The Mechanism of Action of Xanthine Oxidase

THE RELATIONSHIP BETWEEN THE RAPID AND VERY RAPID MOLYBDENUM ELECTRON-PARAMAGNETIC-RESONANCE SIGNALS

By ROBERT C. BRAY,* STEVEN GUTTERIDGE,* DAVID A. STOTTER††
and STEPHEN J. TANNER*

*School of Molecular Sciences, University of Sussex, Falmer, Brighton BN1 9QJ, Sussex, U.K., and

†Department of Chemistry, University of Essex, Wivenhoe Park, Colchester CO4 3SG, Essex, U.K.

(Received 19 September 1978)

On the basis of the work of Gutteridge, Tanner & Bray [*Biochem. J.* (1978) 175, 887–897] and of other data in the literature, a mechanism for the reaction of xanthine oxidase with reducing substrates is proposed. In the Michaelis complex, xanthine is bound to molybdenum via the N-9 nitrogen atom. Coupled transfer of two electrons to molybdenum and the C-8 proton to the enzyme yields (Enzyme)–Mo–SH. Concerted with this process, reaction of the xanthine residue with a nucleophile in the active centre yields a covalent intermediate that breaks down to give the product by alternative pathways at high and at low pH values.

Olson *et al.* (1974) put forward a comprehensive mechanism explaining the processes of reduction and reoxidation of the enzyme xanthine oxidase by its substrates, xanthine and oxygen. The main points, involving the binding and reaction at the molybdenum centre of the enzyme of up to three successive xanthine molecules, have been widely accepted (see Bray, 1975). Reaction of the xanthine molecules was taken to involve a covalently bound intermediate, with transfer of two electrons from the substrate to the enzyme. These electrons were initially accepted by molybdenum, which was thereby reduced from the six- to the four-valent state, but were then rapidly redistributed among the other reducible centres. It was suggested that the xanthine residue, having lost the hydrogen from its C-8 position, was bound via this carbon atom to the terminal sulphur atom of a persulphide group located near to molybdenum in the active centre. Hydrolysis of the intermediate yielded the product, uric acid, and the free (reduced) enzyme.

Almost all information about molybdenum in xanthine oxidase has been derived from e.p.r. spectroscopy. According to Olson *et al.* (1974), the e.p.r. properties of the intermediate species were supposed to depend on the number of electrons that the enzyme half-molecule had accepted, as well as on the pH of the solution. Thus at high pH values, in the reaction of the first xanthine molecule with the oxidized enzyme, the intermediate gave the Very

Rapid molybdenum(V) e.p.r. signal, but under all other conditions it gave the Rapid signal.

More recently, Gutteridge *et al.* (1978a) emphasized the role of a proton-accepting group in the enzyme active centre in abstracting the hydrogen from the substrate C-8 position. Further, Gutteridge *et al.* (1978b) presented evidence that this accepting group is (Enzyme)–Mo=S, transformed on protonation to (Enzyme)–Mo–SH. They concluded that the sulphur atom of the accepting group is the one liberated as thiocyanate during inactivation of the enzyme by cyanide. This is the sulphur originally presumed by Massey & Edmondson (1970) to be present as a persulphide participating in the catalytic reaction. Finally, Tsopanakis *et al.* (1978) carried out pH-jump studies at subzero temperatures on the Very Rapid signal, generated on treating the enzyme with xanthine in aq. dimethyl sulphoxide. Results did not support the claim of Edmondson *et al.* (1973) and Olson *et al.* (1974) that the Rapid and Very Rapid signals are in pH-dependent equilibrium with one another.

Partial revision of the reaction mechanism of Olson *et al.* (1974) thus becomes necessary. In the present paper we propose a new mechanism for interaction of the enzyme with reducing substrates.

Kinetic Considerations and the Development of a Reaction Scheme

An acceptable reaction mechanism for xanthine oxidase must account for the kinetic relationships

† Present address: Shell Toxicology Laboratory (Tunstall), Sittingbourne Research Centre, Sittingbourne, Kent ME9 8AG, U.K.

Scheme 3. *Proposed chemical nature of the various signal-giving species in reduction of xanthine oxidase by xanthine* The numbering of the species corresponds to that in Scheme 2. **Bold-face** type denotes atoms of, or derived from, the 7-, 8- and 9-positions of the substrate molecule. The Michaelis complex (I) and the intermediate (II) (shown in square brackets) are not detected by e.p.r.; (III) gives the Very Rapid signal and (IV) and (VI) the Rapid; (V) is the product, uric acid. In the signal-giving species, though $\text{Mo}^{(\text{IV})}$ is shown, internal oxidation-reduction equilibria with iron-sulphur and flavin will yield some $\text{Mo}^{(\text{V})}$. In the Rapid species, the strongly coupled proton is the one attached to S and the weakly coupled one that on X; the aquo complex is depicted, although complexes with product, substrate or buffer constituents are also possible. The geometry of the ligands, the nature of the nucleophile, X, and whether other atoms intervene between it and Mo are uncertain; electron transfer is represented schematically.

Edmondson *et al.* (1973) observed experimentally with xanthine as substrate when varying the pH value.

Chemical Interpretation

A chemical interpretation of Scheme 2, is shown in Scheme 3, taking account of (Enzyme)- $\text{Mo}=\text{S}$ as the proton-accepting group in the enzyme (Gutteridge *et al.*, 1978b). Attack on the substrate takes place in the Michaelis complex (I), in which the substrate is co-ordinated, via its N-9 position, to the anion-binding site in the enzyme (Gutteridge *et al.*, 1978a; Bergmann & Levene, 1976). The nucleophile, X, rather than the 'persulphide' group of Massey & Edmondson (1970), is depicted as an undefined species attacking the C-8 carbon of the xanthine. The reaction is, overall, one of coupled transfer of two electrons to molybdenum (probably via the N-9 atom) and of the proton to the acceptor group

(cf. Stiefel, 1973), while the xanthine C-8 carbonium ion reacts with the nucleophile, X.

At low pH, after this initial reaction, hydrolysis of (II) to yield the product (V) and the Rapid species (IV) takes place before the proton transferred to the enzyme has exchanged into the solvent. At high pH, on the other hand, deprotonation to yield the Very Rapid species (III) precedes hydrolysis and product liberation. We are not certain why the intermediate (II) has not been detected by e.p.r. Possibly its existence is so transient that it has so far been missed. Alternatively, its internal oxidation-reduction equilibrium may be such that $\text{Mo}^{(\text{V})}$ is not favoured.

Geometry of the ligands around Mo, the nature of the nucleophile, X, and whether or not other atoms intervene between it and the Mo atom are not certain. However, noting that both the strongly coupled and the weakly coupled proton are present

together in the Rapid signal and that neither is present in the Very Rapid signal, we have assumed, in Scheme 3, that X, when it is not substituted by the xanthine residue, bears the weakly-coupled proton of the Rapid signal.

Further Consideration

A further signal derived from functional enzyme (Edmondson *et al.*, 1972) is Inhibited (Pick *et al.*, 1971; Tanner & Bray, 1978b). This is believed to correspond to an RCO group bound in the active centre, where RCHO or RCH₂OH is the aldehyde or alcohol used to inactivate the enzyme and generate the signal. We now suggest that Inhibited represents the 'wrong-way-round' reaction of a substrate-type molecule at the active centre, with the sulphur atom now acting as a nucleophile rather than as a proton acceptor. Thus, in Inhibited, we would have (Enzyme)–Mo–S–COR. Similarly, since Resting II (Lowe *et al.*, 1976) is regarded (Tanner & Bray, 1978a) as the desulpho enzyme analogue of Inhibited, we propose that in Resting II we have (Enzyme)–Mo–O–COR.

Scheme 3 suggests further studies. Under some conditions the 'invisible' species (II) might become detectable. Also, particular acylating or alkylating agents might give rise to Very Rapid species or to their as yet unknown desulpho analogues. Further work is also required on direct oxygen transfer in xanthine oxidase-catalysed reactions. Murray *et al.* (1966) claimed a small but significant transfer of ¹⁸O from ¹⁸O₂ to uric acid during xanthine oxidase turnover at pH 8.9. We suggest, if direct oxygen transfer is substantiated, that it could only occur via an oxygenated enzyme species, this probably being of the type (Enzyme)–Mo=O.

We thank Professor F. Bergmann for valuable discussions. The work was supported by a Programme Grant from the Medical Research Council. S. J. T. was in receipt of a Studentship from the Science Research Council.

References

- Bergmann, F. & Levene, L. (1976) *Biochim. Biophys. Acta* **429**, 672–688
- Bray, R. C. (1975) *Enzymes* 3rd Ed. **12**, 299–419
- Edmondson, D., Massey, V., Palmer, G., Beacham, L. M. & Elion, G. B. (1972) *J. Biol. Chem.* **247**, 1597–1604
- Edmondson, D. E., Ballou, D., Van Heuvelen, A., Palmer, G. & Massey, V. (1973) *J. Biol. Chem.* **248**, 6135–6144
- Gutteridge, S., Tanner, S. J. & Bray, R. C. (1978a) *Biochem. J.* **175**, 869–878
- Gutteridge, S., Tanner, S. J. & Bray, R. C. (1978b) *Biochem. J.* **175**, 887–897
- Lowe, D. J., Barber, M. J., Pawlik, R. & Bray, R. C. (1976) *Biochem. J.* **155**, 81–85
- Massey, V. & Edmondson, D. (1970) *J. Biol. Chem.* **245**, 6595–6598
- Murray, K. N., Watson, J. G. & Chaykin, S. (1966) *J. Biol. Chem.* **241**, 4798–4801
- Olson, J. S., Ballou, D., Palmer, G. & Massey, V. (1974) *J. Biol. Chem.* **249**, 4363–4382
- Pick, F. M., McGartoll, M. A. & Bray, R. C. (1971) *Eur. J. Biochem.* **18**, 65–72
- Stiefel, E. I. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 988–992
- Tanner, S. J. & Bray, R. C. (1978a) *Biochem. Soc. Trans.* **6**, 230–232
- Tanner, S. J. & Bray, R. C. (1978b) *Biochem. Soc. Trans.* **6**, 232–234
- Tanner, S. J., Bray, R. C. & Bergmann, F. (1978) *Biochem. Soc. Trans.* **6**, 227–229
- Tsopanakis, A. D., Tanner, S. J. & Bray, R. C. (1978) *Biochem. J.* **175**, 879–885