Biochimica et Biophysica Acta, 571 (1979) 195—200

BBA 68860

AUTOINACTIVATION OF XANTHINE OXIDASE

THE ROLE OF SUPEROXIDE RADICAL AND HYDROGEN PEROXIDE

ROBERT E. LYNCH * and IRWIN FRIDOVICH

Division of Hematology-Oncology, University of Utah Medical Center, Salt Lake City, UT 84132, and Department of Biochemistry, Duke University Medical Center, Durham, NC 27710 (U.S.A.)

(Received March 23rd, 1979)

Key words: Xanthine oxidase autoinactivation; Superoxide radical; Hydrogen peroxide

Summary

Xanthine oxidase suffers autoinactivation in the course of catalyzing the oxidation of acetaldehyde. When no special efforts were made to maintain a high pO_2 in these reaction mixtures catalase protected the xanthine oxidase, but superoxide dismutase did not. However, when oxygen depletion was slowed or prevented by working at lower concentrations of xanthine oxidase, at lower temperatures or by vigorous agitation under an atmosphere of 100% oxygen, superoxide dismutase or catalase protected markedly when added separately and protected almost completely when added together. This result correlates with the greater production of O_2^-, relative to H_2O_2, by xanthine oxidase, at elevated pO_2. Since histidine also provided some protection and the high levels of acetaldehyde used would have precluded any significant effect of $OH^{\cdot}$, we conclude that singlet oxygen, or something with similar reactivity, was generated from O_2^- plus H_2O_2 and contributed significantly to the observed autoinactivation.

The toxicity of molecular O_2 is mediated largely by intermediates of its reduction. The first of these, O_2^-, damages certain biological molecules and structures and is scavenged by superoxide dismutases. These enzymes are essential components of the defense against the toxicity of O_2. The data which support some portions of this view of the role of O_2^- in the toxicity of dioxygen are firm. Thus, superoxide is produced during the autoxidations of many

* Present address: Howard Hughes Medical Institute, University of Utah Medical Center, Salt Lake City, UT, U.S.A.

biologic compounds, including leucoflavins [1,2], hydroquinones [2], catecholamines [3,4], tetrahydropterins [5], cytochrome [6], ferredoxins [7] and hemoglobins [8,9] as well as during the action of several oxidases and flavin containing dehydrogenases [10]. Furthermore, certain organelles and even whole cells, including respiring submitochondrial particles [11,12], illuminated chloroplast fragments [13—15] and activated granulocytes [16] have been shown to release O_2^-. In a soluble extract of *Streptococcus faecalis*, 17% of the total oxygen uptake could be accounted for in terms of O_2^- generated [17]. Clearly the biological production of O_2^- is a reality. Cells, in which the activity of superoxide dismutase has been increased, resist both killing by hyperbaric oxygen and the oxygen-enhancements of the toxicity of streptonigrin and of methyl viologen [18—21]. Furthermore, mutants with defects in superoxide dismutase exhibited the expected intolerance for oxygen [22].

The deleterious effects of O_2^- have been partially explored. Fluxes of O_2^-, generated photochemically or enzymatically, have been shown to oxidize linolenate [23], lyse erythrocytes [24] and vesicles formed of erythrocyte stroma [25], depolymerize hyaluronate [26] inactive virus [27], kill bacteria [27,28] and damage mammalian cells in culture [29]. There has been little dy of the effects of O_2^- on enzymes beyond reports of the inactivation of papain [30] and of glyceraldehyde-3-phosphate dehydrogenase [31] by a radiochemical source of these radicals. During investigations of the effects of an enzymatic source of O_2^- upon human erythrocytes, it was noted that xanthine oxidase was subject to a self-inactivation which was partially prevented by superoxide dismutase [24,25]. The effect of O_2^- upon xanthine oxidase may be taken as a model for its potential effects upon diverse enzymes and thus seemed worthy of more thorough study.

Materials and Methods

Acetaldehyde, from Eastman Organic Chemicals, was stored at —20°C and was freshly distilled prior to use. Xanthine (Sigma), bovine erythrocyte superoxide dismutase (Truett), bovine liver catalase (Boehringer or Calbiochem), EDTA, mannitol and histidine (Sigma) were obtained from the indicated sources and were used without special purification. Xanthine oxidase was prepared from raw cream [32] and was stored at —20°C in 50 mM potassium phosphate, 0.1 mM EDTA at pH 7.8.

Xanthine oxidase activity was estimated spectrophotometrically in terms of the rate of conversion of xanthine to urate followed by the increase in absorbance at 295 nm [33]. O_2^- produced during the xanthine oxidase reaction was also measured spectrophotometrically in terms of the superoxide dismutase-inhibitable reduction of cytochrome *c* at 550 nm [34]. The inactivation of xanthine oxidase during its action upon acetaldehyde was assessed by removing samples at intervals and diluting them into buffered xanthine and following the rate of increase in absorbance at 295 nm at 25°C.

Results

Autoinactivation of xanthine oxidase

Xanthine oxidase (1.5 μM) was progressively inactivated when allowed to act

upon 10 mM acetaldehyde in 50 mM potassium phosphate, 0.1 mM EDTA (pH 7.8) and at 25°C. Superoxide dismutase did not provide significant protection, but did increase the substantial protection offered by catalase. These results, shown in Fig. 1A, were obtained in reaction mixtures which were open to the air, but not agitated or bubbled. Since the initial oxygen concentration in solution (0.24 mM) was only 2.5% of the initial acetaldehyde concentration and since diffusion across a still air-water interface is a slow process, we must expect that the pO_2 declined rapidly in these reaction mixtures. Xanthine oxidase can cause both univalent and divalent oxygen reduction but the univalent process is favored by high pO_2 [35]. It follows that the rate of O_2^- production, relative to the rate of H_2O_2 production, must have declined rapidly with the declining pO_2 in these reaction mixtures. It seemed likely that the small effect of superoxide dismutase may have been due to this circumstance.

Protection by superoxide dismutase

The rapid decline in pO_2, and hence in O_2^- production, might be avoided by slowing the rate of the xanthine oxidase reaction or by increasing the aeration. Both approaches were tested. The rate of oxidation of xanthine was reduced 10-fold by an equivalent reduction in the concentration of xanthine oxidase (Fig. 1B) or by lowering the temperature to 5°C (Fig. 2). In both cases superoxide dismutase or catalase, added separately, afforded significant protection and added together protected almost completely. Furthermore, in accord with the expected gradual decrease in O_2^- production relative to H_2O_2 production, due to gradually decreasing pO_2, superoxide dismutase protection was most evident during the early part of the reaction and catalase protection was most effective during the late phase.

A high concentration of dissolved oxygen was also maintained, in spite of a

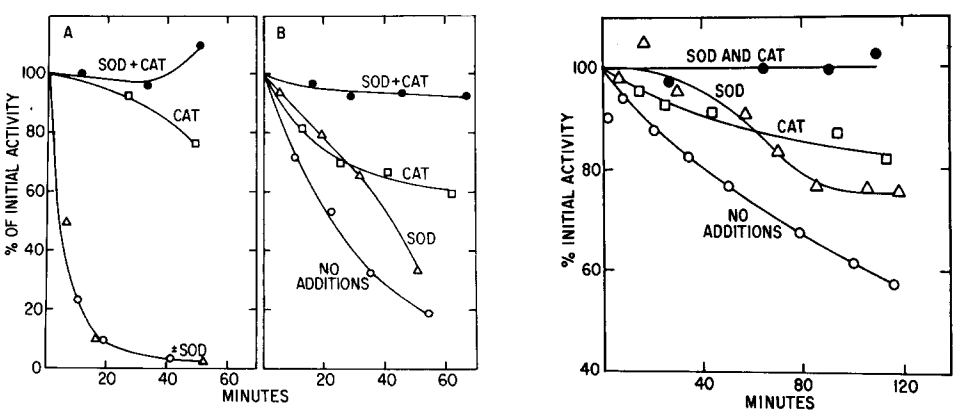


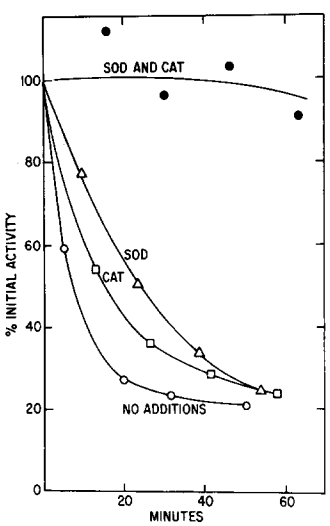
Fig. 1. Autoinactivation of xanthine oxidase at 25°C in air. Reaction mixtures contained 10 mM CH_3CHO, 50 mM potassium phosphate, 0.1 mM EDTA and where indiced 10 μg/ml of superoxide dismutase (SOD) and/or of catalase (CAT) at pH 7.8 and at 25°C. The concentration of xanthine oxidase was 1.5 μM in A and 0.15 μM in B. Although reaction mixtures were open to the air, no attempt was made to agitate during the reaction.

Fig. 2. Autoinactivation of xanthine oxidase in air at 5°C. Reaction mixtures contained 10 mM CH_3CHO, 50 mM potassium phosphate, 0.1 mM EDTA, 0.36 μM xanthine oxidase, at pH 7.8, and where indicated 10 μg/ml of catalase (CAT) and&or superoxide dismutase (SOD). No agitation was applied.

rapid xanthine oxidase reaction, by agitating the reaction mixture under an atmosphere of 100% oxygen. In this case superoxide dismutase was more protective than catalase and the combined presence of both of these enzymes afforded full protection (Fig. 3).

Effects of scavengers of OH· and of $O_2(^1\Delta g)$

There have been numerous reports in which O_2^- and H_2O_2 appeared to collaborate in causing an effect. In the first report of this phenomenon, the effect was the generation of ethylene from methional [36] and either superoxide dismutase or catalase prevented the reaction. Since compounds which scavenge OH· or $O_2(^1\Delta g)$, but which do not react with either O_2^- or H_2O_2, have also been seen to inhibit in these cases [6,23,24]; we have supposed that O_2^- and H_2O_2 can conspire in the production of these very reactive species. Iron compounds have recently been reported to facilitate this process [37]. Since the autoinactivation of xanthine oxidase was prevented by superoxide dismutase and catalase a similar cooperative effect was supposed and the effects of mannitol, a scavenger for OH·, and of histidine, a scavenger for $O_2(^1\Delta g)$ were investigated. Because the reaction mixtures already contained 10 mM CH_3CHO, which would effectively scavenge OH·, we could not expect mannitol to exert much effect. As shown in Fig. 4, mannitol did not protect but histidine did. In these reaction mixtures the relative extent of O_2^- production was optimized by allowing the xanthine oxidase to act upon acetaldehyde at 5°C and under 100% oxygen and superoxide dismutase was seen to offer pronounced protection.



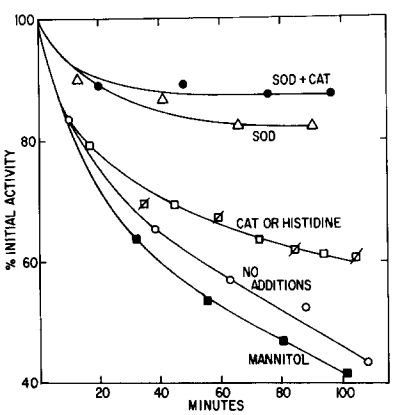


Fig. 3. Autoinactivation of xanthine oxidase in 100% oxygen at 25°C. Reaction mixtures contained 10 mM CH_3CHO, 50 mM potassium phosphate, 0.1 mM EDTA and 1.3 μM xanthine oxidase and where indicated 10 μg/ml of catalase (CAT) and/or superoxide dismutase (SOD). Reaction mixtures were continuously agitated under 100% O_2 at 25°C at pH 7.8.

Fig. 4. Autoinactivation of xanthine oxidase in 100% O_2 at 5°C. Reaction mixtures contained 10 mM CH_3CHO, 50 mM potassium phosphate, 0.1 mM EDTA, 0.33 μM xanthine oxidase and where indicated 10 μg/ml superoxide dismutase (SOD), 10 μg/ml catalase (CAT) 5 mM histidine or 100 mM mannitol. Reaction mixtures were continuously agittated under 100% O_2 at 5°C and at pH 7.8.

Discussion

Xanthine oxidase suffers a syncatalytic inactivation by mercurials [38,39] and by H_2O_2 [40]. This suggests that a reactive sulfur center is transiently generated during the catalytic cycle. Massey and Edmondson [41] presented data supporting the essentiality of a persulfide group for the activity of this enzyme. We have now seen that the aerobic action of this enzyme on acetaldehyde results in an autoinactivation which can be due primarily to H_2O_2 or to O_2^-, depending upon their relative rates of production; which in turn is a function of the pO_2. When the pO_2 was maximized, by working at 5°C with agitation under an atmosphere of 100% O_2, superoxide dismutase was more protective than was catalase (Fig. 4), but the combination of superoxide dismutase plus catalase was most effective. This is yet another instance in which O_2^- and H_2O_2 interact to generate potent oxidants, which have tentatively been identified as $OH^\cdot$ and $O_2(^1\Delta g)$ [23,24,36]. In the present case the high level of CH_3CHO present in the reaction mixture would effectively scavenge $OH^\cdot$, so the inactivation could not have been due to this radical. Indeed, mannitol was not able to protect and even slightly augmented the rate of autoinactivation. Histidine did protect significantly and this suggests that $O_2(^1\Delta g)$, generated from O_2^- plus H_2O_2, may have played a role in the autoinactivation of xanthine oxidase. Another dimension has now been added to considerations of oxygen toxicity. Enzymes, which like xanthine oxidase have a readily oxidized essential component, whether exposed transiently during their catalytic cycles or present at all times, can be inactivated by the oxidant, possibly singlet oxygen, which is generated by the interaction of O_2^- and H_2O_2.

Acknowledgements

This work was supported by research grants from the National Institutes of Health, Betheda, MD, the United States Army Research Office, Research Office, Research Triangle Park, NC and Merck, Sharp and Dohme, Rahway, NJ.

References

1 Ballou, D., Palmer, G. and Massey, V. (1969) Biochem. Biophys. Res. Commun. 36, 898—904
2 Misra, H.P. and Fridovich, I. (1972) J. Biol. Chem. 247, 188—192
3 Misra, H.P. and Fridovich, I. (1972) J. Biol. Chem. 247, 3170—3175
4 Cohen, G. and Hekkila, R. (1974) J. Biol. Chem. 249, 2447—2452
5 Fisher, D.B. and Kaufman, S. (1973) J. Biol. Chem. 248, 4300—4304
6 Cassell, R.H. and Fridovich, I. (1975) Biochemistry 14, 1866—1868
7 Misra, H.P. and Fridovich, I. (1971) J. Biol. Chem. 246, 6886—6890
8 Misra, H.P. and Fridovich, I. (1972) J. Biol. Chem. 247, 6960—6962
9 Gotoh, T. and Shikama, K. (1976) J. Biochem. (Tokyo) 80, 397—399
10 Fridovich, I. (1975) Ann. Rev. Biochem. 44, 147—159
11 Loschen, G., Azzi, A., Richler, C. and Flohé, L. (1974) FEBS Lett. 42, 68—72
12 Boveris, A. (1977) Adv. Exp. Med. Biol. 78, 67—82
13 Asada, K. and Kiso, K. (1973) Eur. J. Biochem. 33, 253—257
14 Epel, B.L. and Neuman, J. (1973) Biochim. Biophys. Acta 325, 520—529
15 Halliwell, B. (1975) Eur. J. Biochem. 55, 355—360
16 Cheson, B.D., Curnutte, J.T. and Babior, B.M. (1977) Prog. Clin. Immunol. 3, 1—65
17 Britton, L., Malinowski, D.P. and Fridovich, I. (1978) J. Bacteriol. 134, 229—236
18 Hassan, H.M. and Fridovich, I. (1977) J. Bacteriol. 129, 1574—1583

19 Hassan, H.M. and Fridovich, I. (1977) J. Bacteriol. 130, 805—811
20 Hassan, H.M. and Fridovich, I. (1977) J. Bacteriol. 132, 505—510
21 Hassan, H.M. and Fridovich, I. (1977) J. Biol. Chem. 252, 7667—7672
22 Hassan, H.M. and Fridovich, I. (1979) J. Infect. Dis. 1, 357—367
23 Kellogg, E.W. III and Fridovich, I. (1975) J. Biol. Chem. 250, 8812—8817
24 Kellogg, E.W. III and Fridovich, I. (1977) J. Biol. Chem. 252, 6721—6728
25 Lynch, R.E. and Fridovich, I. (1978) J. Biol. Chem. 253, 1838—1845
26 McCord, J.M. (1974) Science 185, 529—531
27 Lavelle, F., Michelson, A.M. and Dimitrejevic, L. (1973) Biochem. Biophys. Res. Commun. 55, 350—357
28 Babior, B., Curnutte, J.T. and Kipnes, R.S. (1975) J. Lab. Clin. Med. 85, 235—244
29 Michelson, A.M. and Buckingham, M.E. (1974) Biochem. Biophys. Res. Commun. 58, 1079—1086
30 Lin, W.-S., Armstrong, D.A. and Lal, M. (1978) Int. J. Rad. Biol. 33, 231—243
31 Buchanan, J.O. and Armstrong, D.A. (1978) Int. J. Rad. Biol. 33, 409—418
32 Waud, W.R., Brady, F.O., Wiley, R.D. and Rajagopalan, K.V. (1975) Arch. Biochem. Biophys. 169, 695—701
33 Kalckar, H.M. (1947) J. Biol. Chem. 167, 429—443
34 McCord, J.M. and Fridovich, I. (1969) J. Biol. Chem. 244, 6049—6055
35 Fridovich, I. (1970) J. Biol. Chem. 245, 4053—4057
36 Beauchamp, C. and Fridovich, I. (1970) J. Biol. Chem. 245, 4641—4646
37 McCord, J.M. and Day, E.D., Jr. (1978) FEBS Lett. 86, 139—142
38 Fridovich, I. and Handler, P. (1958) J. Biol. Chem. 231, 899—911
39 Brumby, P.E., Miller, R.W. and Massey, V. (1965) J. Biol. Chem. 240, 2222—2228
40 Betcher-Lange, S.L. (1977) Ph.D. Thesis, Duke University, pp. 99—129
41 Massey, V. and Edmondson, D.E. (1970) J. Biol. Chem. 245, 6596—6598