The Chemistry of Xanthine Oxidase

8. ELECTRON-SPIN-RESONANCE MEASUREMENTS DURING THE ENZYMIC REACTION*

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Anaerobically, xanthine reduces the molybdenum, the flavin and probably also some of the iron of xanthine oxidase (Bray, Pettersson & Ehrenberg, 1961). In order to demonstrate that these changes are essential for the enzymic reaction, it is necessary to show that they occur at rates compatible with the overall kinetics of the enzymic reaction; the recent development of rapid-reaction techniques for electron-spin-resonance measurements has now made this feasible. This paper describes the application of the rapid-freezing technique (Bray, 1961) to the detection of enzyme intermediates during the enzymic oxidation of xanthine by molecular oxygen. Measurements, under conditions which would be expected to minimize interference by 'inactive' forms of the enzyme, have been made, both during the approach to and the decay from the 'steady state'; they provide strong evidence that the molybdenum, the flavin and possibly also the iron participate in the electron-transport processes of the enzyme and throw further light on the mechanism of the reaction.

EXPERIMENTAL

Xanthine-oxidase solutions. The samples, which were similar to those used by Bray *et al.* (1961), were dialysed against pyrophosphate buffer (pH 8·0–8·2) before use; calculations of the percentages of the different forms of the enzyme were made as described by Bray *et al.* (1961). Two samples, designated UXO-37 and UXO-43, were used for most of the experiments; they contained about 50 and 40% respectively of xanthine oxidase-*a*.

Rapid-reaction experiments. Resting xanthine-oxidase and oxygen-saturated solutions of xanthine were allowed to react together, from separate syringes, by the techniques described by Bray (1961) and Bray & Pettersson (1961). The reaction was quenched by squirting the reaction mixture into *iso*pentane at about 130°K. Electron-spin-resonance measurements were made at 77°K. No evidence of changes in the signals during storage of the samples in

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liquid nitrogen was observed; the time intervals from squirting to measuring ranged from about ¼ to 20 hr. The spectrometer was standardized with a solution of copper–ethylenediaminetetra-acetic acid complex and the signals were measured as described by Bray *et al.* (1961). Corrections for variations in the tube diameters and also, where necessary, for incomplete coverage of the cavity by the packed sample, were applied.

The procedure was apparently without any harmful effects on the enzyme. On thawing out the frozen samples under *iso*pentane, no signs of precipitation could be detected; the ratio activity/E_{450} after thawing was at least 90% of that of the starting solution in measurements on UXO-37.

RESULTS

Electron-spin-resonance signals of varying intensities were obtained under all the conditions tested. Fig. 1 shows a typical trace, in which the signals, previously designated as *A*, *B* and *C* and attributed respectively to the flavine–adenine dinucleotide free radical (FADH), Mo(v) and perhaps Fe (II) (Bray *et al.* 1961), are clearly visible. In addition, weak signals (marked as *C′* in Fig. 1) are visible on the low-field side of signal *A*; like signal *C*, these resembled components of the unknown signal of Beinert & Sands (1960; see particularly their Fig. 3*e*), and presumably the two signals are closely related to one another. Signals *C* and *C′* could be distinguished clearly in almost all the rapid-reaction experiments that have been carried out, including some at short reaction times, but quantitative measurements of their intensities were not attempted.

Figs. 2–4 show the variations in the intensity of the Mo(v) signal as a function of the reaction time for the two preparations of xanthine oxidase. In Figs. 2 and 3 xanthine was present at a concentration comparable with that of the enzyme and the oxygen was present in excess. In Fig. 4 the substrate was present in excess over both enzyme and oxygen.

The striking difference between the two preparations (Figs. 2 and 3) was unexpected, since the concentrations of the enzyme, substrate and electron
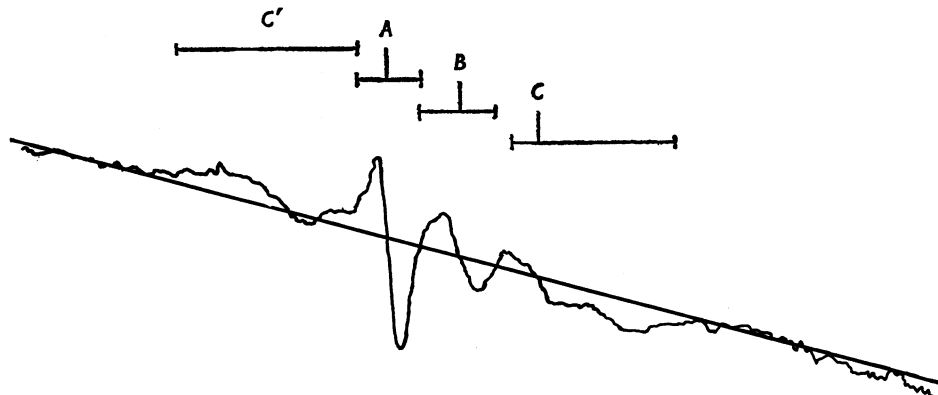
Fig. 1. Electron-spin-resonance curve (first derivative) for a sample of xanthine oxidase (UXO-43) frozen 100 msec. after mixing with oxygenated xanthine. For meaning of A, B, C, C' see text.
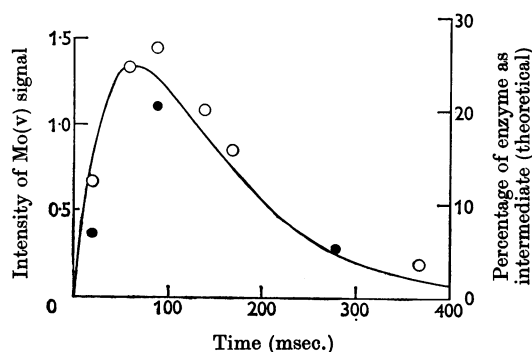

Fig. 2. The intensity of the Mo(v) signal (signal B) for samples of xanthine oxidase (UXO-43) frozen at various times after mixing with oxygenated xanthine at 24°. The signal-intensity units are arbitrary but are approximately the same and refer to the same concentration of total xanthine oxidase in Figs. 2–4. The concentrations immediately after mixing were: total xanthine oxidase, 0·08 mM; xanthine, 0·06 mM; oxygen, approx. 0·6 mM. The open (O) and full (●) circles refer to two different series of experiments; the theoretical curve was calculated as described in the Discussion from the velocity constants of Gutfreund & Sturtevant (1959).
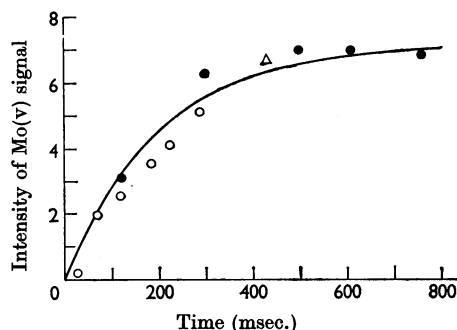
Fig. 3. As for Fig. 2, but with UXO-37 (concentration of total xanthine oxidase, 0·11 mM), at 30°. The theoretical curve was calculated for a unimolecular reaction with $k = 5$ sec.$^{-1}$ △; O and ● refer to different series of experiments.

acceptor were similar, as also were the compositions of the enzyme samples. Further, the histories of the two samples were almost identical; possibly the most significant difference was that the dialysis of UXO-37 before use was less exhaustive than that given to UXO-43. Since it was possible that the difference between the preparations might be due to an inhibitor in UXO-37, the effect of substrate concentration on the enzymic activities was investigated, with xanthine as substrate and oxygen as electron acceptor (Bray, 1959). In the presence of about 0·03 μM-xanthine oxidase, reaction rates were maximal at about 0·05 and 0·15 mM-xanthine

respectively for UXO-43 and UXO-37. This difference is strong evidence for the presence of an inhibitor in UXO-37 which is absent from UXO-43 (Bray, 1959).

In addition to the Mo(v) signals, free-radical signals were also observed in all the rapid-reaction runs. In general, the form of the free radical/time curves followed the corresponding molybdenum curves fairly closely. The approximate average free radical/Mo(v) ratios for the points in Figs. 2–4 were respectively 1·0, 0·1 and 0·1 (these ratios have been corrected to correspond to equal areas under the absorption curves and therefore approximate to molecular ratios). The maximum observed concentrations of Mo(v) calculated for the experiments in Figs. 2–4 corresponded respectively to about 0·02, 0·1 and 0·1 g.atom/mole of total xanthine oxidase. Since the Mo(v) signals, particularly for UXO-43, were thus relatively weak, it was thought advisable to check that added inorganic molybdenum
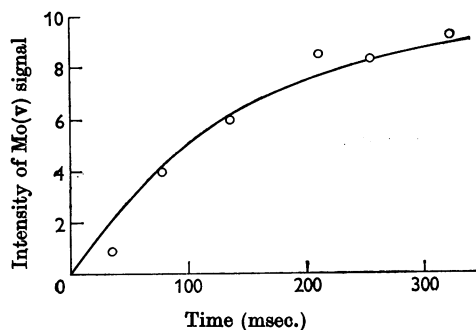
Fig. 4. As for Fig. 3, but with the xanthine concentration increased to 1·9 mM. The theoretical curve was calculated for a unimolecular reaction with $k = 7$ sec.$^{-1}$

did not give signals under the conditions of the experiments. Accordingly a sample of the enzyme was incubated with 1 mol.prop. of sodium molybdate for two days at 5° before running fast-reaction experiments side by side with runs on the untreated enzyme. The fairly weak signals obtained appeared identical in the control and treated samples.

Further evidence that the observed electron-spin-resonance signals are related to the enzymic activity and not to artifacts is provided by the observation that, after thawing samples for a few minutes and then refreezing, signals could no longer be detected. This observation was made with UXO-37, by using an excess of oxygen over xanthine.

DISCUSSION

Gutfreund & Sturtevant (1959) used rapid spectrophotometric techniques to follow changes in the absorption at 450 mμ during the xanthine oxidase-catalysed oxidation of xanthine by molecular oxygen. They concluded that at 25° a rapid combination of enzyme and substrate was followed by a unimolecular reduction with $k_{red.}$ 10·5 sec.$^{-1}$, the reduced enzyme then combining rapidly with oxygen and being reoxidized with a unimolecular constant, $k_{ox.}$, of 21·5 sec.$^{-1}$

If it is assumed that there are two active centres/mole of xanthine oxidase-a (Bray et al. 1961), then in Figs. 2 and 3 the concentration of enzyme active centres was the same as, or slightly greater than, the substrate concentration; further, the electron acceptor was present in excess. No true steady state would be expected under these conditions; if the Gutfreund & Sturtevant mechanism is accepted, the reaction would be represented simply by two successive unimolecular reactions:

$$\text{ES} \xrightarrow{k_{red.}} \text{EH} \xrightarrow{k_{ox.}} \text{E},$$

where ES is the enzyme–substrate complex, EH is a reduced form of the enzyme and E is the resting, reoxidized enzyme. For such a system, the concentration of the reduced form, [EH], can readily be plotted as a function of time if the velocity constants and the initial concentration of the enzyme–substrate complex $[ES]_0$ are known (Frost & Pearson, 1956). With Gutfreund & Sturtevant's velocity constants, the maximum concentration of EH should occur at 65 msec. with

$$[EH]/[ES]_0 = 25\%$$

at this point. The theoretical curve calculated in this manner is shown in Fig. 2 (solid line), with an arbitrary concentration scale. Considering the relatively low precision of the measurements on the rather weak Mo(v) signals from UXO-43 and the fact that the velocity constants were obtained by a totally different technique in another Laboratory, the agreement between the experimental points and the theoretical curve is surprisingly good; it provides strong evidence that the Mo(v) signals in this instance are associated with the reduced form of the enzyme.

As stated above the experimental maximum in Fig. 2 corresponded to about 0·02 g.atom of Mo(v)/mole of total xanthine oxidase. It was reported by Bray et al. (1961) that the peak signals associated with the reduced form of xanthine oxidase-a, designated 'EH$_n$', corresponded in one experiment to 0·23 g.atom of Mo(v)/mole of total enzyme. Thus if it is assumed that the molybdenum signals arise, as previous work would lead one to expect, from this 'first reduced level' of the enzyme, 'EH$_n$', then the maximum proportion of the active enzyme in this form in Fig. 2, after correction for the differing contents of xanthine oxidase-a in the two experiments, would be $100 \times 0·02/0·23 \times 60/40$, i.e. 13%. As shown above, the theoretical figure from Gutfreund & Sturtevant's constants is 25%. Since the calculations are approximate, the agreement between the experimental and theoretical values may be regarded as satisfactory.

Since for UXO-43 the data on the Mo(v) signal are entirely consistent with the metal's participating in the electron transport, it is all the more surprising that, for UXO-37, a very similar sample which was examined under similar conditions, the signal, instead of showing a maximum in the region of 65 msec., continued to rise up to about 400 msec. and then remained constant for at least a further 400 msec. (Fig. 3). The plateau corresponded to about 0·1 g.atom of Mo(v)/mole of total xanthine oxidase, which is very roughly what would be expected if all of the xanthine oxidase-a was in the 'first reduced level'. Thus it seems that here, for some reason, reoxidation of the enzyme is not taking place, or rather, since the signals disappeared

on thawing and refreezing, that reoxidation is taking place only slowly. Gutfreund & Sturtevant (1959) also observed that reoxidation of the reduced enzyme at the end of a steady-state period was slow, but they interpreted this as being due to hydrogen peroxide formed in the reaction. A negligibly low rate of reoxidation in the present fast-reaction experiments is not necessarily inconsistent with the observed high activity in the ordinary assay procedure, since the fast-reaction work was done at an abnormally high enzyme concentration. At such concentrations, the presence of endogenous inhibitors, which could be almost without effect in the assay of the xanthine oxidase-*a* content of UXO-37, might stop the reaction almost completely (Bray, 1959).

The proposition that for UXO-37 the plateau in Fig. 3 represents complete reduction of xanthine oxidase-*a* to the first reduced level is strengthened by the fact that almost exactly the same curve was obtained when xanthine was in excess over oxygen (Fig. 4). Further, the rate of appearance of the Mo(v) signal was not greatly affected by variations in substrate concentration (Figs. 3, 4). This is consistent with the rate-limiting step's being reduction of the enzyme–substrate complex, as the theory requires.

It was stated by Bray *et al.* (1961) that xanthine oxidase-*a* can be reduced to a 'second reduced level' designated as 'EH_{n+m}', which did not give electron-spin-resonance signals. However, since all the results in the present paper can be explained satisfactorily in terms of reduction to the first level only, no further evidence on this can be deduced. Since, in the presence of excess of xanthine (Fig. 4), the signals showed no signs of decreasing, up to 300 msec. at 30°, reduction to this second level must be a relatively slow process, although previous work showed it to be complete within 2 min. at 4° (Bray *et al.* 1961). Possibly sluggishness in the reactions of the second reduced level could account for the well-known phenomenon of inhibition of xanthine-oxidase activity by excess of substrate.

No explanation has been given here for the variations in the free radical/Mo(v) ratio, and the nature of the inhibitor postulated to explain the batch variations is still unknown. Further, the weakness of the Mo(v) signals during the reaction was explained by equilibria within the 'first reduced level' (Bray *et al.* 1961), but the nature of the equilibria is still not understood. Nevertheless, the appearance and disappearance of molybdenum signals has been correlated with published kinetic data and the signals were obtained with an excess of enzyme over substrate, by using high concentrations of the electron acceptor, i.e. under conditions that should preclude interference by 'inactive' forms of the enzyme. Incubation of the enzyme with inorganic molybdate before the experiments had no influence on the results. It is concluded that the results represent the first direct evidence for a redox function for the molybdenum of xanthine oxidase. Since signals believed to arise from iron reduction were also observed, this metal may also participate in the electron transport. No evidence on the possible sequence of the reduction of the metals and flavin of xanthine oxidase is at present available.

SUMMARY

1. Electron-spin-resonance signals obtained from xanthine oxidase during the enzymic oxidation of low concentrations of xanthine by molecular oxygen have been studied with a fast-reaction technique.

2. Two preparations of xanthine oxidase were examined. For one, the kinetics of the appearance and disappearance of the Mo(v) signal correlated well with the known kinetics of the overall reaction, thus providing the first direct evidence for the participation of the metal in the electron-transport processes of the enzyme. Reoxidation of the second sample at the high enzyme concentrations needed for electron-spin-resonance measurements was slow; this may have been due to the presence of an inhibitor.

3. The detection of signals possibly arising from the reduction of iron may indicate a redox function for this metal in addition to flavin and molybdenum in xanthine-oxidase action.

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