A RELATIONSHIP BETWEEN THIOLS AND THE SUPEROXIDE ION

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1. Introduction

Many in vivo processes such as phagocytosis [1] or the oxidation of haemoglobin [2] produce superoxide ions. Further reduction of these ions can produce the very reactive hydroxyl radical and this process is believed to be one cause of the tissue destruction in chronic inflammatory disease [3]. The control of superoxide-ion concentration is believed to be by a range of superoxide dismutase proteins (SODs) present in many tissues and intracellular fluids [1]. However, the production of superoxide ions often occurs in bursts and the lifetime of the ion is $\sim 10^{-5}$ s [1]. Under these conditions, it is questionable whether the superoxide dismutase proteins are present in sufficient quantity at the correct part of the cell to completely control the metabolism of superoxide ions and, consequently, it is possible that the biological matrix of the cell may play a key role in the control process.

Since the production of superoxide ion in vivo is believed to be by a non-specific free-radical chain reaction, the most obvious component of the matrix to be implicated would be free-radical trapping systems such as glutathione or other thiols. We report the effect of glutathione and other related thiols in two dismutase assays, in one of which dismutase activity is measured by an increase in reaction rate and in the second of which it is measured by an inhibition of reaction rate. Since haemolysate is available in this laboratory as a consequence of clinical studies, we have related our experiments particularly to the copper/zinc superoxide dismutase found in the cytosol. In the cytosol from an erythrocyte, ~3 μmol/l superoxide dismutase and 3000 μmol/l free, non-protein-bound thiol would be expected [1,4]. The thiol is believed to be mainly glutathione.

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2. Materials and methods

Gluthathione, cysteine and bovine superoxide dismutase were obtained from Sigma Chemicals and d-penicillamine from Dista Ltd.

Superoxide dismutase activity was assayed by observing changes in superoxide-dependent reactions In an assay according to [5], the superoxide ion is generated by photoreduction of oxygen sensitised by riboflavin. The augmentation of the oxidation of dianisidine on removal of O_2^- was detected. In an assay according to [6], superoxide is involved in the radical chain process, resulting in the oxidation of epinephrine to adrenochrome. SOD inhibits this process.

Haemolysate was obtained from heparinised human blood by centrifugation and lysis of the cells in distilled water, as detailed in [7]. Haemoglobin was precipitated with chloroform/ethanol mixture.

3. Results

Both assays were calibrated using standard solutions of bovine superoxide dismutase. The augmentation assay was linear up to at least 1.2 μg SOD/ml ($r = 0.99$ $p < 0.001$) and the inhibition assay up to 2 μg/ml ($r = 0.997$ $p < 0.001$) (fig.1).

Glutathione produced an inhibition in the inhibition assay in the absence of any SOD and at concentrations equivalent to those expected in the cytosol. Perhaps more surprisingly, it also produced an inhibition in the augmentation assay (table 1). This latter result was investigated further using other thiols and a smaller effect, with a rather different concentration dependence, was found for both cysteine and penicillamine (table 2).

Glutathione was added to solutions containing

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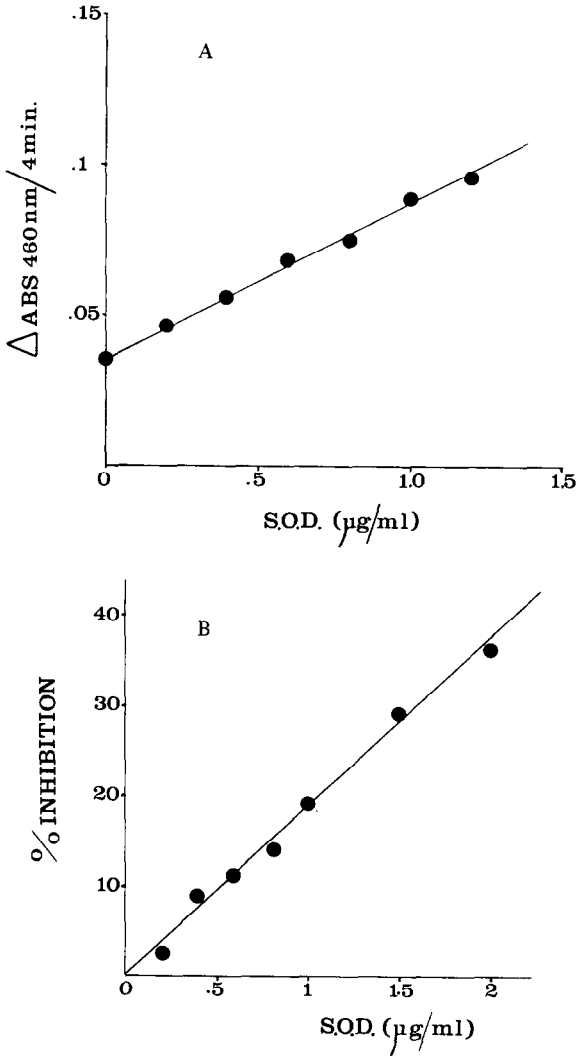

Fig.1. A comparison of calibration graphs for two assays for superoxide dismutase activity: (A) augmentation assay from [5]; (B) inhibition assay from [6].

100 μg bovine SOD/ml and the net dismutase activity was measured in the augmentation assay. The result was not affected by the added thiol up to a molar ratio of ~50:1 but, at the ratio expected in the cytosol (1000:1), the augmentation effect of the SOD was completely removed (fig.2).

Table 1
The effect of glutathione on the augmentation and inhibition assays

Inhibition (%)	200 μmol/l	500 μmol/l	800 μmol/l	1000 μmol/l
Augmentation assay	54	74	87	87
Inhibition assay	9	12	14	23

The SOD activity in a human haemolysate was 110 μg/ml (3.4 μmol/l). The effect of lysis and of haemoglobin precipitation produces a considerable reduction in thiol concentration but residual thiol at 111 μmol/l was recorded in this case. Successive additions of 111 μmol/l of glutathione reduces the SOD activity until, at a level of 500–1000 μmol/l, the effect of the photo-oxidation is completely suppressed. Above this concentration, the thiol inhibits the naturally occurring oxidation of dianisidine, i.e., a level below the blank of fig.1 (Δ = 0.035 absorbance units) is achieved.

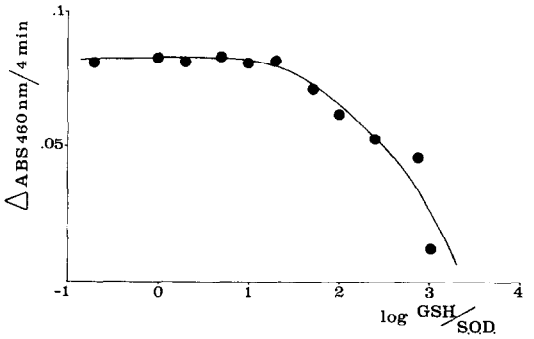

Fig.2. The effect of increasing thiol on the activity of bovine superoxide dismutase in the inhibition assay. Thiol levels are expressed as a ratio per mol superoxide dismutase.

Table 2
The effect of penicillamine and cysteine in the augmentation assay

Inhibition (%) with	100 μmol/l	200 μmol/l	400 μmol/l	600 μmol/l	800 μmol/l	1000 μmol/l
Penicillamine	0	0	14	20	27	38
Cysteine	5	8	13	18	21	26

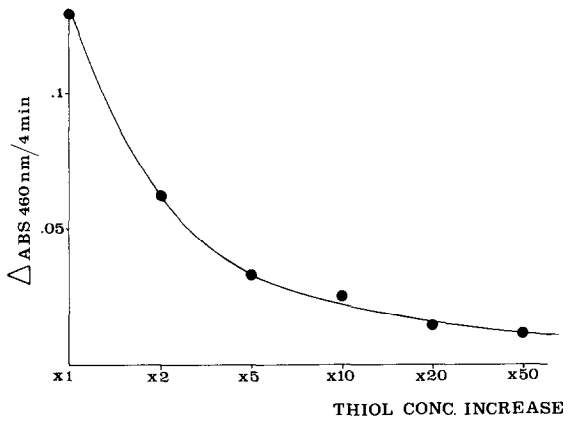

Fig.3. The effect of added glutathione on the superoxide dismutase of a sample of human haemolysate. The naturally occurring thiol level was 111 μmol/l and successive aliquots of 111 μmol/l glutathione were added. The abscissa is expressed in terms of the increased thiol content of the sample.

4. Discussion

Most clinical and pharmacological assays of superoxide dismutase activity are carried out in lysates which would, in general, contain a residual thiol level. A clear practical implication of this work is that this thiol level can interfere with the analysis (fig.2) and must be ascertained to be below the limit at which an appreciable interference is produced in the assay chosen. Studies in which this is not done must be regarded as suspect.

There are, however, more fundamental implications. O_2^- is a free-radical anion and its production in vivo may involve free-radical chain reactions in some circumstances [1]. Oxygen can be reduced to peroxide by cytochrome oxidase entirely within the protein and in a way that no other entity comes in contact with the intermediate metabolites [8] but there is no such specific protection in the generation of O_2^-. Thus, in vitro methods of generating O_2^- by free-radical chain reactions may be regarded as models for the in vivo situation. The molar ratio of glutathione to SOD in the cytosol of erythrocytes (~1000:1) is such that the augmentation assay used here would cease to record any activity from SOD (fig.1) and in the inhibition assay, glutathione would produce a greater inhibition than SOD at in vivo concentrations. Thus, it seems likely that thiols play a major role in controlling O_2^- concentrations, with SOD playing a more subtle part, e.g., in the production of the correct metabolites for protein synthesis [8].

The action of thiols is not as a dismutase; since they cause an inhibition in the augmentation assay and since SOD can prevent their action up to a certain ratio, it would seem that they act on the superoxide-generating system or on superoxide itself but in a slower fashion than SOD. It is the difference in relative amount in vivo that makes the thiol effect a possibility. Since glutathione and other thiols are known to form relatively long-lived free radicals which often require specific reactions to remove them [9], it seems most likely that the action of the thiols is as free-radical scavengers in the in vitro and possibly in the in vivo free-radical reactions required to produce O_2^-.

Thus, we suggest that the role of thiols in the control of superoxide-ion concentration requires further consideration and, in a practical sense, superoxide dismutase activity can only be assessed in the pure protein or in lysates in which the effect of the matrix has been investigated and defined or eliminated.

Acknowledgements

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