plus 14 μg/ml superoxide dismutase; (▲) no menadione; (△) no xanthine oxidase. The % conversion to oxyhaemoglobin was calculated from absorption changes as in [6].

The semiquinone of benzoquinone contrasted with that of menadione by reacting only very slowly with O_2, and not at all with methaemoglobin. The reaction of benzoquinone with superoxide did not therefore cause any methaemoglobin reduction; but it was possible to detect the ESR signal of the semiquinone.

Hence, with menadione, we have observed both the forward and backward reaction of eq. (2):

$$O_2 + Q^{\cdot-} \rightleftharpoons O_2^{\cdot-} + Q \qquad (2)$$

($Q^{\cdot-}$ = semiquinone, Q = quinone)

We have also found that the semiquinone reacts with methaemoglobin (reaction 3) in preference to O_2 (reaction 2) in air-saturated solution:

$$Hb^{3+} + Q^{\cdot-} \longrightarrow Hb^{2+} + Q \qquad (3)$$

However, when superoxide dismutase was present, the reduction of methaemoglobin by the menadione radical was drastically inhibited (fig.2). This was not due to its preventing the production of the semiquinone, since methaemoglobin was reduced under N_2, when no superoxide would be produced (fig.2). Under these conditions superoxide dismutase had little effect. Also, since the reduction of methaemoglobin by superoxide is much too slow [8] to account for the observed rate, superoxide dismutase was not preventing a direct reaction of superoxide. The result can be explained by the removal of superoxide by superoxide dismutase inhibiting the reverse of reaction (2) and displacing the equilibrium to the right. The concentration of the semiquinone would be decreased, and reduction of methaemoglobin thereby inhibited. In effect superoxide dismutase allowed O_2 to compete much more efficiently for the semiquinone. This is an example of superoxide dismutase inhibiting a reaction of a precursor of superoxide, in this case the menadione radical.

A similar result was obtained with NQS. Mild heating produced semiquinone radicals which reduced methaemoglobin. In the presence of O_2, superoxide dismutase completely inhibited this reduction. Methaemoglobin reduction still took place under N_2,

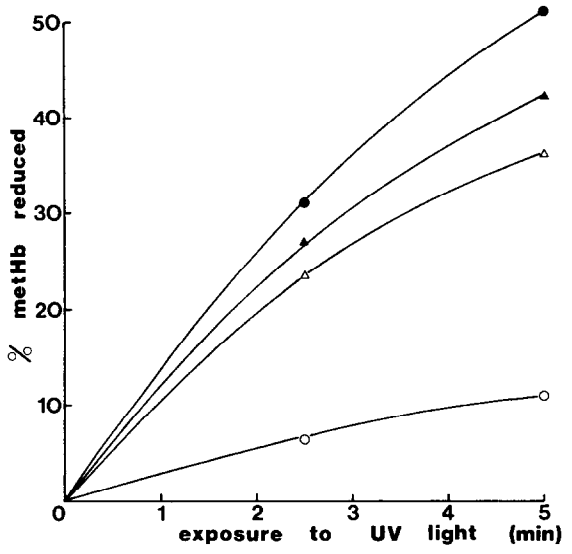

Fig.2. Effect of superoxide dismutase on the reduction of methaemoglobin by menadione radicals. Solutions contained 12.5 μM methaemoglobin and 100 μM menadione, and were irradiated at 365 nm, at 20°C. The % reduction of methaemoglobin was calculated from $\Delta A_{577} = 0.0475$. Air was introduced into the N_2-bubbled solutions before measuring the absorbance. (●) In air; (○) in air + 7 μg/ml superoxide dismutase; (▲) in N_2; (△) in N_2 + 7 μg/ml superoxide dismutase.

so superoxide was not required for production of the radical.

This mechanism, whereby superoxide dismutase inhibits the reactions of a free radical, is likely to operate where the radical can react reversibly with oxygen, i.e., the equilibrium constant for reaction (2) is not far from 1, and both forward and backward reactions are fast. It can be calculated from the redox potentials in [9] that under the conditions of our studies k_2 for menadione should be ~1, and the rate constants for both forward and backward reactions are $\sim 10^8$ [10]*. These conditions should therefore be met with menadione, and also with NQS, with k_2 calculated to be ~60. However, with benzoquinone, the radical does not react readily with O_2 and k_2 calculated from the data in [9] is about 3×10^4. Superoxide dismutase would therefore be expected to have less effect on its reactions. The mechanism need not necessarily be restricted to semiquinones. Reactions of other radicals with redox potentials similar to the O_2/O_2^- couple should also be inhibited by superoxide dismutase.

This mechanism could have broader implications with respect to both the interpretation of experiments examining effects of superoxide dismutase, and the biological role of the enzyme. There have been a variety of reactions described that can be inhibited by superoxide dismutase. However, in only a few has it been possible to demonstrate a direct reaction of superoxide [2]. Many are also inhibited by catalase, and the superoxide dismutase effect has been explained in terms of its preventing the production of hydroxyl radicals from H_2O_2 and superoxide (the Haber-Weiss reaction), and subsequent reactions of these radicals [3–5]. However, the Haber-Weiss reaction has been shown to occur at a reasonable rate only in the presence of chelated iron [11,12] and whether enough of this catalyst would be present in the above examples is yet to be shown. In many of the systems in which superoxide dismutase exhibits an inhibitory effect, the source of superoxide is the reaction of a free radical, frequently a semiquinone, with O_2 (e.g., reactions involving dihydroxyfumarate [3,13], paraquat [14], alloxan and 6-hydroxydopamine [5]). This is a similar situation to the one we have been studying with menadione; therefore consideration should be given to the possibility that these reactions are also reversible, and that superoxide dismutase may be lowering the concentrations and preventing competing reactions of the intermediate radicals. In particular, reactions that are inhibited by superoxide dismutase or catalase may require a product of the reaction of H_2O_2 with one of these free radicals.

Concerning the biological role of superoxide dismutase, it is interesting to speculate that it could be involved in controlling levels of other free radicals as well as superoxide. It may be significant that two commonly occurring quinones in living systems are ubiquinone (co-enzyme Q) and menadione (vitamin K). Roles for ubiquinone as a link in the electron transport chain and menadione in the synthesis of prothrombin are well established, but it is generally regarded that these are unlikely to be their only functions. Reactions of menadione ($E_o = -0.20$) we

* A true equilibrium would not be established because of the dismutation of O_2^-, but these figures indicate that the reaction should be readily reversible

have shown to be influenced by superoxide dismutase, and ubiquinone, with $E_0 = -0.08$ (calculated from [9,15]) should also fall into this category. An interesting possibility is that superoxide dismutase, superoxide and these semiquinones are all involved in some regulatory role.

Acknowledgements

This work was supported by the Medical Research Council of New Zealand and the Canterbury Medical Research Foundation. We are grateful to Dr H. C. Sutton and Dr R. W. Carrell for helpful discussions.

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