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Properties of xanthine oxidase preparations dependent on the proportions of active and inactivated enzyme

Recent work from this laboratory[1] has confirmed earlier conclusions (refs. 2, 3; *cf.* ref. 4) that milk xanthine oxidase (xanthine:O_2 oxidoreductase, EC 1.2.3.2) as normally prepared is a mixture of the active enzyme with two inactive forms. These are de-molybdo xanthine oxidase, a natural constituent of milk which may be removed by selective denaturation and inactivated xanthine oxidase, a preparation or storage artefact. We now demonstrate that certain properties of xanthine oxidase samples are a function of their contents of active and inactivated enzyme. Though PALMER AND MASSEY[5] and MASSEY *et al.*[6] earlier questioned the existence of inactivated xanthine oxidase, they have since presented[7] new evidence for it and proposed a

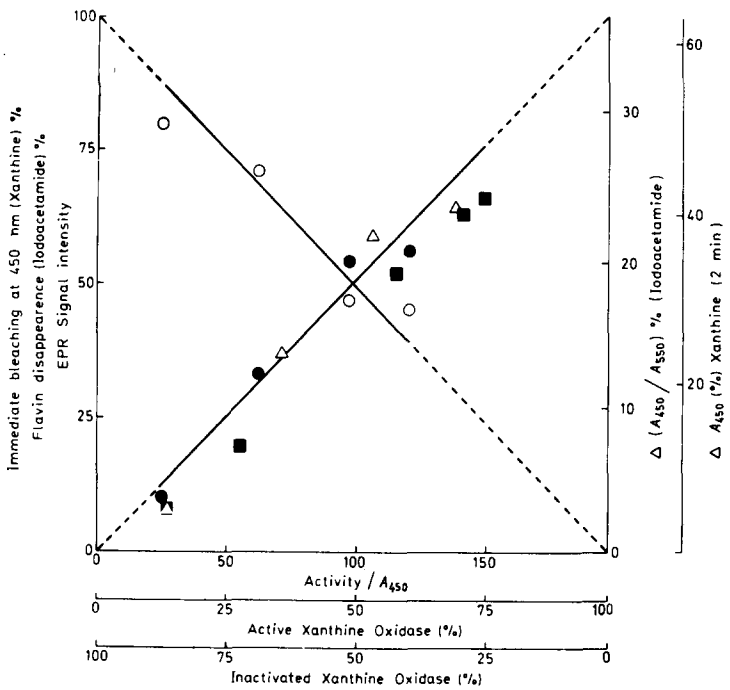

Fig. 1. Properties of xanthine oxidase samples as a function of their activity (23.5°)/$A_{450\ nm}$ ratios. Measurements of four parameters, each on several different enzyme samples are summarised. Relative intensity in arbitrary units of the Slow Mo(V) EPR signal[10,11] (○) was measured after 20-min reduction at about 20°, pH 8.2, by 25 moles dithionite. The Rapid Mo(V) EPR signal[10,11,13] (●) was measured after 1.5-min reduction at the same pH and temperature by 2.0 moles xanthine. "Immediate" anaerobic 450-nm bleaching (■) was measured after 2 min at about 22° and pH 8.2 with about 7 μM xanthine oxidase and 0.2 mM xanthine. The limiting value for complete bleaching was $\Delta A_{450\ nm} = 63\%$ (attained after 40 h by a sample with activity/$A_{450\ nm} = 149$). Inactivation of enzyme (0.02 mM) with iodoacetamide (1.0 mM) was at 25°, pH 5.6, in the presence of xanthine (0.2 mM) for 40 min. Reaction was started by adding enzyme last and stopped by adding excess of cysteine. 92–95% inactivation was achieved. FAD disappearance from the products was estimated (△) from $A_{450\ nm}/A_{550\ nm}$, assuming complete flavin disappearance corresponded to 37% decrease of this ratio[16].

method of quantification based on reaction with iodoacetamide. The present work is an extension of our own and of the findings of MASSEY *et al.*

Enzyme samples were prepared by the 0.1 M salicylate denaturation method (Step 2(a)ii of ref. 1) and hence were low in the de-molybdo form. Partial inactivation was achieved[8] by incubation, *e.g.* at 37° in 0.1 M phosphate (pH 6.2). Samples were dialysed and centrifuged before use and had FAD/Mo about 1.1 (fluorimetric determination[1]) and an $A_{450\ nm}/A_{550\ nm}$ ratio of 3.28 ± 0.09. Xanthine oxidase activity was expressed as activity/$A_{450\ nm}$ at 23.5° (ref. 1). Improved anaerobic techniques using argon containing <1 ppm oxygen (*cf.* ref. 9) were employed.

In Fig. 1 results of four types of measurement on xanthine oxidase samples of differing specific activities are expressed as a function of activity/$A_{450\ nm}$. In the absence of de-molybdo enzyme this ratio is directly related to relative amounts of active and inactivated enzyme[1]. Three parameters show a clear positive correlation with activity/$A_{450\ nm}$, indicating them to be properties of active xanthine oxidase molecules. A fourth parameter, however, shows a negative correlation indicating it to be a specific property of inactivated xanthine oxidase, the first such property observed. This parameter is the intensity of one of the molybdenum (V) EPR signals, namely the Slow signal[10]. This develops[11] over periods of minutes when the enzyme is reduced with dithionite but was earlier assumed to depend on slow changes in active enzyme molecules. Other recent data of BEINERT AND ORME-JOHNSON[12] are consistent with the Slow signal being due to inactivated xanthine oxidase, though this form of the enzyme has been widely overlooked.

The other EPR signal in Fig. 1 is the Rapid molybdenum (V) signal[10,11,13] obtained after relatively short incubation (1.5 min) with low concentrations of xanthine. In confirmation of BRAY *et al.*[3] the signal under these conditions is clearly seen to be derived from active xanthine oxidase molecules only. The form of this signal was identical for all the four enzyme samples used. Additional experiments with a larger excess of xanthine (20 moles) and 65-min incubation at 18°, revealed more complicated behaviour. The shape of the Rapid signal was the same for all four samples and, as expected[13], was slightly different from that at the lower substrate concentration. There was some increase in signal intensity with increasing activity/$A_{450\ nm}$, but here intensities appeared to extrapolate, at zero activity/$A_{450\ nm}$, to a finite signal intensity. A possible interpretation is that both active and inactivated xanthine oxidase contribute and give identical signals under these conditions, but further work is required to test this.

MORELL[4] first observed rapid and slow phases in the bleaching at 450 nm of xanthine oxidase by xanthine, the extent of the rapid phase being related to activity/$A_{450\ nm}$. Comparable results have been obtained more recently[1,7]. Data on the present samples are included in Fig. 1 for comparison with the other properties. Finally, data on the FAD content of enzyme samples, following treatment with iodoacetamide in the presence of xanthine, are presented. It was established earlier[13,14] that part of the FAD (determined fluorimetrically or from $A_{450\ nm}/A_{550\ nm}$ of the product) disappears from the enzyme during this reaction. Though it was assumed[14] that iodo[^{14}C]acetamide alkylated the protein, while FAD was liberated, more recent work by KOMAI AND MASSEY[7] has shown that FAD becomes alkylated while remaining bound to the protein. The alkylated flavin has no absorption at 450 nm (ref. 7) and presumably no fluorescence under the conditions of measurement. The alkylation procedure[14] involved

anaerobic addition of enzyme to a mixture of iodoacetamide and xanthine. It has been pointed out[7] that under such conditions inactivated enzyme would not be extensively reduced and so would presumably not react, though this does not appear to have been checked with samples of differing activity/$A_{450\ nm}$. Fig. 1 illustrates that extent of apparent flavin disappearance during alkylation shows a good positive correlation with activity/$A_{450\ nm}$. Further, when the enzyme was preincubated with the xanthine (16 h at 22°), to ensure reduction of inactivated as well as active enzyme prior to adding the alkylating agent, we found the flavin reaction was much more extensive (up to 98%). Our results thus strengthen evidence[7] that incomplete reaction with iodoacetamide is not due to nonequivalence[14] of the two FAD molecules of active xanthine oxidase. Rather, as pointed out by KOMAI AND MASSEY[7], the incompleteness serves to confirm the existence[1] of inactivated enzyme. It seems that fairly exact stoichiometry for 50% FAD disappearance obtained in two series of experiments[13,14] was due to fortuitous combination of incomplete inactivation, some contamination with de-molybdo enzyme and some small loss of molybdenum accompanying alkylation.

In principle, values of all the parameters in Fig. 1, except the intensity of the Rapid EPR signal, could be extrapolated to obtain the theoretical value for activity/$A_{450\ nm}$ of active xanthine oxidase uncontaminated by the inactivated form. In fact, errors in such a procedure would be relatively large, particularly since the present work was carried out with enzyme samples inferior to the best of ref. 1. Consequently, in Fig. 1 a limiting activity/$A_{450\ nm}$ of 197 at 23.5° has been assumed. This corresponds to the maximum activity/Mo value (6.9 units/μmole) actually found for specially prepared crude xanthine oxidase by HART AND BRAY[15], divided by the extinction coefficient[1] for enzyme free from the de-molybdo form ($\varepsilon^{Mo}_{450\ nm}$ 0.035 $l \cdot \mu mole^{-1} \cdot cm^{-1}$). Fig. 1 makes it clear that such samples must be substantially free from inactivated enzyme. Similar or slightly lower limiting activity/$A_{450\ nm}$ values for xanthine oxidase have been deduced by KOMAI AND MASSEY[7]. The highest activity/$A_{450\ nm}$ value actually obtained in this laboratory is 177 (R. T. PAWLIK AND R. C. BRAY, unpublished). This work emphasises the importance of contributions by inactivated xanthine oxidase molecules to the properties of even the best samples of the enzyme.

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