Rapid Type 2 Molybdenum(V) Electron-Paramagnetic Resonance Signals from Xanthine Oxidase and the Structure of the Active Centre of the Enzyme

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Rapid type 2 molybdenum(V) e.p.r. signals from reduced functional xanthine oxidase have been further investigated. These signals, which show strong coupling of two protons to molybdenum, have been obtained under a variety of new conditions: specifically either at pH 8.2 in the presence of borate ions, or at pH 10.1–10.7 with or without various other additions. Parameters of the signals were obtained with the help of computer simulations. In at least some of these signals, the coupled protons must be located on the enzyme rather than on bound species. The relationship between type 1 and type 2 Rapid signals is discussed. They may represent geometrical isomers, or alternatively, hydroxyl uptake as a ligand of molybdenum may be involved in formation of type 2 species.

The chemical natures of the molybdenum centres of the various molybdenum-containing enzymes and the mechanisms of their enzymic reactions have been the subject of considerable interest in recent years. E.p.r. studies of enzyme-bound molybdenum in the quinquevalent state have provided much relevant information (Bray, 1975; Bray, 1979; Gutteridge & Bray, 1979).

In the case of milk xanthine oxidase, reduction of the functional enzyme by a wide variety of substrates and other reducing agents yields a family of e.p.r. signals given the name 'Rapid' by Bray & Vänngård (1969). These signals all have g_{av} about 1.975 and show strong coupling of molybdenum to exchangeable protons. These workers distinguished Rapid type 1 from Rapid type 2 signals. Type-1 signals showed strong coupling of molybdenum to one proton and (at least in some cases) considerably weaker coupling to a second proton, also (see also Gutteridge *et al.*, 1978a). On the other hand, the type 2 signals showed strong coupling to two approximately equivalent protons.

The nature of the groups binding the strongly coupled protons is of particular interest, since the proton strongly coupled in the Rapid type 1 signal is the one that is transferred from the C-8 position of purine substrates during the enzymic reaction

Abbreviations used: Caps, 3-(cyclohexylamino)propanesulphonic acid; Ches, 2-(cyclohexylamino)ethanesulphonic acid; Bicine, *NN*-bis-(2-hydroxyethyl)glycine.

(Gutteridge *et al.*, 1978a). Because of the magnitude of the proton splittings, their apparent near-isotropic nature and the absence of observable nitrogen hyperfine structure, it is highly likely (Bray & Vänngård, 1969; Gutteridge *et al.*, 1978b) that these protons are separated from molybdenum by either a single oxygen or a single sulphur atom.

In the Rapid type 1 signal, not only the strongly coupled proton, but also the one that is weakly coupled, is located on the enzyme rather than on any bound substrate or product molecule or buffer species, since splittings from both protons are clearly observed in a nitrate complex of the enzyme (Gutteridge *et al.*, 1978a). On the other hand, there is no definite information on the location of the protons in the Rapid type 2 signal. Type-2 signals have been reported so far only from complexes of the enzyme with purine substrates or product molecules (Bray & Vänngård, 1969; Pick & Bray, 1969; Bray *et al.*, 1978). It was concluded by Bray *et al.* (1978) that the type 2 signal obtained in the presence of xanthine represents a non-productive complex of this substrate with the enzyme. The possibility was therefore seriously considered that at least one of the protons of this signal might be located on the purine molecule rather than on the enzyme.

We now report the generation of Rapid type 2 signals in the absence of purine molecules and discuss the implications of these findings.

Materials and Methods

Milk xanthine oxidase was prepared by the salicylate-denaturation method of Hart *et al.* (1970). Final concentrations of the enzyme (as concentrations of functional active centres, calculated from activity measurements; Bray, 1975) were 0.1 to 0.2 mM. Caps, Ches and Bicine were obtained from Sigma, Kingston upon Thames, Surrey, U.K. All buffers were adjusted with NaOH and contained 1 mM-EDTA.

Acetaldehyde was distilled below 22°C under N₂ and stored under liquid N₂. Solutions were prepared in water cooled to 4°C and made anaerobic by bubbling N₂ through them. An appropriate amount of undiluted acetaldehyde was added by using a syringe pre-cooled to 4°C. Concentrations of acetaldehyde were determined by using ϵ_{278} (25°C) of 8.55 M⁻¹·cm⁻¹ (Lienhard & Jencks, 1966). Removal of buffers from xanthine oxidase was achieved by gel filtration with Sephadex G-25 equilibrated with approx. 0.1 mM-NaOH, pH about 9.3 (CO₂ not excluded) and elution with the same solvent.

Procedures for mixing enzyme and substrate anaerobically were as described by Barber *et al.*, (1976). Rapid-freezing techniques were as described by Gutteridge *et al.* (1978a). Conditions were selected such that only the molybdenum of the functional and not of the desulpho enzyme was reduced. In all experiments at pH 10.0 or above, reduction was carried out at about pH 8.2 and the pH was then raised by appropriate addition of buffer or NaOH and the samples were frozen immediately. Reduction by acetaldehyde (about 5 mM) was for 5 min at about 20°C. Reduction by dithionite was as follows. For the experiment of Fig. 1(g), approx. 1 mM-dithionite was used for 1 min in the presence of Bicine and borate. For that of Fig. 1(a), 4 mM-dithionite was used for 15 s at pH 8.2 and the pH was then raised and the sample frozen after 6 ms (rapid freezing).

E.p.r. running conditions (cf. Gutteridge *et al.*, 1978a), under which there is no significant saturation of Mo(V) signals, were: microwave power 10 mW, modulation 0.16 mT, temperature 120 K. Simulations used the program of Lowe (1978). Difference spectra were obtained by the procedures of Bray *et al.* (1978). Relatively small amounts of Rapid type 1 spectra were subtracted from the experimental spectra until features obviously due to them just disappeared.

Results

We used the reducing agent dithionite or the substrate acetaldehyde to generate Rapid signals from functional xanthine oxidase. In previous work, aldehydes (Bray & Meriwether, 1966) or dithionite (Gutteridge *et al.*, 1978b) have yielded only Rapid type 1 signals. However, as shown in Fig. 1, we now find that they give, instead, Rapid type 2 signals under particular conditions, namely either at high pH values or in the presence of certain buffer ions.

Fig. 1(g) was obtained by reduction with dithionite in Bicine buffer, pH 8.2, with the addition of 50 mM-borate. This type 2 Rapid spectrum may be contrasted with the type 1 spectrum obtained under similar conditions, but in the absence of borate, by Gutteridge *et al.* (1978a). The corresponding computer-simulated spectrum, obtained by using the parameters listed in Table 1, is shown in Fig. 1(h). Fig. 1(f) shows, for comparison, the rather different xanthine type 2 difference spectrum, replotted from the work of Bray *et al.* (1978) (see also Table 1).

As Fig. 1(d) shows, we obtained a further variant of the type 2 Rapid spectrum, when working at the relatively high pH value of 10.7 in the presence of high concentrations of Ches buffer, with acetaldehyde as reductant. The simulation of this spectrum is shown in Fig. 1(e) (see also Table 1). Under otherwise similar conditions, however, but at lower

Table 1. E.p.r. parameters of various Rapid type 2 spectra
Proton hyperfine coupling constants [$A(^1H)$] and half-linewidths (Δ) are given in mT.

Conditions	g_1	g_2	g_3	$g_{av.}$	A_1	A_2	A_3	$A_{av.}$	$\Delta_{av.}$	Reference
Xanthine	1.9951	1.9712	1.9616	1.9760	$\begin{Bmatrix} 1.07 \\ 1.07 \end{Bmatrix}$	$\begin{Bmatrix} 0.98 \\ 0.98 \end{Bmatrix}$	$\begin{Bmatrix} 0.93 \\ 0.93 \end{Bmatrix}$	$\begin{Bmatrix} 1.01 \\ 1.01 \end{Bmatrix}$	0.22	Bray <i>et al.</i> (1978)
Borate	1.9911	1.9695	1.9630	1.9745	$\begin{Bmatrix} 1.40 \\ 1.00 \end{Bmatrix}$	$\begin{Bmatrix} 1.52 \\ 1.07 \end{Bmatrix}$	$\begin{Bmatrix} 1.65 \\ 1.40 \end{Bmatrix}$	$\begin{Bmatrix} 1.52 \\ 1.16 \end{Bmatrix}^*$	0.19†	Present work
Ches	1.9913	1.9697	1.9603	1.9738	$\begin{Bmatrix} 1.10 \\ 1.10 \end{Bmatrix}$	$\begin{Bmatrix} 1.28 \\ 0.75 \end{Bmatrix}$	$\begin{Bmatrix} 1.37 \\ 1.17 \end{Bmatrix}$	$\begin{Bmatrix} 1.25 \\ 1.01 \end{Bmatrix}^*$	0.23‡	Present work

* Note that the combination of hyperfine splittings given is an arbitrary one. Thus, though we can say for instance that with borate there are two protons giving splittings of 1.40 and 1.00 mT respectively in the A_1 direction, we cannot say which of these protons is giving the larger splitting and which the smaller in the A_2 and A_3 directions.

† Values of Δ_1 , Δ_2 and Δ_3 were 0.185, 0.167 and 0.230 respectively.

‡ Values of Δ_1 , Δ_2 and Δ_3 were 0.26, 0.22 and 0.22 respectively.

pH values, or with lower concentrations of buffer, Rapid type 1 spectra could be obtained (J. P. G. Malthouse & R. C. Bray, unpublished work).

Under the conditions of Figs. 1(d) and 1(g), only the type 2 spectra, as illustrated, were obtained. We also obtained type 2 spectra accompanied by type 1 spectra, under a variety of other conditions. Such mixed spectra could be resolved into components by difference-spectral techniques, as described by Bray *et al.* (1978). Type 2 difference spectra so obtained are illustrated in Figs. 1(a), 1(b) and 1(c). A type 2 spectrum closely similar to that of Fig. 1(d) was obtained with dithionite as reductant in place of acetaldehyde (compare particularly Fig. 1a with Fig. 1b). Similarly, essentially the same spectrum was obtainable with Ches, with Caps or with no buffer at all present (compare Figs. 1d, 1b and 1c).

From Table 1 and Fig. 1 it is clear that relatively small variations in the parameters of the Rapid type 2 has a noticeably higher g_1 value than do the overall lineshape. g -Values for the species listed are relatively close to one another, except that xanthine type 2 has a noticeable higher g_1 value than do the others. [Absolute accuracy of g -values in the present work is ± 0.0005 , though relative accuracy is better than this; in the work of Bray *et al.* (1978), accuracy was almost certainly somewhat less good than the ± 0.0005 that was claimed.] $A(^1\text{H})_{\text{av}}$ (proton hyperfine coupling constant) values are rather larger for the buffer signals than for the xanthine signal. This is particularly so for the borate signal, for which the splittings approach those of the Slow signal (Gutteridge *et al.*, 1978b).

It is important that in the present simulations slight non-equivalence of the two interacting protons has been assumed on at least two of the principal

Fig. 1. Rapid type 2 signals obtained under various conditions

Reduction with dithionite was used for (a) and (g) and with acetaldehyde for (b)–(d). (e) and (h) respectively are computer simulations (using the parameters of Table 1) of (a)–(d) and (g). (f) shows for comparison the Rapid type 2 signal obtained with xanthine (reproduced from Bray *et al.*, 1978). Final pH values and buffer concentrations were as follows: (a) pH 10.1 (100 mM-Caps/5 mM-Bicine); (b) pH 10.5 (100 mM-Caps/5 mM-Bicine); (c) pH 10.7 (no buffer added; 40 mM- Na^+); (d) pH 10.7 (1.1 M-Ches/5 mM-Bicine); (g) pH 8.2 (50 mM-borate/50 mM-Bicine). (d) and (g) are experimental spectra. (a)–(c) were obtained by difference techniques (see the Materials and Methods section; see also this section for details of the reduction, e.p.r. and computer-simulation procedures). The field scale corresponds to a microwave frequency of 9.26 GHz. Stick diagrams under the simulated spectra indicate the principal g -values and the proton splittings.

axes. This non-equivalence is very clearly apparent on inspection, for example, of the g_1 feature of the borate complex (Fig. 1g; see also Fig. 1h and the stick diagram). Although, in previous simulations of the xanthine type 2 Rapid signals (Bray & Vännegård, 1969; Bray *et al.* 1978), the protons were taken to be equivalent, additional attempts (not illustrated) to simulate this signal indicated that incorporating some non-equivalence into the splittings would probably give an even better fit than the one reported by Bray *et al.* (1978).

Discussion

Our results provide new information relating to the structure of the active centre of xanthine oxidase, particularly when this is in the configuration giving rise to the Rapid type 2 spectrum. In previous work (Bray & Vännegård, 1969; Pick & Bray, 1969; Bray *et al.*, 1978), type 2 signals were observed only when a purine molecule (specifically xanthine, uric acid or 1-methyluric acid) was bound in the signal-giving species. We now find (Fig. 1) that these signals may be obtained under quite a wide variety of conditions, in the absence of purines. Furthermore, two different reducing agents generated the signals and buffers could be omitted from the system. Thus specific binding in the signal-giving species of any one type of reductant, product or buffer ion as a prerequisite for type 2 signal formation, is excluded. Furthermore, particularly for the experiment of Fig. 1(d), but presumably also generally for type 2 spectra, the interacting protons must be present on the enzyme molecule itself, rather than on other molecules or ions (except perhaps hydroxyl) bound to it. Rapid type 2 signals are thus in this respect analogous to type 1 signals (*cf.* Gutteridge *et al.*, 1978a).

The relationship between Rapid type 1 and type 2 signals is of particular interest. Further work will be required to elucidate fully the factors determining whether the enzyme yields under particular conditions a type 1 spectrum, a type 2 spectrum, or a mixture of both. We may nevertheless speculate on the relevant structures. We might assume that the two non-equivalent protons of the Rapid type 1 signal are borne by the same groups in the enzyme (presumably terminal oxygen or sulphur ligands of molybdenum when in the unprotonated state) as are the two corresponding but more nearly equivalent protons in the type 2 signal. If this were so, then the two signal-giving species would have to be regarded as isomers. For instance, in the type 2 species the two proton-binding groups might be equatorial ligands of molybdenum, whereas in the type-1 species, one of these groups might have migrated to an axial position. Transformation from one such species to the other might then, correspondingly, be promoted by different anions binding

to the enzyme in alternative sites adjacent to molybdenum.

On the other hand, a further important point, which may not be immediately reconcilable with this hypothesis, must be raised. The finding that high pH values favour the type 2 signal (*cf.* Figs. 1a–d), raises the possibility that hydroxyl uptake by the enzyme may be involved, thus the second strongly coupled proton of the type 2 signal might be that of a hydroxyl ion co-ordinating to molybdenum, particularly at high pH values and perhaps as an additional ligand of the metal. This possibility is especially interesting in relation to proposals (see e.g. Yamanouchi & Enemark, 1979) that oxygen transfer may be involved in the mechanisms of certain molybdenum-containing enzymes. Further work, especially by X-ray absorption extended fine structure (e.x.a.f.s.) (*cf.* Tullius *et al.*, 1979; Bordas *et al.*, 1979) and by ^{17}O -substitution (*cf.* Gutteridge *et al.*, 1979), is clearly required on these problems.

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