Molybdenum(V) e.p.r. signals obtained from xanthine oxidase on reduction with aldehyde substrates and with 2-amino-4-hydroxy-6-formylpteridine

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2-Amino-4-hydroxy-6-formylpteridine, a known 'slow' substrate and inhibitor of xanthine oxidase, is unusual in that it gives rise under suitable conditions to all types of molybdenum(V) e.p.r. signals obtainable from the enzyme, namely Very Rapid, Rapid, Inhibited and Slow. The Very Rapid signal appears in a slightly modified form. The Inhibited signal, originally thought to be unique to reaction of methanol or of formaldehyde with xanthine oxidase, is now shown to be obtainable with several other aldehydes. These include, in addition to 2-amino-4-hydroxy-6-formylpteridine, acetaldehyde and glycolaldehyde. Parameters of the signals, obtained with the help of computer simulations, are presented. The appearance of Very Rapid and of Inhibited signals with these additional substrates may be of importance in elucidating the structure of the enzyme active centre. In agreement with previous work, the Very Rapid signal is attributed to an obligatory intermediate in turnover. On the other hand, the Inhibited signal is attributed to a side reaction, presumably inhibitory in nature, occurring during the catalytic process.

The mechanism of action of xanthine oxidase has been extensively studied by e.p.r. of molybdenum(V) in the active centre of the enzyme (Gutteridge & Bray, 1979; Bray, 1980*a,b*). Four classes of e.p.r. signals from the metal may be detected under appropriate conditions. Signals given the name Rapid represent the normal reduced form of the enzyme. Slow signals represent the corresponding reduced form of the desulpho enzyme, this being a degradation product of the active enzyme. The Very Rapid signal is believed to represent an intermediate in turnover of substrates, having the substrate residue covalently bound to the active centre (Gutteridge & Bray, 1980; Malthouse & Bray, 1980). However, it has been detected for a few substrates only, e.g. xanthine and 1-methylxanthine. Finally, the Inhibited signal is believed to provide evidence for an inhibitory side reaction, of unknown nature, that occurs during the turnover processes. This side reaction occurs during oxidation of the substrate formaldehyde (Pick *et al.*, 1971) as well as during the extremely slow turnover of ethylene glycol (Tanner & Bray, 1978*a,b*). It seemed of

importance in seeking to understand the mechanism of action of the enzyme to know if the Very Rapid and Inhibited signals were obtainable from a wider range of substrates. We now present information on this point.

Materials and methods

Materials

Xanthine oxidase was prepared by the salicylate denaturation procedure of Hart *et al.* (1970). Concentrations of functional active centres were calculated from activity and A_{450} measurements (Bray, 1975). 2-Amino-4-hydroxy-6-formylpteridine was a gift from Professor A. Albert and Dr. W. L. F. Armarego, John Curtin School of Medical Research, Australian National University, Canberra, A.C.T., Australia; 2-amino-4-hydroxy-6-hydroxymethylpteridine (85% purity) was a gift from Dr. J. A. Montgomery, Southern Research Institute, Birmingham, AL, U.S.A. Acetaldehyde was redistilled and solutions of it were standardized as described by Malthouse *et al.* (1980). [^2H]Formaldehyde was prepared by hydrolysis (Pick *et al.*, 1971) of the corresponding para-formaldehyde [98% isotopic purity, from Merck, Sharpe and Dohme (Canada), c/o Cambrian Chemicals, Croydon, Surrey CR0 4XB, U.K.].

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Glycolaldehyde was from Koch–Light Laboratories, Colnbrook, Bucks. SL3 0BZ, U.K.

E.p.r. samples

Xanthine oxidase (final concentration 0.1 to 0.6 mM) was reduced in e.p.r. tubes by addition of the appropriate substrates before being frozen in liquid N₂. In some cases, as indicated, reduction was conducted anaerobically, whereas in others air or O₂ was bubbled gently through the sample immediately before it was frozen. Rapid freezing was performed as described by Gutteridge *et al.* (1978).

E.p.r. procedures

E.p.r. spectra were recorded with a Varian E9 spectrometer linked to a computer (Bray *et al.*, 1978). Recording conditions were as follows: temperature about 150 K; microwave power 10 mW; microwave frequency 9.3 GHz; modulation amplitude 0.16 mT, except for Figs. 1(a), 4(a) and 4(d), for which it was 0.5, 0.2 and 0.2 mT respectively. A dual-sample cavity was employed. Cu²⁺–EDTA complex was used as standard for absolute intensity measurements. Computer-generated difference spectra were obtained as described previously (Bray *et al.*, 1978). Computer simulation of e.p.r. spectra employed the program of Lowe (1978) or an iterative version of this (D. J. Lowe, unpublished work).

Results

Signals from 2-amino-4-hydroxy-6-formylpteridine

2-Amino-4-hydroxy-6-formylpteridine has long been known to be a very 'slow' substrate for xanthine oxidase as well as being a potent inhibitor of the oxidation of xanthine and other substrates by the enzyme (Lowry *et al.*, 1949). The compound was reported to be oxidized by the enzyme in the presence of O₂ at a rate corresponding to 0.0007 of the rate of xanthine oxidation. The product, though not fully characterized, was presumed to be the corresponding pteridinecarboxylic acid. 2-Amino-4-hydroxy-6-hydroxymethylpteridine has also been reported to be a potent xanthine oxidase inhibitor (Petering & Schmitt, 1950).

Fig. 1 shows e.p.r. signals that were observed on treatment of the enzyme under various conditions with the formylpteridine. Parameters associated with some of the signals are given in Table 1. Under most conditions mixtures of signals were obtained (e.g. Fig. 1a), but under specific reaction conditions or by using difference techniques individual signals could be obtained. It was in no sense unexpected to find that Rapid signals (Fig. 1c) and Slow signals (not illustrated, but cf. Fig. 1a) were obtainable. On the other hand, the signal of Fig. 1(b) was less expected. The high value of g_1 and the absence of proton

Fig. 1. Molybdenum(V) e.p.r. signals from treatment of xanthine oxidase with 2-amino-4-hydroxy-6-formylpteridine

Spectrum (a) shows a mixture of signals in which Inhibited and Very Rapid signals are prominent, though contributions from the Slow signal are also present. Spectra (b), (c) and (d) show respectively Very Rapid, Rapid and Inhibited signals. Spectra (a), (c) and (d) are spectra recorded directly, whereas (b) is a difference spectrum. pH values (measured with a micro electrode after the spectra had been recorded), final substrate concentrations and reaction times (anaerobic conditions) in spectra (a)–(d) were respectively as follows: (a) pH 7.5, 0.7 mM, 30 min; (b) pH 7.2, 4.0 mM, 1 min; (c) pH 8.5, 0.7 mM, 45 min; (d) pH 9.8, 1.0 mM, 5 min. For spectrum (c) the sample was kept anaerobic, but for spectra (a), (b) and (d) air or O₂ was bubbled briefly through the samples immediately before they were frozen. Buffers were 60 mM-sodium pyrophosphate for (a), (c) and (d), and 20 mM-Bicine [NN-bis-(2-hydroxyethyl)glycine]/NaOH for (b). A small amount of the Inhibited signal was subtracted from spectrum (b) to give the spectrum shown. Here and in Figs. 2 and 4 the arrow corresponds to $g = 2.0037$.

splittings leads us to attribute this signal to the Very Rapid class. Fig. 1(d) shows that 2-amino-4-hydroxy-6-formylpteridine gives rise to an Inhibited signal also, this being of the type that lacks proton splittings, thus resembling the Inhibited signal from glycol (Tanner & Bray, 1978b) rather than that from formaldehyde (Pick *et al.*, 1971). The g -values of the new signal (Table 1) differ slightly from those of the Inhibited signals from these other substrates.

We made limited studies of the conditions required to obtain the Very Rapid and Inhibited signals. The Very Rapid signal was detected at

Table 1. *E.p.r. parameters of molybdenum(V) signals from xanthine oxidase*

Parameters were determined with the help of computer simulations. *g*-values are believed to be accurate to ± 0.0003 . Half-linewidths are given in mT.

Signal	Substrate	<i>g</i> -values (half-linewidths)				Reference
		1	2	3	Average	
Very rapid	Xanthine	2.0252 (0.19)	1.9550 (0.16)	1.9494 (0.19)	1.9765 (0.18)	Tanner <i>et al.</i> (1978)
Alloxanthine	Alloxanthine*	2.0269	1.9593	1.9444	1.9769	Williams & Bray (1981)
Very rapid	2-amino-4-hydroxy-6-formylpteridine	2.0221 (0.24)	1.9551 (0.20)	1.9466 (0.27)	1.9746 (0.24)	Present work
Inhibited	2-amino-4-hydroxy-6-formylpteridine	1.9970 (0.20)	1.9800 (0.18)	1.9541 (0.20)	1.9770 (0.19)	Present work
Inhibited	Formaldehyde†	1.9911 (0.20)	1.9772 (0.14)	1.9513 (0.17)	1.9732 (0.17)	Tanner <i>et al.</i> (1978)
Inhibited	Acetaldehyde	1.9936 (0.22)	1.9783 (0.15)	1.9546 (0.19)	1.9775 (0.19)	Present work
Inhibited	Glycolaldehyde	1.9931 (0.25)	1.9777 (0.18)	1.9547 (0.21)	1.9752 (0.21)	Present work
Inhibited	Glycol*	1.9932 (0.21)	1.9780 (0.15)	1.9547 (0.18)	1.9753 (0.18)	Tanner & Bray (1978b)

* An added reducing agent was required to generate the signal.

† These values were confirmed in the present work. Coupling to a non-exchangeable proton is also observed in this signal, which is obtainable on treatment with either formaldehyde or methanol.

maximum amplitude after incubation of the enzyme with the substrate and with air for a few minutes at pH 7–8. We failed to detect the signal under anaerobic conditions. The Inhibited signal, though sometimes detectable after incubation of the enzyme and substrate in the presence of air for as little as 1 min (particularly if the pH was above 9), increased steadily in amplitude when the incubation time was extended. Additional experiments in which 2-amino-4-hydroxy-6-formylpteridine was replaced by 2-amino-4-hydroxy-6-hydroxymethylpteridine showed that this compound, too, could give rise to the Very Rapid and Inhibited signals. The forms of the signals with the two pteridine derivatives could not be distinguished from one another.

Signals from aldehydes

It has long been known (Pick & Bray, 1969) that simple aldehyde substrates of xanthine oxidase can give rise to the Rapid molybdenum(V) signal. In the present work we studied particularly acetaldehyde. Signals obtained with this substrate are shown in Fig. 2 and the time course of their development is presented in Fig. 3. In agreement with work on other aldehyde substrates (Bray *et al.*, 1967), Very Rapid signals were not detected. Conditions tried included short reaction times (6 ms) as well as pre-reduction of the enzyme with xanthine, with subsequent addition of the aldehyde followed by aeration (cf. work with alloxanthine by Williams & Bray, 1981). The Slow signal was not detected with acetaldehyde

Fig. 2. Rapid e.p.r. signal obtained from reaction of xanthine oxidase with acetaldehyde

The reaction times in spectra (a) and (b) under anaerobic conditions with 5.5 mM-acetaldehyde were respectively 0.65 s (rapid freezing) and 62 min. An FADH[•] signal is also present in spectrum (a). The experiment was performed as described in the caption to Fig. 3.

at reaction times up to 2 h under anaerobic conditions.

Under particular reaction conditions with concentrated acetaldehyde we generated the Inhibited signal (Fig. 4c). Figs. 4(a) and 4(b) show for comparison Inhibited signals from [³H]formaldehyde and from 2-amino-4-hydroxy-6-formylpteridine respectively. We also found that the

Fig. 3. Time course of development of Rapid molybdenum(V) and of $FADH^\bullet$ signals during anaerobic reduction of xanthine oxidase by acetaldehyde

The vertical scale represents fractional conversion of molybdenum and flavin in the functional enzyme into the signal-giving species ($\bullet$, molybdenum; $\circ$, $FADH^\bullet$), and the horizontal scale represents reaction time at pH 8.2 in 0.11 M-Bicine/NaOH buffer at 22°C with 5.5 mM-acetaldehyde. The xanthine oxidase concentration was 0.2 mM, and EDTA (1.5 mM) was also present. Rapid freezing was used for times up to 300 s.

Inhibited signal could be generated with another good aldehyde substrate of xanthine oxidase, namely glycylaldehyde (Fig. 4d). Parameters of the various Inhibited signals are listed in Table 1.

Discussion

2-Amino-4-hydroxy-6-formylpteridine (together with the corresponding 6-hydroxymethyl compound) is unique among xanthine oxidase substrates in giving rise to molybdenum(V) e.p.r. signals belonging to all four classes. In considering these signals, the possibility of the compound reacting at the pteridine 7-position ought to be considered, in addition to oxidation of its aldehyde function. The observation of a Very Rapid signal from this substrate is particularly noteworthy. Parameters of the signal with the pteridine substrate are closer to those of the Alloxanthine signal (Williams & Bray, 1981) or the high-pH version of the xanthine Very Rapid signal (Gutteridge & Bray, 1980) than to those of the standard Very Rapid signal (Tanner *et al.*, 1978). Nevertheless, ^{33}S -substitution work (J. P. G. Malthouse & R. C. Bray, unpublished work) confirms the allocation of the pteridine signal to the Very Rapid class.

The observation of the Very Rapid signal with the pteridine substrate is consistent with, and provides some further support for, the proposal that this signal represents an obligatory intermediate in

Fig. 4. Inhibited e.p.r. signal from reaction of various aldehydes with xanthine oxidase

The aldehydes used for spectra (a)–(d) were respectively $[^2\text{H}]$ formaldehyde, 2-amino-4-hydroxy-6-formylpteridine, acetaldehyde and glycylaldehyde. Spectrum (b') is a simulation of (b), based on the parameters of Table 1. Reaction conditions were as follows, the medium being in all cases 20 mM-Bicine/NaOH buffer, pH 8.2. In spectra (a) and (d) the aldehyde at a final concentration of 200 mM was added to the enzyme and the solution was occasionally shaken gently with air for 4 h at about 20°C. For spectrum (c) the enzyme was first reduced with acetaldehyde (1 mM) for 5 min, then the concentration of the substrate was raised to 760 mM and the sample was frozen after a further 2 min. For spectrum (b) enzyme was first reduced anaerobically for 1 min with xanthine (1 mM), 2-amino-4-hydroxy-6-formylpteridine (5 mM) was then added and air was bubbled through the sample, which was frozen 1 min after the second addition.

turnover of the enzyme. Detailed kinetic work including product-release studies is obviously desirable, but a turnover time of about 2 min is expected.

A requirement for oxygen might indicate that one-electron reoxidation of the initially produced two-electron-reduced enzyme molecule is required before the signal can be seen, in contrast with the situation with xanthine (Olson *et al.*, 1974).

Our observations on the Inhibited signal increase substantially the number of aldehyde substrates that are now known to give rise to this signal. That glycolaldehyde gives the signal strengthens the view (Tanner & Bray, 1978a) that this compound is an intermediate in the reactions of xanthine oxidase with ethylene glycol yielding the Inhibited signal (Tanner & Bray, 1978b). In fact *g*-values for the Inhibited signals for glycolaldehyde (Table 1) and for glycol (Tanner & Bray, 1978b) are not distinguishable.

With the Inhibited signal derived from methanol or formaldehyde, it has been shown by ^{13}C - and by ^2H -substitution work (Tanner *et al.*, 1978; Pick *et al.*, 1971) that a $-\text{CHO}$ group or the equivalent, derived from the substrate, interacts with molybdenum in the signal-giving species. For the other aldehydes yielding the signal, the lack of proton splittings indicates that the group bound must be $-\text{RCO}$. Small changes in the *g*-values for the different Inhibited signals (Table 1) clearly reflect the presence of different R groups in the signal-giving species.

Though we have not made activity measurements or detailed kinetic studies, previous proposals on the origin of the Inhibited signal remain acceptable. With acetaldehyde or with glycolaldehyde, as with formaldehyde (Pick *et al.*, 1971), long reaction times or high substrate concentrations or both are required to generate this signal. On the other hand, with 2-amino-4-hydroxy-6-formylpteridine low concentrations and short times sufficed, presumably reflecting the much lower apparent K_m (Lowry *et al.*, 1949) for the pteridine derivative. It is considered that the Inhibited signal provides evidence for a side reaction to the turnover processes, in which partly reduced enzyme has reacted with a substrate molecule in a manner that does not lead to product release, but rather to the formation of a relatively stable intermediate incorporating the $-\text{RCO}$ residue. Such a 'wrong' reaction might perhaps involve (cf. Bray *et al.*, 1979) an interchange in the roles of the proposed nucleophilic group and proton-acceptor group of the catalytic centre. On the other hand, apparently only aldehyde and not purine substrates are capable of undergoing this side reaction, and this presumably indicates specific involvement of the carbonyl group. Normal turnover of aldehyde substrates involves their non-hydrated forms (Gregory *et al.*, 1972). It might be speculated that the Inhibited signal involves reaction of the aldehydes, instead, in the hydrated form and conceivably in a reaction involving both the nucleo-

phile and the proton acceptor of the enzyme. ^{17}O -substitution studies should throw further light on the structure of the Inhibited-signal-giving species.

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