Inactivation of 3 α -hydroxysteroid dehydrogenase by superoxide radicals

Modification of histidine and cysteine residues causes the conformational change

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3 α -Hydroxysteroid dehydrogenase (EC 1.1.1.50) from *Pseudomonas testosteroni* was inactivated by superoxide radicals generated by the aerobic xanthine oxidase reaction. Superoxide dismutase, NAD⁺, bovine serum albumin and histidine and cysteine as free amino acids partially protected the enzyme from inactivation. NADH-binding properties were determined by fluorescence spectroscopy, and no variation was found between native enzyme and the unmodified fraction of the partly inactivated one. The fluorescence emission maximum for the completely inactivated enzyme was shifted 10 nm to a longer wavelength when compared with the native one, and it seems possible that the modification of histidine and cysteine residues by superoxide radicals causes the conformational change of the enzyme and the consequent loss of catalytic activity.

INTRODUCTION

Cellular production of superoxide radicals (O₂^{•-}) is an inevitable process in aerobic organisms; electron-transport chains in microsomal fractions, mitochondria, chloroplasts and several cytoplasmic oxidases are sites for generation of the radicals [1, 2]. The enzymic or non-enzymic production of O₂^{•-} has also been studied: xanthine/xanthine oxidase [3], nitrofurantoin/oxyhaemoglobin [4], u.v. irradiation [5] and NADH/phenazine methosulphate system [6].

The deleterious effects of O₂^{•-} have been partially explored. It is widely considered that these radicals are directly or indirectly responsible for aging, mutagenesis and inflammation [7, 8]. The effects of oxidative modification by oxygen free radicals in enzymic function and in intracellular proteolytic degradation of glutamine synthetase have been demonstrated [9, 10].

In our laboratory we have worked on the regeneration of NADH with phenazine methosulphate as an electron carrier. From an economic point of view, the regeneration of cofactor is very important in the enzymic synthesis of organic compounds. When 3 α -HSDH was incorporated in the above regeneration system, inactivation of this enzyme was observed, and SOD partially prevented the inactivation [11]. This is the evidence that 3 α -HSDH was inactivated by O₂^{•-} generated by the NADH/phenazine methosulphate system. The inactivation of enzymes by activated forms of oxygen is becoming a key point in enzyme technology.

In the present paper we report the study of the inactivation of 3 α -HSDH by O₂^{•-} generated by the aerobic xanthine oxidase reaction.

MATERIALS AND METHODS

3 α -HSDH from *Pseudomonas testosteroni*, xanthine oxidase from buttermilk, crystalline bovine liver catalase, bovine blood SOD and horse heart cytochrome *c* were

purchased from Sigma Chemical Co., and these were used directly without further purification. Androsterone, NAD⁺, NADH, xanthine and all amino acids were also obtained from Sigma Chemical Co., and acetaldehyde was obtained from Eastman. All other reagents were of analytical grade.

Enzyme activity was calculated by measuring the initial rate of increase in absorbance by NADH at 340 nm ($\epsilon_{340} = 6220 \text{ M}^{-1} \cdot \text{cm}^{-1}$), and all reactions were conducted in cuvettes of 1 cm pathlength in a Beckman double-beam spectrophotometer equipped with ultrathermostat. All reactions were carried out at 25 °C. Unless otherwise noted, reaction mixtures contained 0.13 mM-androsterone, 0.4 mM-NAD⁺ and 0.1 mM-EDTA in 0.1 M-bicarbonate buffer, pH 10.0. Androsterone was dissolved in methanol, and the final concentration of methanol in reaction mixtures was 3.3% (v/v).

Xanthine oxidase activity was measured in terms of the rate of conversion of xanthine into urate, detected at 300 nm, and SOD activity was assayed by its ability to inhibit the reduction of cytochrome *c*, by O₂^{•-}. The reduction of cytochrome *c* was calculated by using an absorption coefficient of $21\,600 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 550 nm.

Unless otherwise noted, the inactivation of 3 α -HSDH was carried out by incubating 10 nM enzyme with the acetaldehyde/xanthine oxidase reaction system containing substrate at pH 10 for the times indicated, and residual enzyme activity was measured by adding NAD⁺ solution. The concentration of acetaldehyde was 27 mM in all experiments. To monitor the time course of inactivation, 0.5 μM -3 α -HSDH was incubated with the acetaldehyde/xanthine oxidase reaction system, and at intervals 25 μl portions of incubation mixture were removed for assay of residual enzyme activity; this assay was performed in 0.1 M-sodium phosphate buffer, pH 7.0.

Fluorimetric titration was performed with a spectrofluorimeter (Jobin Yvon 3) fitted with an ultrathermostat at 25 °C. In the titration of binding sites, microlitre amounts of a concentrated solution of enzyme were

Fig. 1. Rate of production of $O_2^{\bullet-}$ by the aerobic xanthine oxidase reaction

The reaction was carried out in 0.1 M-bicarbonate buffer, pH 10.0. The concentration of cytochrome *c* was 0.15 mM; the results shown by ● symbols were obtained when O_2 -saturated buffer was used.

Fig. 2. Inactivation of 3 α -HSDH by the aerobic xanthine oxidase reaction

Reaction mixtures were as described in the Materials and methods section in 0.1 M-bicarbonate buffer, pH 10.0. Reactions were initiated at time zero by adding 3 α -HSDH. Curve 1 was the time course of the 3 α -HSDH reaction without xanthine oxidase, and curve 2 was obtained when SOD (0.14 μ M) was added at the beginning and xanthine oxidase (0.1 μ M) was added at arrow I. Curve 3 was obtained after adding xanthine oxidase (0.1 μ M) at arrow I followed by SOD (0.14 μ M) at arrow II. Curve 4 was obtained when xanthine oxidase (0.1 μ M) was added at arrow I.

added to a fixed concentration of NADH in 2.0 ml of buffer. The concentrations of both enzyme and NADH were corrected for dilution. For titrations with partly inactivated enzyme, the enzyme was partly inactivated by incubation with the acetaldehyde/xanthine oxidase

Fig. 3. Time course of inactivation of 3 α -HSDH at different pH values and xanthine oxidase concentrations

Inactivation was performed at pH 10.0 (□ and ○) or at pH 7.0 (●). The xanthine oxidase concentration was 0.02 μ M (□) or 0.15 μ M (○ and ●).

reaction system, and 150 nM-SOD was added to prevent further inactivation during titration (indeed, no further inactivation during titration was observed). All experiments were performed with a 10 nm passing band for both excitation and emission. The dissociation constant and number of binding sites of NADH were determined on a subunit basis by Stockell plot [12, 13].

The completely inactivated 3 α -HSDH was separated from the inactivation reaction mixtures by gel filtration. This separation was achieved on a Sephacryl S-300 gel (Pharmacia Fine Chemicals) column (1.2 cm $\times$ 50 cm) pre-equilibrated with 0.1 M-sodium phosphate buffer, pH 7.0, at 4 $^{\circ}$ C.

Fig. 4. Effect of SOD on inactivation of 3 α -HSDH

Reaction mixtures were the same as that for Fig. 2 except for the indicated concentrations of SOD. Enzyme activity was measured after 10 min incubation. The xanthine oxidase concentration was 0.02 μ M (●), 0.1 μ M (○) or 0.2 μ M (■).

RESULTS

Rate of production of O₂^{•-} by the xanthine oxidase reaction

The aerobic xanthine oxidase reaction, known to generate large amounts of O₂^{•-}, was used as the source of this radical [3]. Acetaldehyde was used as an electron donor in place of xanthine in order to avoid interference due to a strong u.v. absorbance by xanthine and urate. The rate of O₂^{•-} production was calculated by measuring the rate of univalent reduction of cytochrome *c* by O₂^{•-}. As shown in Fig. 1, the production rate increased linearly with xanthine oxidase concentration up to 0.15 μ M; O₂ depletion was observed beyond 0.15 μ M in our experimental conditions. Unless otherwise noted, the concentrations of xanthine oxidase were used within this range.

Inactivation of 3 α -HSDH by O₂^{•-}

As shown in Fig. 2, initiation of the xanthine oxidase reaction rapidly decreased the 3 α -HSDH activity. When SOD was added at the time of the initiation, inactivation was almost totally prevented, but enzyme activity could not be completely recovered when SOD was added 2 min after the initiation of the xanthine oxidase reaction. Inhibition by acetaldehyde was not observed. Higher concentrations of xanthine oxidase increased the inactivation of 3 α -HSDH. The inactivation of 3 α -HSDH at the same concentration of xanthine oxidase was greater at pH 10.0 than at pH 7.0 (Fig. 3). Kono & Fridovich have shown that catalase was more inhibited at the higher pH by O₂^{•-} [14]. Increased stability of O₂^{•-} at higher pH has been reported [15].

Effect of SOD

The SOD protection was tested at different concentrations of xanthine oxidase (Fig. 4). SOD prevented the inactivation of 3 α -HSDH completely at low concentration of xanthine oxidase (0.02 μ M), but at high concentration of xanthine oxidase (0.2 μ M) the inactivation was not completely prevented. In a search for other possible causes of inactivation (non-specific protein, OH[•] radicals and H₂O₂), 375 μ M-bovine serum albumin without xanthine oxidase, 10 mM-mannitol and 0.1 μ M-catalase were tested separately, and in no case was an effect observed. This indicates that the inactivation of 3 α -HSDH was largely, if not exclusively, due to O₂^{•-}.

Effects of NAD⁺ and related compounds on inactivation

As shown in Table 1, NAD⁺ gave more protection than did the other compounds tested. Adenine also protected the enzyme, but in contrast adenosine gave more

Table 1. Effect of NAD⁺ and related compounds on the inactivation of 3 α -HSDH by O₂^{•-}

Enzyme activity was measured after 10 min incubation with 0.15 μ M-xanthine oxidase at pH 10.0.

Addition	Concn. (mM)	Activity (%)	Inhibition (%)
None	—	50.0	0
NAD ⁺	0.7	93.0	0
ADP	1.0	47.0	22.0
Adenosine	1.0	30.0	40.0
Adenine	1.0	74.0	70.0

Table 2. Effect of bovine serum albumin on the inactivation of 3 α -HSDH by O₂^{•-}

Enzyme activity was measured after 10 min incubation with 0.15 μ M-xanthine oxidase at pH 10.0.

Concn. of bovine serum albumin (μ M)	Enzyme activity (%)
0	55.0
10	64.0
25	67.0
50	78.0
125	85.0
250	90.0
375	93.0

inactivation. When tested for inhibition of 3 α -HSDH activity, adenine gave more inhibition than did the other compounds.

Effect of bovine serum albumin on inactivation

Bovine serum albumin was added to the xanthine oxidase reaction system in order to examine the effect of non-specific protein on inactivation by O₂^{•-}. When the concentration of bovine serum albumin was increased to 375 μ M, 3 α -HSDH was almost completely protected (Table 2). No effect of bovine serum albumin on xanthine oxidase or 3 α -HSDH activity was observed.

Effect of amino acids on inactivation and cytochrome *c* reduction

In an attempt to determine whether the protection of bovine serum albumin is linked to its macromolecular structure or only to the presence of certain amino acid residues, 13 typical amino acids were tested for ability to protect 3 α -HSDH from inactivation or to inhibit cytochrome *c* reduction by O₂^{•-}. Effective protection of 3 α -HSDH and inhibition of reduction of cytochrome *c* by histidine and cysteine were observed (Table 3).

Table 3. Effects of amino acids on the inactivation of 3 α -HSDH and reduction of cytochrome *c* by O₂^{•-}

Enzyme activity was measured after 10 min incubation with 0.15 μ M-xanthine oxidase. 'Other amino acids' were phenylalanine, tyrosine, tryptophan, proline, cystine, methionine, serine, alanine, glycine, asparagine and leucine. Their concentrations were 10 mM, except for tyrosine and cystine, which were 2 mM and 0.1 mM respectively because of limited solubility. The concentration of cytochrome *c* used was 0.15 mM. Each value was the average for triplicate runs.

Addition	Enzyme activity (%)	Inhibition of cytochrome <i>c</i> reduction (%)
None	47.0	0
Histidine	68.0	30.0
Cysteine	70.0	32.0
Other amino acids	45–49	Not determined

Fig. 5. Fluorescence titration of NADH with native 3 α -HSDH and partly inactivated 3 α -HSDH

NADH (0.7 μ M) was titrated with both native enzyme (curve 1) or partly inactivated enzyme (curve 2) in 0.1 M-bicarbonate buffer, pH 10.0. Excitation was at 340 nm and emission was at 438 nm.

Fluorescence titration

The fluorescence titration curves of both native and partly inactivated enzyme against a 0.7 μ M-NADH are shown in Fig. 5. A decrease of fluorescence intensity was observed in the titration with partly inactivated enzyme.

To examine the NADH-binding properties, 0.23 μ M- and 0.13 μ M-NADH were titrated with native and partly inactivated enzyme respectively (detailed results not shown). For native enzyme, a dissociation constant of 0.59 μ M and 0.80 NADH binding sites per subunit were obtained. With partly inactivated enzyme, the unmodified fraction of the partly inactivated enzyme was determined by measuring the residual enzyme activity after inactivation reaction. By the Stockell plot for the native enzyme fraction, the values obtained were a dissociation constant of 0.55 μ M and 0.74 NADH binding sites per subunit.

Fluorescence spectrum

The fluorescence spectra of the completely inactivated enzyme and the native one were obtained at an excitation

Fig. 6. Fluorescence spectra of native 3 α -HSDH (—) and completely inactivated 3 α -HSDH (-----)

wavelength of 270 nm (Fig. 6). The maximum emission for the completely inactivated enzyme was shifted 10 nm to a longer wavelength when compared with the native one. The maximum emission for the native enzyme was at 330 nm.

DISCUSSION

3 α -HSDH was inactivated rapidly and irreversibly by $O_2^{\cdot-}$ generated by the aerobic xanthine oxidase reaction. At higher concentrations of xanthine oxidase the enzyme was inactivated to a greater extent. This could be due to a higher production rate of $O_2^{\cdot-}$ and a higher steady-state concentration of this radical. When increasing xanthine oxidase concentration, a saturation of the SOD catalytic capacities was observed and 3 α -HSDH activity was not fully protected by SOD. This might be due to competition between both enzymes, 3 α -HSDH and SOD, for the $O_2^{\cdot-}$, with a minor proportion of 3 α -HSDH reacting with $O_2^{\cdot-}$ more rapidly than SOD. A similar result has been reported for hydrogenase from *Alcaligenes eutrophus* H 16 [16]. The inactivation of SOD by H_2O_2 produced by dismutation of $O_2^{\cdot-}$ might contribute to this phenomenon [17, 18]. It has also been shown by Kono & Fridovich that catalase was inhibited by $O_2^{\cdot-}$ by two different mechanisms [14]: a rapid inhibition, prevented and reversed by SOD; a slow irreversible inhibition, prevented by SOD.

The cofactor-binding properties of both native enzyme and the partly inactivated one were examined by fluorescence titration studies. The number of binding sites and the dissociation constant of NADH were found not to be modified in the active fraction of partly inactivated enzyme when compared with the native one. This means that the unmodified fraction of partly inactivated enzyme remains intact. The fluorescence change in the case of partly inactivated enzyme could be accounted for by the contribution of unmodified (active) enzyme, implying that modified (inactivated) enzyme is capable of binding NADH only very weakly.

Protection of 3 α -HSDH by bovine serum albumin against the inactivation by $O_2^{\cdot-}$ was observed in the present work. The stabilization of immobilized enzymes within bovine-serum-albumin-cross-linked supports could be consistent with this observation. The $O_2^{\cdot-}$ produced by activated phagocytes during phagocytosis could exacerbate and prolong the inflammatory process. There is ordinarily very little SOD in extracellular fluids, and so the anti-inflammatory effect by serum albumin might be of relevance in this respect.

In an attempt to determine whether the protection was linked to the bovine serum albumin macromolecular structure itself or only to the presence of certain amino acids residues, a protective effect of histidine and cysteine was also shown. On the basis of the concentrations used, bovine serum albumin was much more efficient than the free amino acids in the protection of the enzyme against inactivation, and it is considered that the macromolecular structure of bovine serum albumin itself could play some role. The protective effects of histidine and cysteine could be due to the competition for $O_2^{\cdot-}$ and a resulting decrease in the concentration of these radicals. The inhibition by histidine and cysteine of the cytochrome c reduction could be related to that possibility.

Greater exposure of the chromophore buried in the interior of the protein to a hydrophilic environment

causes a lowering of the chromophore energy level; the emission maximum of proteins is shifted to longer wavelength [19]. The emission maximum for completely inactivated enzyme was shifted 10 nm to longer wavelength, and this could be due to the fact that modification of histidine and cysteine residues by O₂^{•-} causes the conformational change of the enzyme.

Protection by NAD⁺ was observed, but inhibition of cytochrome *c* reduction by NAD⁺ was not. The mechanism of protection by the cofactor is not known, but it has been proposed that the binding of NAD⁺ could shield the catalytic sites (target sites) from attack by the free radicals and thus protect the enzyme [20]. NAD⁺ can bind with the residues of amino acids at catalytic sites and it gave more protection than adenine (Table 1). In view of the inactivation mechanism and the above result, NAD⁺ could protect the enzyme in two ways: by shielding catalytic sites (target sites) from attack by O₂^{•-} and/or by preventing the conformational change of the enzyme.

REFERENCES

1. Fridovich, I. (1982) in *Pathology of Oxygen* (Auer, A. P., ed.), pp. 1–19, Academic Press, New York
2. Freeman, B. A. & Crapo, J. D. (1982) *Lab. Invest.* **47**, 412–426
3. Fridovich, I. (1970) *J. Biol. Chem.* **245**, 4053–4057
4. Dershwitz, M. & Novak, R. F. (1982) *J. Biol. Chem.* **257**, 75–79
5. Roberts, P. D. (1973) *Int. J. Radiat. Biol.* **24**, 143–152
6. Nishikimi, M., Rao, N. A. & Yagi, K. (1972) *Biochem. Biophys. Res. Commun.* **46**, 849–854
7. Fridovich, I. (1978) *Science* **201**, 875–880
8. Halliwell, B. & Gutteridge, J. M. C. (1984) *Biochem. J.* **219**, 1–14
9. Levine, R. L., Oliver, C. N., Fuls, R. M. & Stadtman, E. R. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 2120–2124
10. Levine, R. L. (1983) *J. Biol. Chem.* **258**, 11823–11827
11. Legoy, M. D., Larreta Garde, V., Ergon, F. & Thomas, D. (1979) *J. Solid-Phase Biochem.* **4**, 143–148
12. Stockell, A. (1959) *J. Biol. Chem.* **234**, 1286–1292
13. Luedtke, A. & Karush, F. (1982) *Biochemistry* **21**, 5738–5744
14. Kono, Y. & Fridovich, I. (1982) *J. Biol. Chem.* **257**, 5751–5754
15. Chevalet, J., Rouelle, F., Lambert, J. P. & Gierst, L. (1972) *J. Electroanal. Chem.* **39**, 201–216
16. Schneider, K. & Schlegel, H. G. (1981) *Biochem. J.* **193**, 99–107
17. Hodgson, E. K. & Fridovich, I. (1975) *Biochemistry* **14**, 5294–5299
18. Blech, D. M. & Borders, C. L. (1983) *Arch. Biochem. Biophys.* **224**, 579–586
19. Brand, L. & Witholt, B. (1967) *Methods Enzymol.* **11**, 826–838
20. Buchanan, J. D. & Armstrong, D. A. (1978) *Int. J. Radiat. Biol.* **33**, 409–418

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