ON THE FORMATION OF THE SUPEROXIDE ANION RADICAL DURING THE REACTION OF REDUCED IRON-SULFUR PROTEINS WITH OXYGEN*

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The formation of a free radical species, having the characteristics of the superoxide anion radical, (O_2^-), during aerobic catalysis involving milk xanthine oxidase, is confirmed. In the presence of erythrocuprein, which has been shown to catalyze dismutation of O_2^-, no radical was observed. Flavin-depleted xanthine oxidase, although reducible by xanthine, did not produce O_2^- radicals under conditions where native enzyme did. Similarly, on reoxidation of a number of flavin-free iron-sulfur proteins of the plant ferredoxin type, no O_2^- radicals were detected. With ferredoxin from Clostridium pasteurianum, however, O_2^- radicals were observed.

Knowles, Gibson, Pick and Bray (1) recently brought forth convincing evidence that a free radical form of oxygen, namely the superoxide anion, O_2^-, is formed in substantial quantity when xanthine oxidase catalyzes the oxidation of xanthine by molecular oxygen at elevated pH (pH 10.5). Previous studies on the initiation of sulfite oxidation (2), the production of luminescence in the presence of suitable organic substances (3, 4) and more directly, those on the chemiluminesence of oxygen itself (5) during the xanthine oxidase reaction, had strongly suggested that oxygen free radicals are involved in this enzymatic process. Handler and his colleagues (6) had made a survey of oxidizing enzymes which initiated sulfite oxidation and came to the conclusion that among flavoproteins only those containing non-heme iron - which are now known to contain the characteristic groupings of iron-sulfur proteins - are effective initiators. These observations, taken together, raise the question,

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whether the formation of O_2^- on interaction of the reduced form with O_2 may be a general property of iron-sulfur proteins or may in some way also be related to the flavoprotein character of these enzymes. The availability in our laboratory of a number of iron-sulfur proteins (cf. Table I), which do not contain flavin, prompted us to study this question. An additional stimulus was the possibility - opened up by recent work in two laboratories (7)[1] - of studying flavin-free xanthine oxidase with respect to oxygen radical formation.

Our results are summarized in Figures 1 and 2 and Table I. Figure 1 shows that we have fully confirmed the results of Knowles et al. (1). The EPR signal observed by these investigators with $g_{\parallel} = 2.08$ and $g_{\perp} = 2.00$ and attributed to the O_2^- radical was readily seen on oxidation of a dithionite solution (Fig. 1 A) and of xanthine oxidase previously reduced by dithionite (Fig. 1 B), and on reaction of xanthine with xanthine oxidase in the presence of oxygen (Fig. 1 C), all at pH 10.5.

In addition we have now seen the characteristic EPR signal - albeit at diminished intensity - on reaction of xanthine with xanthine oxidase in the presence of oxygen at pH 8 (Fig. 1 D), which is within the pH range in which most studies on this enzyme have been carried out in the past. Figure 1 E shows that the same signal was obtained when ferredoxin from Clostridium pasteurianum, previously reduced with dithionite, was reoxidized with oxygen[2]. In those instances when dithionite was used as reductant, the quantity added to the protein was such (cf. legend to Table I and Fig. 1) that the radical observed by EPR could not have been due to the oxidation of dithionite as in Figure 1 A. In contrast to these findings, on reoxidation of all other iron-sulfur proteins listed in Table I, after these proteins had been reduced with dithionite, we could not detect the formation of the typical EPR signal attributed to O_2^- [2].

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1 Unpublished experiments by M. Uozumi, L. H. Piette, W. H. Orme-Johnson, R. E. Hansen, and H. Beinert.

2 The formation of the O_2^- radical on reoxidation of a different bacterial ferredoxin (Clostridium welchii) and the failure of spinach ferredoxin to produce O_2^- under similar conditions has also been shown by R. Nilsson, F. M. Pick and R. C. Bray, submitted to Biochim. Biophys. Acta.

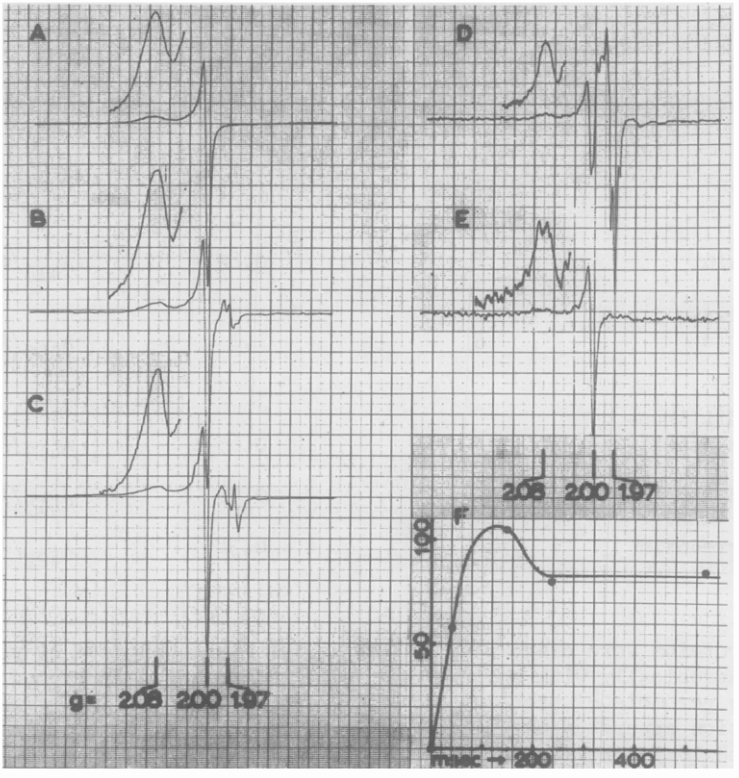

FIGURE 1: A - E, EPR spectra observed 150 msec after mixing various reducing mixtures with an equal volume of oxygenated buffer. A. 0.31 mM sodium dithionite in 0.01 M potassium phosphate of pH 7.4 was mixed with 1 M glycine buffer of Ph 10.5. B. 0.053 mM xanthine oxidase in 0.01 M pyrophosphate of pH 8.5 was reduced anaerobically with sodium dithionite, corresponding to 14 eq per mole of enzyme, and mixed and frozen as in A. The xanthine oxidase used in the experiments depicted here was prepared according to Hart and Bray (13)[3], and was the sample more fully described in (15). The activity had declined approximately 20% from the value quoted for the best preparations (13). C. 0.025 mM xanthine oxidase dissolved as in B was mixed with oxygenated glycine buffer as in A, which contained 2 mM xanthine. D. Enzyme as in C was mixed with 0.05 M pyrophosphate buffer of pH 8.0, which contained 2 mM xanthine and was equilibrated with oxygen gas. E. 0.40 mM ferredoxin from Clostridium pasteurianum (A_{390}/A_{280} = 0.78) dissolved in 0.01 M potassium phosphate buffer of pH 7.4 and reduced anaerobically with sodium dithionite corresponding to 1 eq per mole of protein, was mixed with glycine buffer and frozen as in A. The conditions of EPR spectroscopy were: microwave power, 45 mwatt; modulation amplitude, 2 gauss; scanning rate, 200 gauss per min; time constant, 0.5 sec; and temperature, 102° K. The enlarged low field portion at g = 2.08 was recorded at an amplification 4 fold higher than that used for the complete spectra and at 6 gauss modulation amplitude. The relative amplification used for recording spectra A - E were: 1, 2, 1.25, 3.2, and 8. At g = 1.97 signals of Mo(V) are seen with xanthine oxidase, particularly at the lower pH (D). Under the experimental conditions used flavin and Mo signals are partly saturated. The signal observed at g = 2.00 in D has contributions from flavin semiquinone, Mo(V) and O_2^-, whereas in B and C this signal is largely due to O_2^-. F shows the time course of formation of the O_2^- radical in the experiment with clostridial ferredoxin (E). The ordinate refers to signal amplitude in arbitrary units.

3 As recently modified by I. Hart, M. A. McGartoll, and R. C. Bray, submitted to Biochem. J.

TABLE I

Iron sulfur proteins tested for O_2^- production with negative results.

Protein*	Reference	Concentration mM	Purity Criteria
Putidaredoxin	8	0.82	$A_{415}/A_{278} = 0.36$
C. pasteurianum paramagnetic protein	9	1.2	$A_{550}/A_{280} = 0.29$
A. vinelandii iron-sulfur protein II	10	0.65	$A_{419}/A_{280} = 1.0$
Pig adrenodoxin**	11	0.72	$A_{415}/A_{280} = 0.85$
Spinach ferredoxin	12	0.38	$A_{422}/A_{280} = 0.45$

* All proteins were dissolved in 0.01 M potassium phosphate of pH 7.4 and were reduced anaerobically with solid-diluted dithionite (18) corresponding to 0.9 reducing eq per mole of protein. After anaerobic transfer into syringes of the rapid reaction apparatus they were mixed with an equal volume of 1 M glycine buffer of pH 10.5, previously equilibrated with oxygen gas at 22 - 25° C. Samples were frozen 0.15 and 1.5 seconds after mixing.

** This experiment was carried out both in the presence and in the absence of 7 μM methylviologen.

It is of interest that all of these proteins contain only two iron atoms per molecule, which are presumably in equivalent environments (14), whereas xanthine and aldehyde[4] oxidases as well as Clostridial ferredoxin have been shown to contain iron in at least two different environments (15). We conclude from these experiments that the concentration of O_2^- formed from the iron-sulfur proteins listed in Table I, under the conditions of our experiments, must have been at least an order of magnitude lower than that produced with clostridial ferredoxin, if O_2^- was formed at all. If the concentration of O_2^- formed depended on the number of iron atoms present, then we would not expect, according to our results on the clostridial ferredoxin, that the g_1 absorption should have been detectable at the concentrations of protein used in most instances. We

4 W. H. Orme-Johnson and H. Beinert, unpublished.

should have found the much stronger $g_\perp$ absorption, had O_2^- been formed, however.

Figure 2 shows results of experiments on the formation of O_2^- by xanthine oxidase depleted of flavin. In the experiment of Figure 2 A the depleted enzyme was reconstituted with flavin and rapidly mixed with xanthine under the conditions of Figure 1 A. Molybdenum, iron (not shown) and O_2^- signals were observed as with native enzyme showing that the flavin-depleted protein had remained viable. The flavin-depleted enzyme itself, however, did not produce any detectable O_2^- radicals, although iron and molybdenum signals appeared on addition of xanthine (Fig. 2 B) as had been shown before (7)[1]. The experiment of Figure 2 C was patterned after that of Figure 1 B. The flavin-depleted protein was reduced with dithionite and then mixed with oxygenated buffer. No O_2^- radical was detected.

The experiments of Figure 2 D and E adduce additional support for the assignment of the assymmetric EPR signal at $g_\parallel = 2.08$, $g_\perp = 2.00$ to the O_2^- radical. In the experiment of Figure 2 E erythrocuprein, lately shown by McCord and Fridovich (19) to contain a powerful superoxide dismutase activity, was present in xanthine oxidase under O_2^--forming conditions. Figure 2 D depicts the results of mixing xanthine oxidase with 2 mM xanthine at pH 10.5, and Figure 2 E shows the results of a repetition of this experiment in the presence of erythrocuprein. The inclusion of erythrocuprein in the mixture completely abolished the signal of the O_2^- radical, while the signals attributable to Mo(V) and iron (not shown) were essentially unchanged. This strongly supports the concept that the signal with $g_\parallel = 2.08$, $g_\perp = 2.0$ is indeed due to the superoxide anion.

The results described in the present paper confirm that xanthine oxidase does produce superoxide radicals in the course of aerobic oxidation of xanthine, suggest that the simpler iron-sulfur proteins are autoxidized at a sufficiently slow rate that superoxide is either not produced at all or (as in the case of clostridial ferredoxin) at a low level, and support the conclusion drawn by Komai *et al.* (7), namely, that in metalflavoproteins which produce O_2^- in the course of aerobic catalysis, the flavin functional group is essential to this activity.

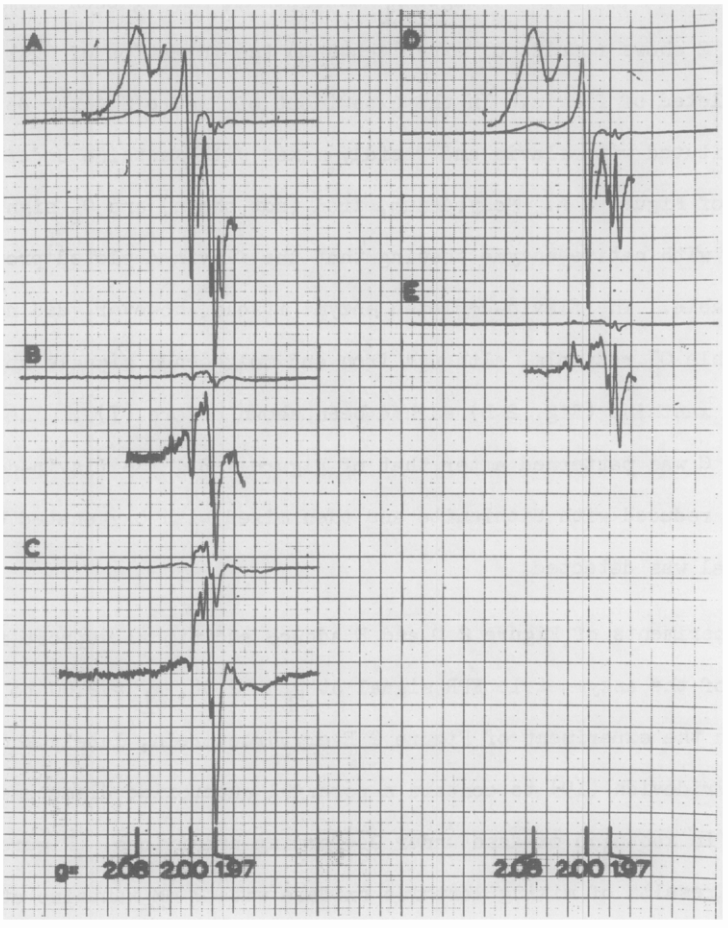

FIGURE 2: EPR spectra observed 150 msec after mixing various reducing mixtures with an equal volume of oxygenated buffer. In A - C flavin-depleted or reconstituted xanthine oxidase was used, in D and E native enzyme with and without erythrocuprein. A. Xanthine oxidase prepared by the procedure of Massey et al. (16) with an AFR (23.5° C) of 90 was treated according to Komai (7) to remove flavin. The protein so obtained had a spectrum evidently identical to that reported by Komai et al. and had a flavin content of 0.1 nmole per mg according to the fluorometric method of Burch (17)[3] while the undepleted xanthine oxidase showed a flavin content of 5.8 nmole per mg. by the same procedure. The enzyme was reconstituted with FAD according to Komai et al. (7) and the resulting protein was separated from most of the excess FAD by ammonium sulfate precipitation. The protein was dissolved in 0.01 M pyrophosphate, pH 8, to give an enzyme concentration of 0.025 mM. This solution was then mixed with an equal portion of 2 mM xanthine in 1 M glycine buffer of pH 10.5, saturated with O_2 at 25° C, and frozen after 0.15 sec. B. Flavin-depleted xanthine oxidase as in A (but not reconstituted) and at a concentration of 0.025 mM, based on a molar absorptivity (450 mμ) of 50,000 $M^{-1} \times cm^{-1}$ (7). C. Flavin-depleted xanthine oxidase as in B was reduced anaerobically with 16 electron eq of solid diluted sodium dithionite (18) per mole of enzyme, and subsequently mixed with an equal portion of 1 M glycine buffer, pH 10.5, saturated with O_2 at 25° C. D. Xanthine oxidase (16) at a concentration of 0.1 mM, in 0.01 M pyrophosphate, pH 8, was mixed with an equal volume of 2 mM xanthine in 1 M glycine, pH 10.5, saturated with O_2 at 25° C. E. Repetition of the experiment of D in the presence of

erythrocuprein of high purity (0.35% Cu). The concentration of erythrocuprein after mixing corresponded to 0.24 µg of copper per ml. The conditions of EPR spectroscopy were as in Fig. 1. The main spectra shown directly below the capital letters A - E represent a 1000 gauss scan at amplification suitable for demonstration of the O_2^- radical. The molybdenum signals at g = 1.97 are shown below the main curves at an increased amplification (x 10 for A, B, D, and E, and x 4 for C).

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