Biochimica et Biophysica Acta, 430 (1976) 182–188

BBA 47072

THE ACCUMULATION OF SUPEROXIDE RADICAL DURING THE AEROBIC ACTION OF XANTHINE OXIDASE

A REQUIEM FOR $H_2O_4^-$

ELLEN K. HODGSON and IRWIN FRIDOVICH

Department of Biochemistry, Duke University Medical Center, Durham, N. C. 27710 (U.S.A.)

(Received September 15th, 1975)

SUMMARY

The action of xanthine oxidase upon acetaldehyde or xanthine at pH 10.2 has been shown to be accompanied by substantial accumulation of O_2^- during the first few minutes of the reaction. H_2O_2 decreases this accumulation of O_2^- presumably because of the Haber-Weiss reaction ($H_2O_2+O_2^- \rightarrow OH^-+OH+O_2$) and very small amounts of superoxide dismutase eliminate it. This accumulation of O_2^- was demonstrated in terms of a burst of reduction of cytochrome *c*, seen when the latter compound was added after aerobic preincubation of xanthine oxidase with its substrate. The kinetic peculiarities of the luminescence seen in the presence of luminol, which previously led to the proposal of $H_2O_4^-$, can now be satisfactorily explained entirely on the basis of known radical intermediates.

INTRODUCTION

The chemiluminescence of luminol (3-aminophthalhydrazide) in aqueous solutions appears always to involve O_2^- [1, 2]. The xanthine oxidase reaction is known to produce O_2^- and H_2O_2 and on this basis one can readily understand why the xanthine oxidase reaction has been observed to initiate the luminescence of luminol [3, 4]. There are however aspects of this luminescence which have seemed surprising and which led to the proposal of a new intermediate, formed by a reaction of O_2^- with H_2O_2, i.e. $H_2O_4^-$ [5]. Thus, the intensity of luminescence increased gradually during the first few minutes of the reaction, as though some reactive intermediate were accumulating. Furthermore, incubation of xanthine oxidase with xanthine, for a few minutes prior to the addition of luminol, gave an immediately high level of luminescence, indicating that the xanthine oxidase reaction accumulated a reactive compound. Superoxide dismutase completely inhibited this luminescence, whether present during the preincubation or whether added with the luminol, yet it did not seem likely that O_2^- itself would be sufficiently stable to accumulate during several min of reaction. Furthermore attempts to demonstrate the accumulation of O_2^-, through the use of cytochrome *c*, were not successful [5]. Since H_2O_2 augmented the initial luminescence

in this system without eliminating the time-dependent increase in luminescence, it seemed possible that O_2^- reacted reversibly with H_2O_2 to give $H_2O_4^-$, which accumulated and thus accounted for the observed time-dependent increases in luminescence [5].

The technical difficulties, which previously interfered with attempts to demonstrate the accumulation of O_2^- in the xanthine oxidase reaction [5] have now been overcome. Furthermore the confusions arising from the ability of the copper-zinc superoxide dismutase to catalyze the peroxidation of luminol, with attendant luminescence, have been recognized and surmounted through the use of the manganese-containing superoxide dismutase [6]. It is now possible, therefore, to demonstrate that O_2^- does accumulate during the xanthine oxidase reaction and thus to fully account for the details of the luminescence seen in the presence of luminol. We may now lay to rest the proposal [5] of the chemical species $H_2O_4^-$.

MATERIALS AND METHODS

The manganese-containing superoxide dismutase was prepared from *Escherichia coli* as previously described [7]. Xanthine oxidase was prepared from cream by a procedure which avoids proteolysis [8]. Catalase was from the Sigma Chemical Company and was purified of contaminating superoxide dismutase [9] by repeated washings through an XM100A Diaflo ultrafiltration membrane from the Amicon Corporation. Catalase activity was assayed according to the method of Beers and Sizer [10]. Cytochrome *c* Type VI was obtained from the Sigma Chemical Company. Luminol was obtained from the Aldrich Chemical Company, Inc. and was recrystallized from dilute HCl. Hydrogen peroxide of high purity and at a concentration of at least 98 % was kindly provided by Dr. Peter Smith, Department of Chemistry, Duke University. Acetaldehyde solutions were freshly distilled daily. Luminescence was measured with the photometer described by Mitchell and Hastings [11]. Spectrophotometric measurements were recorded on a Gilford Model 2000 recording spectrophotometer using Yankeelov cuvettes [12]. All reactions were performed aerobically in aqueous solutions buffered at pH 10.2 by 0.05 M sodium carbonate containing 10^{-4} M ethylene diamine tetraacetic acid (EDTA).

RESULTS

Time-dependent charges in luminol luminescence

Addition of xanthine oxidase to reaction mixtures, containing xanthine and luminol, caused a luminescence whose intensity increased gradually during the first 3 min of the reaction. H_2O_2 increased both the maximum luminescence and the rate at which it was achieved, while superoxide dismutase completely inhibited light production. Fig. 1 illustrates these effects. Since the xanthine oxidase reaction generates H_2O_2 as well as O_2^-, the effect of catalase was explored. As shown in Fig. 2, catalase diminished the luminescence but did not eliminate the time-dependent increase in its intensity. When superoxide dismutase was present, in addition to catalase, it entirely inhibited light production. These effects are shown in Fig. 2.

When the xanthine oxidase reaction was allowed to proceed for a short time prior to the addition of luminol, an intense luminescence was immediately evident

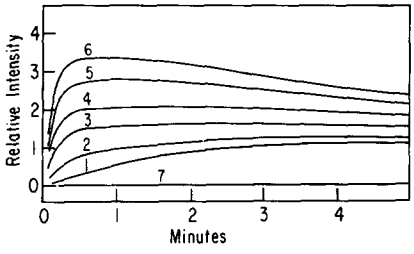

Fig. 1. Luminescence of luminol catalyzed by the xanthine oxidase reaction. Reaction mixtures contained $2 \cdot 10^{-4}$ M xanthine, $1.5 \cdot 10^{-5}$ M luminol, $6.6 \cdot 10^{-9}$ M xanthine oxidase, $1 \cdot 10^{-4}$ M EDTA, 0.05 M sodium carbonate and the following concentrations of hydrogen peroxide: (1) None; (2) $3.3 \cdot 10^{6}$ M; (3) $5.0 \cdot 10^{-6}$ M; (4) $6.6 \cdot 10^{-6}$ M; L (5) $8.3 \cdot 10^{-6}$ M, and (6) $10 \cdot 10^{-6}$ M. Curve (7) was obtained in the presence of $10 \cdot 10^{-6}$ M H_2O_2 and 0.9 μg/ml superoxide dismutase. The reaction mixture was buffered at pH 10.2 and 25 °C, and the total volume was 3 ml. Xanthine oxidase was the last component added.

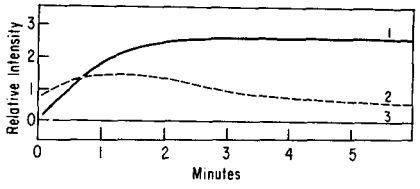

Fig. 2. Effect of catalase on the xanthine oxidase-catalyzed luminol luminescence. Reaction mixtures contained $2 \cdot 10^{-8}$ M xanthine oxidase. Other conditions and components were as described in Fig. 1. In addition the reaction mixture in (2) contained 23 units/ml of catalase and that in (3) contained 23 units/ml catalase and 0.6 μg/ml superoxide dismutase. Xanthine oxidase was the last component added.

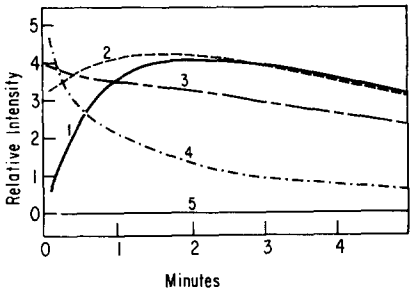

Fig. 3. Effects of preincubation of xanthine oxidase with xanthine upon luminescence seen when luminol is subsequently added. Reaction mixtures contained $6.6 \cdot 10^{-8}$ M xanthine oxidase and other conditions and components were as described in Fig. 1. Curve (1) was obtained when xanthine oxidase was the last component added. Curve (2) was obtained when xanthine oxidase was allowed to act upon xanthine for 0.5 min before the addition of luminol. Curves (3), (4), and (5) were obtained when xanthine oxidase was preincubated with xanthine for 3 min prior to the addition of luminol. Also present during the preincubation were 23 units/ml of catalase (curve (4)) and 0.6 μg/ml superoxide dismutase (curve (5)). Curves similar to (4) and (5) were obtained when the catalase (4) or superoxide dismutase (5) was added subsequent to the preincubation at the same time as the luminol was added.

upon addition of the luminol. This was followed by a gradual decline in luminescence. Catalase, present from the outset, did not inhibit the initial burst of light production but did hasten its subsequent decline. In contrast, superoxide dismutase whether present from the outset, or added with the luminol, completely suppressed luminescence. Fig. 3 illustrates these effects.

The accumulation of O_2^-.

The results described above suggested that an accumulation of O_2^- might fully account for the time-dependent increase in luminescence and for the effects seen when the xanthine oxidase reaction was allowed to proceed for several minutes, prior to the addition of luminol. If O_2^- did accumulate during the xanthine oxidase reaction, then subsequent addition of ferricytochrome *c* should result in a burst of reduction of the cytochrome *c*, which could be followed at 550 nm. This might be hard to detect because the sudden increase in absorbance due to reduction of cytochrome *c* would be small compared to the increase due to the addition of ferricytochrome *c*. This technical difficulty was surmounted through the use of Yankeelov cuvettes [12]. Thus compartment A of the cuvette contained acetaldehyde and buffer, while compartment B contained acetaldehyde, ferricytochrome *c* and buffer. At zero time xanthine oxidase was added to compartment A and the absorbance at 550 nm was recorded. At some interval thereafter the cuvette was removed from the spectrophotometer, its contents were mixed by capping and inversion and the recording of absorbance was immediately resumed. Since all components were in the light beam

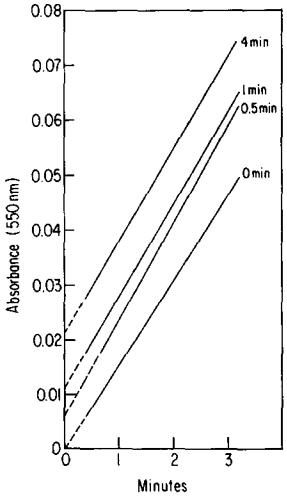

Fig. 4. Burst of cytochrome *c* reduction seen upon preinucbating xanthine oxidase with acetaldehyde. Reactions were performed in Yankeelov cuvettes with compartment A containing 1 ml of 0.01 M acetaldehyde in 0.05 M carbonate buffer containing $1 \cdot 10^{-4}$ M EDTA at pH 10.2, and compartment B containing 1 ml of 0.01 M acetaldehyde and $3 \cdot 10^{-5}$ M cytochrome *c* in the same buffer. 3.6 μg of xanthine oxidase was added to side A and the contents of the cuvette were mixed at the indicated times after addition of the xanthine oxidase. Zero time on the abscissa is the time of mixing and the slope of each curve was extrapolated back to this time of mixing as a measure of the burst of cytochrome *c* reduction. The temperature throughout was maintained at 25 °C, and in each case the base line absorbance was measured before mixing.

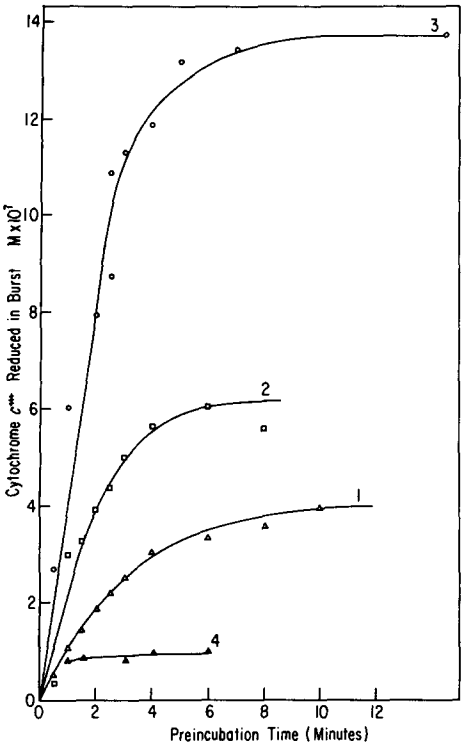

Fig. 5. Increase of burst size with time of preincubation. Reactions were carried out at 25° in Yankeelov cuvettes with the contents of compartments A and B being identical to those in Fig. 4. The concentrations of xanthine oxidase added to side A at zero time were: (1) 0.9 μg; (2) 1.8 μg; (3) 3.6 μg and (4) 0.9 μg. In addition in (4) side A contained $3 \cdot 10^{-5}$ M H_2O_2. Each point was obtained by preincubating the xanthine oxidase with the acetaldehyde for the times indicated on the abscissa, before mixing the contents of the cuvette. As in Fig. 4, the bursts of cytochrome *c* reduction were measured and are plotted here as a function of the time of preincubation.

before inversion, the cuvette served as its own reference and the only changes in absorbance which were observed were those associated with the reduction of ferricytochrome *c*. Fig. 4 illustrates the results which were obtained by this technique. It is apparent that the aerobic xanthine oxidase reaction did cause the accumulation of some compound, which was capable of rapidly reducing cytochrome *c*. Since the reduction of cytochrome *c* by native xanthine oxidase is due to O_2^- [13], this is a clear indication that O_2^- does accumulate for several min during the xanthine oxidase reaction.

The initial burst of cytochrome *c* reduction, which is a measure of the concentration of O_2^- at the moment of mixing, was measured as a function of the time of preincubation under varying conditions. Fig. 5 demonstrates that the concentration of O_2^- continued to rise for several min and that both the rate of accumulation and the steady-state concentration finally achieved were a function of the concentration of xanthine oxidase. Indeed, the concentration of O_2^- after 2 min of incubation was a linear function of the concentration of xanthine oxidase. Line 4 in Fig. 5 demonstrates that 0.03 mM H_2O_2 markedly decreased the steady-state level of O_2^- which

could accumulate in the xanthine oxidase reaction. This level of H_2O_2 did not significantly affect the linear rate of reduction of cytochrome *c*, which followed the initial burst. The initial burst of cytochrome *c* reduction was exquisitely sensitive to superoxide dismutase. Thus 0.005 μg/ml of bovine erythrocyte superoxide dismutase inhibited this burst by 100 %, while it diminished the linear rate of reduction of cytochrome *c* by only 13 %. Catalase, at a level of 75 units/ml, had little effect on the accumulation of O_2^- during the xanthine oxidase reaction. While the data shown in Fig. 4–6 were obtained with acetaldehyde, entirely similar results were seen when xanthine was used as the substrate for xanthine oxidase.

DISCUSSION

The relative stability of O_2^- at elevated pH [14] makes it possible for this radical to accumulate at pH 10.2 during the xanthine oxidase reaction. The steady-state level of O_2^- acheived depends upon a balance between its rate of production and its rate of consumption. As shown in Fig. 5, this balance between the enzymatic production and the spontaneous dismutation allows 1.35 μM O_2^- to accumulate during the first 10 min of the action of $1.8 \cdot 10^{-8}$ M xanthine oxidase upon 10 mM acetaldehyde at pH 10.2. This accumulation of O_2^- and its interaction with H_2O_2 fully account for the kinetics of luminol luminescence. Thus the steady-state level of O_2^- was diminished by the presence of 0.03 mM H_2O_2. This effect shown in Fig. 5, is another indication of the Haber-Weiss reaction in which O_2^- and H_2O_2 react, either directly or under the catalytic influence of trace metals, to yield OH^-, $OH^\cdot$ and O_2. Thus, as the xanthine oxidase reaction progresses the concentrations of both O_2^- and of H_2O_2 rise and therefore the rate of production of $OH^\cdot$, by the Haber-Weiss reaction, also increases. The following reactions then lead to a progressively brighter luminescence:

(A) $LH^- + OH^\cdot \rightarrow LH^\cdot + OH^-$

(B) $LH^\cdot + O_2^- \rightarrow LOOH^- \rightarrow N_2 + AP^* \dashrightarrow AP + h\nu$

(C) $LH^- + H_2O_2 \rightarrow LH^\cdot + OH^\cdot + OH^-$

In reaction (A) the luminol monoanion is oxidized to a luminol radical by $OH^\cdot$. In reaction (B) the luminol radical reacts with superoxide anion to yield an adduct which decomposes to yield electronically excited aminophthalate which, in its return to the ground state, luminesces. Since O_2^- is needed both for the Haber-Weiss reaction, which generates the $OH^\cdot$ and for reaction (B) which makes the adduct, superoxide dismutase inhibits luminescence. H_2O_2 augments luminescence because it is needed for the Haber-Weiss reaction and for reaction (C) but it does not eliminate the time-dependent increase in luminescence because the other reactant, i.e. O_2^-, is still accumulating to a steady-state. Furthermore, catalase did not entirely eliminate luminescence because O_2^- can itself act as an oxidant towards luminol, albeit less effectively than the $OH^\cdot$ which can be made in the presence of H_2O_2. Thus:

(D) $LH^- + O_2^- + 2H^+ \rightarrow LH^\cdot + H_2O_2$

Clearly it is not necessary to postulate the accumulation of a new chemical species, such as $H_2O_4^-$, in order to fully account for the kinetics of luminescence of the xanthine oxidase-luminol system, since the accumulation of O_2^- itself serves to explain this process.

When the xanthine oxidase reaction was generating O_2^- at a rate of $4.7 \cdot 10^{-7}$ M/min, as judged from the linear rate of reduction of cytochrome *c*, it accumulated O_2^- to $3.4 \cdot 10^{-7}$ M as judged from the maximum burst of cytochrome *c* reduction. If we equate the rate of production of O_2^- to a second order decay process at the plateau then $4.7 \cdot 10^{-7}$ M $min^{-1} = k\ (3.4 \cdot 10^{-7}\ M)^2$ and $k = 6.8 \cdot 10^4\ M^{-1}\ sec^{-1}$. This is much greater than the value of approximately $2 \cdot 10^2\ M^{-1}\ s^{-1}$ calculated for this pH from the data given by Behar et al. [14]. This indicated that the dismutation of O_2^- by impurities in our reagents was an important factor in limiting what would otherwise have been a markedly greater accumulation of O_2^-.

The assays which are routinely used for assaying superoxide dismutase depend upon a competition between superoxide dismutase and some indicating scavenger for O_2^- such as cytochrome *c* [13], tetranitro-methane [13], epinephrine [15], pyrogallol [16], or sulfite [17]. These assays vary in sensitivity depending upon the rate of reaction of O_2^- with the indicating scavenger, being more sensitive the slower this reaction. It now appears that an assay of ultimate sensitivity could be devised, based upon the ability of superoxide dismutase to inhibit the burst of cytochrome *c* reduction shown in Fig. 4–6, since in this case the superoxide dismutase would be in competition only with adventitious impurities. An indication of the sensitivity which could be achieved is given by the observation that 0.005 μg/ml of superoxide dismutase completely inhibited the burst of cytochrome *c* reduction.

ACKNOWLEDGEMENTS

This work was supported in full by research grants GM-10287 from the National Institutes of Health, Bethesda, Maryland and RDRP-IP-12410-L from the United States Army Research Office, Durham, North Carolina.

REFERENCES

1 Hodgson, E. K. and Fridovich, I. (1973) Photochem. Photobiol. 18, 451–455
2 Michelson, A. M. (1973) Biochimie. 55, 465–479
3 Totter, J. R., Medina, V. J. and Scoseria, J. L. (1960) J. Biol. Chem. 235, 238–241
4 Greenlee, L., Fridovich, I. and Handler, P. (1962) Biochemistry 1, 778–783
5 McCord, J. M., Beauchamp, C. O., Goscin, S., Misra, H. P. and Fridovich, I. (1973) in Oxidases and Related Redox Systems (King, T. E., Mason, H. S. and Morrison, M., eds.), pp. 51–73, University Park Press, Baltimore
6 Hodgson, E. K. and Fridovich, I. (1975) Biochemistry 14, 5294–5303
7 Keele, B. B., McCord, J. M. and Fridovich, I. (1970) J. Biol. Chem. 245, 6176–6181
8 Waud, W. R., Brady, F. O., Wiley, R. D. and Rajagopalan, K. V. (1975) Arch. Biochem. Biophys. 169, 695–701
9 Halliwell, B. (1973) Biochem. J. 135, 379–381
10 Beers, Jr., R. F. and Sizer, I. W. (1952) J. Biol. Chem. 195, 133–140
11 Mitchell, G. W. and Hastings, J. W. (1971) Anal. Biochem. 39, 243–250
12 Yankeelov, Jr., J. A. (1963) Anal. Biochem. 6, 287–289
13 McCord, J. M. and Fridovich, I. (1968) J. Biol. Chem. 244, 6049–6055
14 Behar, D., Czapski, G., Rabani, J., Dorfman, L. M. and Schwarz, H. A.(1970) J. Phys. Chem. 74, 3209–3213
15 Misra, H. P. and Fridovich, I. (1972) J. Biol. Chem. 247, 3170–3175
16 Marklund, S. and Marklund, G. (1974) Eur. J. Biochem. 47, 469–474
17 McCord, J. M. and Fridovich, I. (1969) J. Biol. Chem. 244, 6056–6063