Electron-Paramagnetic-Resonance Spectroscopy of Complexes of Xanthine Oxidase with Xanthine and Uric Acid

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Molybdenum(V) e.p.r. signals from reduced functional milk xanthine oxidase molecules (the Rapid signals), obtained in the presence of purine substrates and products, were further investigated [cf. Bray & Vänngård, (1969) *Biochem. J.* **114**, 725–734; Pick & Bray (1969) *Biochem. J.* **114**, 735–742]. Xanthine forms two complexes with the enzyme that are believed to correspond to different orientations of the substrate molecule in the active site. Only one complex appears to undergo the catalytic reaction. Non-productive complexes, analogous to the one with xanthine, are not formed by 1-methylxanthine or purine. Uric acid forms more than one e.p.r.-detectable complex, one of which is analogous to the non-productive xanthine complex. The computer program used for handling the e.p.r. data is described briefly.

E.p.r. spectroscopy is in principle capable of yielding much information about the structure of the molybdenum active centre of the enzyme xanthine oxidase. As many as ten different e.p.r. signals from molybdenum(V) in different reduced forms of the active and inactive enzyme are known (Bray, 1975), each signal corresponding to the molybdenum atom in a different chemical environment. Of these signals, the family of signals given the name Rapid by Bray & Vänngård (1969) represents the normal functional enzyme in the reduced state. Formation of complexes of the reduced enzyme with purine substrate molecules results in variations in the Rapid signal (Pick & Bray, 1969). Parameters of this signal from two different complexes of the enzyme with the substrate, xanthine, were reported by Bray & Vänngård (1969), though the nature of the complexes remained uncertain. Although no spectroscopic evidence for complex-formation with uric acid could be obtained by Pick & Bray (1969), more recently potentiometric and stoichiometric e.p.r. titrations have indicated that the reduced enzyme [containing molybdenum(IV) or molybdenum(V)] does indeed form complexes with this product molecule (Barber *et al.*, 1977). However, the molybdenum(V) e.p.r. spectrum for a uric acid complex has not so far been described.

Bray & Swann (1972) reported that the Rapid molybdenum(V) signal is of different form when examined at different points in the anaerobic reductive titration of xanthine oxidase with xanthine, but gave no detailed interpretation of their spectra. A reasonable explanation would be that at low xanthine concentrations the signal is in the uncomplexed ('no complex detected'; Bray & Vänngård, 1969)

form. At higher xanthine concentrations, in the region of maximum conversion of molybdenum into the quinquivalent state, the signal is different, and this would presumably be due to relatively weak binding of uric acid to the reduced enzyme. In the presence of an excess of xanthine, the substrate might compete effectively with the uric acid, so that complexes with xanthine rather than uric acid would then be expected. In agreement with this, close inspection of the data of Bray & Swann (1972) reveals that there were small but significant further changes of signal form in the excess-xanthine region. Xanthine complexes of reduced xanthine oxidase must be regarded as Michaelis complexes, which should of course be transients only. However, they are expected to persist in the five-electron-reduced enzyme, which will be present in this region of the titration. This is because this species is unable to accommodate the two additional electrons that it would have to accept from the bound xanthine molecule, if it were to go through the catalytic cycle (Olson *et al.*, 1974; Barber *et al.*, 1977).

We carried out the work reported below to obtain more information on the role of the two xanthine complexes and to obtain direct e.p.r. evidence for existence of a uric acid complex of the reduced enzyme.

Materials and Methods

Milk xanthine oxidase samples for e.p.r. experiments were obtained as described previously (see, e.g., Barber *et al.*, 1976). The enzyme concentration used was from 0.04 to 0.26 mM (functional active centres). ⁹⁵Mo-substituted xanthine oxidase was prepared by the method of Bray & Meriwether (1966). 1-Methylxanthine (Fluka A.G.) was obtained from Fluorochem, Glossop, Derbyshire, U.K., and 1-methyluric

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acid was prepared from it by aerobic treatment with xanthine oxidase.

For a few experiments, sodium pyrophosphate buffer, which can give decreases of apparent pH of up to 3 units on freezing (Williams-Smith *et al.*, 1977), was used. Other buffers, selected to avoid such changes, were: Mes [4-morpholine-ethanesulphonic acid]; Mops [4-morpholinepropanesulphonic acid]; Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]; Ches [2-(cyclohexylamino)ethanesulphonic acid]. Buffers (usually 0.1 M) were adjusted to the required pH with NaOH or HCl, after addition of EDTA (1 mM). To maintain the pH in reductions with concentrated sodium dithionite, this reagent was made up in Na₂CO₃ solution.

Rapid freezing was carried out (unless otherwise stated, anaerobically, at 20–25°C), as described by Bray *et al.* (1973). The e.p.r. spectrum of reduced xanthine oxidase in the presence of uric acid was obtained by a three-syringe rapid-freezing procedure (Palmer *et al.*, 1964). In the first step enzyme was reduced with dithionite (Bray *et al.*, 1975) and in the second it was treated with uric acid. The signal of FADH[•] in xanthine oxidase, which was required for some difference spectra (Figs. 2 and 3), could be obtained (Bray *et al.*, 1975) by reducing the desulpho enzyme with dithionite, in the rapid-freezing apparatus.

The 'warming-up' procedure involves controlled incubation of samples, prepared by the rapid-freezing method, at temperatures intermediate between the initial trapping temperature (about 130 K) and the freezing point, and was carried out as described previously (Bray & Knowles, 1968; Pick & Bray, 1969; Van Heuvelen, 1975). In the present work, the signals first showed signs of changing on warming to about 205 K. However, most of the observed change (Fig. 2; see below) took place during 20 min incubation at 220 K. Care had to be taken to prevent samples being blown out of the e.p.r. tubes by expulsion of gas from the samples, if the temperature was raised too suddenly.

E.p.r. spectra were recorded on a Varian E9 spectrometer. A dual-sample cavity was used where precise standardization of the field scale was important. While spectra were being recorded by the E9 spectrometer they were digitalized, using 256 field steps per spectrum, and recorded, together with details of the running conditions, for later manipulation. The equipment (from Digital Equipment Co., Galway, Ireland) comprised a PDP 11/10 computer with 16 k words of core store, a Decwriter II, a TA11 cassette tape unit (with two cassette stations) and an AR11 interface unit. The latter unit allowed for 'interrupt driven' recording of spectra from the E9 spectrometer and was also connected to a 1300A X-Y visual display unit (from Hewlett-Packard, Slough, Bucks., U.K.), where four spectra could be displayed

simultaneously. The display driver in the AR11 interface unit could also be used to operate the E9 spectrometer as an X-Y plotter, for the redrawing of spectra after manipulation. A curve-fitting routine interpolated between the points, for plotting and other purposes. Spectra of normal complexity, re-plotted via the computer, were indistinguishable from those recorded directly on the E9 spectrometer, apart from some loss of high-frequency noise where this was present. For manipulation purposes, a specially constructed interface between the AR11 interface unit and the E9 spectrometer and display system enabled four ten-turn potentiometers to be used for input of parameters to the computer. The software for the system was based partly on the Sparta Laboratory Applications modules produced by the Digital Equipment Co. and was written in PDP 11 Assembly Language. Spectra were recalled from the cassettes by using the running date and a sequential spectrum number. While displayed, they could be continuously shifted or expanded in the X- or Y-directions, subtracted from or added to each other in any desired proportions, and doubly integrated (with normalization for running conditions) between moveable cursors. Any of these operations could be performed at the same time as recording of other spectra was taking place. Facilities were incorporated in the program for averaging a number of input spectra.

An important feature of the system was that spectra from both halves of the dual cavity could be recorded at the same time. When difference spectra were required, the field shift that had to be applied to compensate for differences in the microwave frequency between the samples could thus be evaluated from the spectra from the reference cavity, which contained Mn²⁺ and diphenylpicrylhydrazyl standards. Alignment to 0.02 mT or better was found to be essential to obtain valid difference spectra. The procedure involved subtracting increasing proportions of one spectrum from another until selected features obviously common to both spectra had disappeared, and the difference spectrum resulting was thought to correspond to a theoretical lineshape for a single species. The process could then be carried out in the reverse direction, subtracting the second spectrum from the first, to obtain the spectrum of the second component. Thus in Fig. 1 spectrum (c) corresponds to $3 \times [(b) - 0.75(a)]$ and (d) to $2 \times [(a) - 0.56(b)]$. Simulation of e.p.r. spectra was also carried out on the PDP 11/10 computer; details of the program used are given by Lowe (1978).

Results and Discussion

Nature of the two xanthine complexes

The precise form of the Rapid signal obtained on reduction of xanthine oxidase with xanthine was

found to depend on the conditions used (Figs. 1*a*, 1*b* and 2*a*–2*c*). Figs. 1*(a)* and 1*(b)* show the effect of increasing the xanthine concentration at a fixed pH, and Figs. 2*(a)* and 2*(b)* illustrate the effect of pH variation at a fixed xanthine concentration. Difference spectra obtained from Figs. 1*(a)* and 1*(b)* are illustrated in Figs. 1*(c)* and 1*(d)*; these are presumed to correspond to single chemical species, since the line-shapes may be simulated (Figs. 1*c'* and 1*d'*) (see the Materials and Methods section for details of the

subtraction and computer-simulation procedures). The two species will be referred to as xanthine Complex 1 (Figs. 1*c* and 1*c'*) and xanthine Complex 2 (Figs. 1*d* and 1*d'*) (Bray & Vännegård, 1969). In confirmation of the conclusions of these workers, in both species, molybdenum(V) is coupled to two protons, these protons being equivalent in Complex 2 and non-equivalent in Complex 1. Parameters used in the

Fig. 1. Resolution of the Rapid signal, obtained by reduction with xanthine, into the xanthine Complex-1 and the xanthine Complex-2 spectra, and computer simulations (a), (b) Experimental spectra obtained by reducing the enzyme at pH 9.5 (0.1 M-Ches buffer) for 1 min with 0.6 or 3.3 mM-xanthine respectively. The enzyme concentration was about $40 \mu\text{M}$ (functional active centres). (c), (d) Difference spectra, obtained from (a) and (b) as described in the Materials and Methods section. (c'), (d') Computer simulations of the difference spectra. E.p.r. conditions were: 150 K, 10 mW microwave power and 0.1 mT modulation. The field scale corresponds to a microwave frequency of 9.292 GHz. Parameters used in the simulation of spectrum (c') (with proton splittings and full linewidths in mT) were: g_1 1.9906, g_2 1.9707, g_3 1.9654; A_1^H 1.2, 0.47, A_2^H 1.3, 0, A_3^H 1.3, 0; linewidths 0.3, 0.36, 0.36; and in spectrum (d') were: g_1 1.9951, g_2 1.9712, g_3 1.9616; A_1^H 1.07, 1.07, A_2^H 0.98, 0.98, A_3^H 0.93, 0.93; linewidths 0.52, 0.3, 0.52. Stick diagrams below the simulated spectra show vertical lines above, crossing or below the baseline to correspond to the positions of the proton-split g_1 -, g_2 - and g_3 -features respectively.

Fig. 2. Rapid signal obtained under various conditions of reduction in the presence of xanthine, or dithionite and uric acid

Reduction with xanthine (4.8 mM) for 0.15 s was used in (a) and (b), at pH 6.1 (Mes buffer) and 9.5 (Ches buffer) respectively. The enzyme concentration was about $120 \mu\text{M}$ (functional active centres). The sample in (c) is the one from (b), but after warming gradually to a temperature of 242 K (see the Materials and Methods section). The sample in (e) was reduced with dithionite (40 mM) for 1.0 s, at about pH 7.5 (Mops buffer). In (d) the enzyme was first reduced with dithionite (300 mM) for 0.4 s, then mixed with uric acid (final concentration 3.6 mM) and frozen 0.16 s later (see the Materials and Methods section for details of the procedure); the final pH was about 7.1 (Hepes buffer). E.p.r. conditions were as in Fig. 1. To clarify the spectra in the low-field region, an FADH^+ signal has been subtracted from spectra (a), (b), (d) and (e).

simulations (Fig. 1) differ only slightly from those used in the earlier work.

Since in Fig. 1 the amount of Complex 1 increased relative to that of Complex 2 on increasing the xanthine concentrations from 0.6 mM to 3.3 mM, relatively weak binding of xanthine in the Complex-1 signal-giving species at this pH value is suggested. Lower pH values tend to favour Complex 1 relative to Complex 2 (compare Figs. 2*a* and 2*b*), although our studies at low pH values were limited by the solubility of xanthine. On the other hand, we found that reaction time had little effect on the relative amounts of the two species. Computer additions and subtractions (results not shown) revealed that spectra obtained by reduction with xanthine under a wide range of conditions could generally be accounted for as mixtures of the xanthine Complex-1 signal, the xanthine Complex-2 signal and the uncomplexed Rapid signal (Fig. 2*e*; see below). The uncomplexed form was the dominant one at low pH values, with low xanthine concentrations. Additional signals observed under specific conditions were the Very Rapid signal, at high pH values and short reaction times (Bray, 1975), and occasionally signals attributed to uric acid complexes (see below). [The Slow signal from the desulpho enzyme becomes apparent only after reaction periods of hours (Swann & Bray, 1972).]

The 'warming-up' experiment illustrated in Figs. 2*b* and 2*c* is particularly significant. Xanthine oxidase can be shown to undergo in the frozen state at least some of the steps of the turnover cycle, when samples prepared by the rapid-freezing method are suitably warmed (Pick & Bray, 1969; Van Heuvelen, 1975). During the warming procedure, the xanthine Complex-1 signal decreased very substantially in intensity, so that the spectrum remaining was a fairly pure xanthine Complex-2 signal (compare Figs. 1*d* and 2*c*). That the species lost on warming was largely xanthine Complex 1 was confirmed by examination of the difference spectra (results not shown). It is quite unlikely that the rate of turnover of the enzyme in the frozen state under the conditions of the experiment (see the Materials and Methods section) would be adequate to deplete significantly the high xanthine concentration that was used (cf. Figs. 1*a* and 1*b*). Therefore this result implies that only the Complex-1 species can represent an intermediate in the catalytic cycle. We suggest therefore that the xanthine molecule is capable of binding to the active centre of the enzyme in two different orientations, represented respectively by the xanthine Complex-1 and xanthine Complex-2 Rapid molybdenum(V) e.p.r. signals. Of these, only the Complex-1 signal represents productive binding to the active centre. That a substrate molecule could be bound in two different ways is not in any way unreasonable, bearing in mind the extremely low specificity of xanthine oxidase (Bray, 1975). Indeed, independently, Bergmann & Levene (1976) con-

cluded that xanthine may be bound to the enzyme in 'favourable' and 'unfavourable' modes, from entirely different studies.

Further warming of the sample of Fig. 2*c* resulted in some reappearance of the Very Rapid signal, as reported by Pick & Bray (1969). More work is required on the relationship between the Very Rapid and Rapid signals; the former may represent an intermediate in which the xanthine molecule is covalently bound to the enzyme (Bray, 1975, 1977).

E.p.r. signals from uric acid complexes

We succeeded in obtaining signals that we attribute to complexes with uric acid, by reducing the enzyme with dithionite and adding uric acid. Fig. 2*d* shows a spectrum obtained in this way, and Figs. 3*a*–3*d* show the effects of gradually increasing concentrations of uric acid. Though traces of xanthine might be formed by enzymic reduction of uric acid in the presence of dithionite (Stöhrer & Brown, 1969), the rate of this back reaction is expected to be slow (Bray, 1975), so that it is unlikely that enough xanthine would be formed to interfere. In any case, despite the obvious similarities, the spectrum in the presence of uric acid (Fig. 2*d*) clearly contains features that are not attributable either to the xanthine Complexes 1 or 2 (Figs. 1*c* and 1*d*) or to the uncomplexed Rapid signal (Fig. 2*e*). This has been confirmed by experiments in $^2\text{H}_2\text{O}$ at 35 GHz (Barber, 1976), which showed the main g_1 -feature in the presence of uric acid to be at a position intermediate between the g_1 -feature of the xanthine Complex-1 spectrum and the g_1 -feature of xanthine Complex-2 spectrum.

Unfortunately, signals obtained in the presence of uric acid are relatively weak, no doubt because of its preferential binding to the enzyme with molybdenum in the quadrivalent state, and we have not so far analysed the 'uric acid Rapid' spectrum completely. However, the best interpretation seems to be that the spectrum is from a mixture of two species, giving signals comparable with those from the two complexes with xanthine, but having slightly different parameters. Thus the g_1 -feature of Complex 2, giving the characteristic 1:2:1 triplet at low field, is displaced by about 0.3 mT to higher field, for uric acid relative to xanthine (compare Figs. 2*d* and 2*c*).

Detailed comparison confirms that the spectra of Bray & Swann (1972) and their interpretation, as given above in the introduction, are fully consistent with the present work. However, in experiments in which the enzyme was reduced directly with xanthine we have only rarely encountered the 'uric acid Rapid' signal. This is no doubt due to the conditions for its formation being critical, as would be expected if uric acid is more weakly bound to the Mo(V) form of the reduced enzyme than is xanthine. One experiment in which the uric acid complex undoubtedly was formed,

Fig. 3. *Modification of the Rapid signal by uric acid (a–d), by 1-methyluric acid (e) or by 1-methylxanthine (f)* For (a)–(e), the enzyme was first reduced with dithionite, then treated with uric acid or 1-methyluric acid; conditions were similar to those in Fig. 2(d). In (f) the enzyme was reduced directly with 1-methylxanthine for 0.05 s. All experiments were performed in 0.05 M-sodium pyrophosphate buffer, pH 8.2. In (f), the spectrum has been simplified by subtracting successively, an $\text{FADH}^{\bullet}$ signal and a signal from enzyme enriched in ^{95}Mo and also reduced with 1-methylxanthine. E.p.r. conditions were: 120 K, 5 mW microwave power, 0.25 mT modulation; microwave frequency about 9.3 GHz, except in (f), where the power was 10 mW and the modulation 0.1 mT.

since the resulting spectrum was almost identical with that in Fig. 2(d), was carried out under partial anaerobiosis with a moderate excess of xanthine, at the low pH value of 5.6.

Complexes with other purines

The Rapid signal from xanthine oxidase reduced by 1-methylxanthine is illustrated in Fig. 3(f), and shows no signs of features, particularly those at low and at high field, that are attributable, with xanthine, to the Complex-2 spectrum. From additional experiments under a range of conditions we conclude that 1-methylxanthine yields only a Complex-1 type of spectrum. Thus it, and also purine (Bray & Vännård, 1969), apparently bind in one orientation only to the enzyme.

Parameters of the 1-methylxanthine Rapid signal, observed in the presence of an excess of the substrate, appear to be quite like those for the xanthine Com-

plex-1 signal (Fig. 1c'), except that the separation of the g_2 - and g_3 -features is smaller. That formation of the complex is complete at a 1-methylxanthine concentration of 3 mM is suggested by a spectrum, at 35 GHz, of a sample in $^2\text{H}_2\text{O}$ (Barber, 1976). This appeared to correspond to a single species. Some conversion into the 1-methylxanthine Rapid species was detectable at the low substrate concentration of 0.2 mM, when short reduction times were used (e.g. 0.01 s at 13°C and pH 8.2 in pyrophosphate buffer, with enzyme in slight excess). This implies tight binding. On the other hand, under these conditions, with longer reaction times, the spectrum changed to a form like that in Fig. 3(e), which is attributed to the product complex (see below).

We now discuss features of the Complex-1 spectrum, common to xanthine, purine and 1-methylxanthine. It should be noted that the spectrum in the absence of complex-formation (Fig. 2e) needs further study, since Bray & Vännård (1969) reported that

even this apparently simple, though rather broad, signal is due to two species. However, we have found that a particular feature of the Complex-1 spectrum, which in all cases distinguishes it from the uncomplexed spectrum, is the displacement of the g_1 -feature in the complex forms towards higher fields. For 1-methylxanthine the shift amounted to about 0.3 mT.

Another prominent and general feature of the Complex-1 spectrum is the secondary splitting of the g_1 -feature, attributed to interaction with a relatively weakly coupled proton. For the 1-methylxanthine signal, we were able to confirm (Fig. 3f), by using ^{95}Mo -substituted xanthine oxidase and subtracting its spectrum from that of the unenriched form, that this secondary splitting is not an apparent one, caused by interference from ^{95}Mo (and ^{97}Mo) lines. For xanthine (Fig. 1c') the splitting amounts to about 0.47 mT, whereas with purine (Bray & Vänngård, 1969) or with 1-methylxanthine the splitting is slightly less, about 0.3 mT.

Finally, we have obtained direct evidence for an e.p.r.-detectable 1-methyluric acid complex (Fig. 3e), in an experiment analogous to the ones with uric acid, described above. Interestingly, though with 1-methylxanthine the substrate does not give a Complex-2 signal, the product does form one, as is evidenced by the high- and low-field features of the 1-methyluric acid spectrum shown in Fig. 3(e).

Further work will be required, with isotopically labelled substrate or product molecules, if information is to be obtained about the geometry of the binding of these molecules to the active site of xanthine oxidase in the Complex-1 and Complex-2 species. It is possible that some of the interacting protons are located on the enzyme, whereas others are on the bound substrate molecules. On the other hand, if the protons are all on the enzyme, then substrate-induced conformation changes must be involved in converting one signal-giving species into another.

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