Distribution of Reducing Equivalents on Xanthine Oxidase Molecules and the Rates of the Intramolecular Electron-Transfer Reactions

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When xanthine oxidase turns over 1-methylxanthine aerobically at pH 8.2, the time-sequence in development of its electron-paramagnetic-resonance signals is not primarily due to slow intramolecular reactions among its centres. It derives instead from gross differences in electron distribution within enzyme molecules reduced by the substrate in comparison with those that have subsequently been partly reoxidized by one-electron reaction with oxygen.

Bray *et al.* (1964) studied a series of reactions that take place after mixing xanthine oxidase with approx. 1 molar proportion of xanthine in the presence of an excess of O₂. They followed these reactions, which occur within a time comparable with the enzymic turnover time, by means of the rapid-freezing electron-paramagnetic-resonance (e.p.r.) method of Bray (1961). The original observation was that e.p.r. signals corresponding to the reduced centres in the enzyme, i.e. Mo(V), the flavin semiquinone (FADH•) and reduced iron-sulphur, each developed and decayed again with a different time-course; this has been interpreted as indicating that some of the intramolecular electron-transfer reactions among the redox-active centres of the xanthine oxidase molecule take place relatively slowly and may become rate-limiting in turnover of the enzyme (Bray *et al.*, 1964; Bray, 1971; Lowe *et al.*, 1972). The conclusions from these 'single-turnover' experiments have been widely quoted, e.g. by Mahler & Cordes (1966).

We decided to reinvestigate the phenomenon for a number of reasons. Xanthine oxidase molecules may accept from substrate molecules up to at least six reducing equivalents per Mo atom (Massey *et al.*, 1970), but work in this laboratory (Lowe *et al.*, 1972; D. J. Lowe & R. C. Bray, unpublished work) has shown that those molecules that have accepted two reducing equivalents per Mo atom have substantially different properties from those that have accepted more. In the former, there is no e.p.r.-detectable interaction between Mo(V) and Fe-S centres with $g_{av.} = 1.95$. This indicates that electrons from the substrate may at any one time, in any one xanthine oxidase active-centre system, be located either on Mo or on Fe-S but not on both. With more reducing equivalents this condition no longer holds and interaction is observed. Such differences in reduced forms of the enzyme suggest that molecules reduced by one equivalent per Mo atom may differ from those reduced by two equivalents in the statistical distribution of electrons among the various centres. This is further

supported by the results of dithionite titrations on xanthine oxidase at long reaction times (Beinert, 1971). Edmondson *et al.* (1973) suggested that intramolecular electron-transfer processes in xanthine oxidase may be relatively rapid and that the kinetic differentiation of the e.p.r. species may be a consequence of interactions of the oxidant and reductant with the protein. They produced very little evidence in support of this view, however, and we therefore carried out the experiments described below.

Milk xanthine oxidase was prepared and used as described by Swann & Bray (1972); activity/ E_{450} ratios at 23.5°C varied between 120 and 130. Concentrations are expressed as active-centre concentration (two active centres per molecule), based on extinction coefficients and limiting activity/ E_{450} ratios as given previously (Swann & Bray, 1972). Rapid freezing was done as described by Bray *et al.* (1973). E.p.r. measurements were done as described by Lowe *et al.* (1972) and Smith *et al.* (1973). Bovine superoxide dismutase was a gift from Dr. G. Rotilio, Centro di Biologia Molecolare del C.N.R., Istituto di Chimica Biologica, Università di Roma, Rome, Italy.

Since it is likely that purine substrates reduce xanthine oxidase by a two-electron mechanism (see Bray, 1974), studies on a one-electron-reduced form require initial reduction of the enzyme with a substrate, followed by incomplete reoxidation with a one-electron oxidizing agent [see eqns. (1)–(3), in which XO_{ox.} represents the oxidized enzyme and XO_{1e.} and XO_{2e.} represent molecules that are reduced by one or two electron equivalents/Mo atom].

It is well known that O₂ can act, with xanthine oxidase, as either a one- or a two-electron oxidizing

agent [eqns. (2) and (3)]. At high O_2 concentrations eqn. (3) is favoured (Nakamura & Yamazaki, 1969; Fridovich, 1970; see also Knowles *et al.*, 1969). Single-turnover experiments on xanthine oxidase, at pH 8.2, with 1-methylxanthine in place of xanthine as substrate and carried out in the presence of O_2 and superoxide dismutase (Bray *et al.*, 1967; Lowe *et al.*, 1972), yield only the Rapid Mo(V) signal (Bray & Swann, 1972), the $FADH^{\cdot}$ signal and Fe-S signals. In confirmation of earlier work (Bray *et al.*, 1967; Lowe *et al.*, 1972), we found the sequence of appearance of the peaks to be Mo(V), followed by $FADH^{\cdot}$, followed by the two Fe-S signals together. Fig. 1

Fig. 1. Effect of O_2 concentration on the time-course of the Fe-S ($g_{av.} 1.95$) and Rapid Mo(V) e.p.r. signals, during single-turnover experiments with xanthine oxidase and 1-methylxanthine at pH 8.2.

The ratio of intensities of the two signals is plotted as a function of reaction time, with signals expressed as integrated intensities, i.e. as unpaired electrons/mol of active centre. Final concentrations were as follows: O_2 , 0.25 mM (○) or 0.75 mM (●); 1-methylxanthine, 0.07 mM; xanthine oxidase (active centres), 0.19 mM; superoxide dismutase, 0.003 mM. The temperature was 22°C.

shows that O_2 concentration has a substantial effect on the single-turnover kinetics. Not only do decreased O_2 concentrations slow down the overall reaction, as expected from its apparent K_m (Ackerman & Brill, 1962), but also under these conditions, Mo signals are increased relative to the Fe-S signals at all reaction times except the very shortest. Thus, as shown in Fig. 1, the slope of graphs of relative intensity of these two signals against time is quite sensitive to the initial O_2 concentration. Such an effect of O_2 concentration is not readily explicable in terms of the original scheme of Bray *et al.* (1964), which involved slow intramolecular electron transfer from Mo to the other constituents of the enzyme. It may, however, readily be understood with relatively fast intramolecular reactions and on the basis of eqns. (1)–(3), provided that it is further assumed that in XO_{1e} reducing equivalents are distributed relatively more in favour of reduced Fe-S and less in favour of Mo(V) than they are in XO_{2e} , i.e. it has to be assumed that the ratio Fe-S/Mo(V) is substantially higher for XO_{1e} than it is for XO_{2e} .

If intramolecular reactions are indeed fast, then no changes in the Fe-S/Mo(V) ratio would be expected during the time-course of anaerobic reduction of xanthine oxidase by low concentrations of 1-methylxanthine, since XO_{1e} would not be formed. [In reduction of the enzyme by excess of substrate, as studied by Edmondson *et al.* (1973), it cannot be assumed that successive substrate molecules all react with the enzyme at the same rate, so that interpretation of the kinetics is complicated.] In experiments under the best anaerobic conditions available to us for rapid-freezing work (Bray *et al.*, 1973) we were able to show that Fe-S/Mo(V) ratios did indeed change substantially less than they did at the lower O_2 concentration of Fig. 1. However, changes in the ratio up to 150 ms reaction time were quite significant, though the possibility cannot be excluded that with more rigorous anaerobiosis changes would become negligible.

Table 1. Reduction of xanthine oxidase by 1-methylxanthine at low concentration, followed by partial reoxidation with phenazine methosulphate at two different concentrations (three-syringe rapid-freezing system)

Intensities of the Fe-S ($g_{av.} = 1.95$) and the Rapid Mo(V) e.p.r. signal are recorded. Reaction time (at 21°C and pH 8.2) in the controls was 150 ms. In the experiments reduction was for 115 ms, followed by 35 ms reoxidation after the introduction of phenazine methosulphate. Additional experiments indicated reoxidation to be about 90% complete at this time. Final concentrations were as follows: 1-methylxanthine, 0.15 mM; xanthine oxidase (active centres), 0.19 mM.

Reduction	Reoxidation	Electrons per active centre		
		Mo	Fe-S	Fe-S/Mo ratio*
1-Methylxanthine	—	0.135	0.411	3.04 ± 0.05 (2)
1-Methylxanthine	Phenazine methosulphate (0.07 mM final)	0.049	0.253	5.16 ± 0.17 (3)
1-Methylxanthine	Phenazine methosulphate (0.14 mM final)	0.014	0.101	7.21 (1)

* Errors are average deviations for the numbers of samples indicated in parentheses.

To obtain more definite information, we then carried out experiments with a one-electron oxidizing agent, phenazine methosulphate, in place of O_2 . We used the three-syringe system of Palmer *et al.* (1964), first to reduce the enzyme with a low concentration of 1-methylxanthine and then to reoxidize it by addition of graded amounts of phenazine methosulphate with a fixed total reaction time of 150ms (Table 1). Though perfect anaerobiosis was apparently not attained, it is nevertheless quite clear that addition of phenazine methosulphate to enzyme reduced by 1-methylxanthine increases the Fe-S/Mo(V) ratio significantly. This result, namely changes in the ratio in response to partial reoxidation at a fixed reaction time, is quite incompatible with the slow intramolecular reaction model but fully compatible with the newer model. Combining our results with the titration results of Beinert (1971) we conclude that most, if not all, of the time-course differences in the e.p.r. signals in single-turnover experiments with 1-methylxanthine at pH 8.2 are due to differences between the properties of XO_{1e} and XO_{2e} and not to slow intramolecular reactions.

It then has to be considered whether or not intramolecular electron-transfer reactions of xanthine oxidase, even if they are not rate-limiting, take place slowly enough under any conditions to be effectively quenched by the rapid-freezing procedure. Indeed, it must be considered whether molybdenum, as is generally assumed, is the component of the enzyme that first receives reducing equivalents from the substrate. The latter question can almost certainly be answered in the affirmative, even if evidence from the time-sequence of e.p.r. signals is discounted (see Bray & Swann, 1972). More work, particularly with improved anaerobiosis, will be required to answer the first question. Measurements with xanthine at pH 10 might be particularly revealing, in view of the differences in behaviour of the two Fe-S signals that have been reported (Lowe *et al.*, 1972; Edmondson *et al.*, 1973). Under these conditions at 10°C and with enzyme in excess over substrate, we found that the Very Rapid signal developed quickly ($t_{\frac{1}{2}}$ 8ms) and was stable up to 100ms reaction time. Since no Rapid signal was detected, these conditions should be useful

for further work on the rates of the intramolecular reactions.

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