Oxygen-17 splitting of the Very Rapid molybdenum(V) e.p.r. signal from xanthine oxidase

RATE OF EXCHANGE WITH WATER OF THE COUPLED OXYGEN ATOM

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Studies have been carried out of effects of ^{17}O substitution on a Mo(V) e.p.r. signal from xanthine oxidase, known as Very Rapid. This transient signal is believed to represent an intermediate in enzymic turnover. When Very Rapid was developed from enzyme equilibrated with ^{17}O -enriched water, strong coupling of Mo(V) to a single oxygen atom was observed, with $A(^{17}\text{O})_{1,2,3}$ 1.34, 1.40, 1.36 mT. The isotropic character of the splittings is interpreted as favouring a structure of the type Mo–O–C. The rate of exchange with water of the oxygen atom detected in the signal was studied. In oxidized enzyme, which contains a terminal oxygen ligand, the exchange rate constant was $2\text{--}4\text{ h}^{-1}$ (pH 5.9–7.8 and about 20°C). However, if the exchange was allowed to take place whilst the enzyme was turning over a substrate, then the process occurred within a few seconds. The present and previous results are interpreted as favouring an enzymic mechanism in which a terminal oxygen ligand reacts, as a nucleophile, with a substrate carbonium ion. To complete the reaction, product liberation, by hydrolysis of the enzyme-bound species, occurs in such a way as to cleave the Mo–O bond, thus explaining the fast oxygen exchange in the presence of the substrate.

The mechanism of action of xanthine oxidase and the structure of the molybdenum centre of the enzyme, at which most reducing substrates of the enzyme react, has been extensively studied (Bray, 1975, 1980*a,b*; Gutteridge & Bray, 1979, 1980). Most information has been derived from e.p.r. spectroscopy. Particularly important was the finding (Gutteridge *et al.*, 1978*a*) that the hydrogen atom of substrate molecules such as 1-methyl-xanthine, replaced in the catalytic reaction by a hydroxy group, is transferred during the catalytic process to the enzyme. The proton-accepting group for this reaction may be a terminal sulphur ligand on the molybdenum atom, converted to a molybdenum-bound-SH group (Gutteridge *et al.*, 1978*b*). Additional information was provided recently by e.x.a.f.s. spectroscopy (Bordas *et al.*, 1980). This supported the presence of a terminal sulphur ligand of the metal in the oxidized functional xanthine oxidase molecule. This group is, however, not present in the non-functional desulpho modification of the enzyme (Bordas *et al.*, 1979, 1980); see also Tullius *et al.*, 1979).

Abbreviation used: e.x.a.f.s., X-ray absorption extended fine structure.

A further important question on the mechanism of action of the enzyme concerns whether or not an oxygen ligand of molybdenum, known to be present from e.x.a.f.s. studies, is involved in the catalytic processes.

The isotope oxygen-17 has a low natural abundance and possesses a magnetic nucleus with I (nuclear spin) = $5/2$. Thus if it replaces normal oxygen as, for example, a ligand of molybdenum when the metal is in the quinquivalent state, then oxygen-17 splitting of the molybdenum e.p.r. signal should be observed. Preliminary work with ^{17}O -enriched water on xanthine oxidase (Gutteridge *et al.*, 1979), sulphite oxidase and other molybdenum-containing enzymes (S. Gutteridge, T. Lamy & R. C. Bray, unpublished work; Cramer *et al.*, 1979) has indicated that considerable information should be forthcoming from studies of ^{17}O coupling to Mo(V). Unfortunately, ^{17}O is not readily available at enrichments greater than about 50% and ^{17}O hyperfine splittings tend to be large, so that the resulting split e.p.r. signals are of low amplitude. A further complication is that, for many of the enzyme signals, proton hyperfine splitting will be superimposed on ^{17}O splitting, yielding very complicated spectra of still lower amplitude. Because of this,

Gutteridge *et al.* (1979) were not able fully to interpret ^{17}O splitting of the Rapid Mo(V) signal from xanthine oxidase.

We therefore turned our attention to another molybdenum(V) e.p.r. signal from xanthine oxidase, lacking proton splittings, namely Very Rapid. This signal, originally described by Palmer *et al.* (1964), is a transient, detected only by rapid freezing during the time range from a few milliseconds to a few tens of milliseconds of reaction between the enzyme and the substrate xanthine, at ambient temperatures (Olson *et al.*, 1974). Very Rapid is favoured by high pH values (see Tsopanakis *et al.*, 1978). The signal is believed (Bray *et al.*, 1979) to represent an intermediate in which the xanthine residue, having lost a hydride equivalent from the C-8 position, has become covalently bound to a nucleophilic group in the enzyme active centre.

We now describe the effect of ^{17}O substitution on the Very Rapid signal. A single coupled oxygen atom in the signal-giving species is revealed. We have studied the kinetics of exchange of this oxygen with water, and results provide new information about the structure of the active centre and the mechanism of action of the enzyme.

Materials and methods

Xanthine oxidase

Xanthine oxidase was partially purified by the salicylate-denaturation method of Hart *et al.* (1970). Concentrated enzyme solutions in dilute (e.g. 1 mM) Bicine [*NN*-bis-(2-hydroxyethyl)glycine]/NaOH buffer, pH 8.2, were frozen as beads by dropping into liquid N_2 . The beads were then freeze-dried, without thawing. Samples were re-dissolved, as necessary, in buffers made up in ordinary water or in ^{17}O -enriched water. Some loss of enzymic activity occurred in the freeze-drying process. Enzyme samples used were approx. 60% functional (calculated from activity/ A_{450} measurements; see Bray, 1975).

Rapid freezing

The rapid-freezing method of Bray (1961), which involves quenching samples by squirting into cold isopentane, was used as described by Gutteridge *et al.* (1978a). For most of the experiments three syringes and two mixers were employed. This permitted, for example, dilution with ordinary water at a predetermined pH value, of a solution of xanthine oxidase in enriched water. After a suitable reaction period under these conditions, addition of xanthine in concentrated buffer at a high pH value could be made. The reaction with the xanthine could then be continued, e.g. for 11 ms, to allow the Very Rapid signal to attain optimum intensity before the process was terminated by freezing the sample. Details of the

flow systems are given in Schemes 1 and 2 below (see the Results section). In all cases the enzyme solution (concentration of functional active centres 0.6 mM) in ^{17}O -enriched water contained dilute Bicine buffer (about 0.5 mM; pH about 8.0). The enzyme syringe used had a capacity of 0.25 ml. Also, in all experiments, the final addition was of xanthine (10 mM) in carbonate buffer (approx. 1 M, so adjusted that in all cases the final pH after mixing was 10.2 ± 0.2). In those experiments that involved reduction of xanthine oxidase with salicylaldehyde during the dilution step, the substrate concentration before mixing was about 0.5 mM.

Solutions in ^{17}O -enriched water

Enriched water containing up to 52.7 atom% of ^{17}O was obtained from Mound Facility, Monsanto Research Corporation, Miamisburg, OH 45342, U.S.A. Appropriate solutions were made up in small volumes of this solvent. Where it was important to know pH values precisely on the actual concentrated enzyme samples used in rapid freezing experiments, appropriate small portions of the individual solutions from the syringes were mixed, using the same volume ratios as were employed in the experiments, and the pH values of these mixed solutions were then determined with a cup-type microelectrode (Radiometer type G2221C, from V. A. Howe and Co., London SW6 3EP, U.K.).

E.p.r. measurements

These were carried out using a Varian E9 spectrometer linked to a computer and visual display system (Bray *et al.*, 1978). Spectra were generally recorded at 130 K with 20 mW power and 0.2 mT modulation amplitude. To estimate percentage exchange of ^{17}O in the kinetic experiments, three scans of each spectrum were recorded and the total amplitudes of the hyperfine feature occurring at the highest field position were measured (see arrows in Fig. 2e) and the average calculated. This amplitude was divided by the average of the total amplitudes of the main $g_{\perp}$ features of each spectrum. The values of this ratio were expressed relative to the value of the ratio for the starting solution. Simulations of e.p.r. spectra employed the programs of Lowe (1978).

Results

Optimum conditions for observing the Very Rapid Mo(V) e.p.r. signal

Edmondson *et al.* (1973) described the kinetics of development of the Very Rapid and Rapid e.p.r. signals from functional xanthine oxidase during reduction of the enzyme by xanthine at pH 8.5 and 10.0. At the former pH, both signals were observed together at the shortest reaction times employed. At the higher pH, the Very Rapid signal could be

observed briefly on its own, with a very short induction period before the appearance of Rapid. However, Very Rapid did not reach maximum intensity until well after Rapid had appeared.

As a preliminary to studying the effects of ^{17}O enrichment on Very Rapid, we sought to obtain this signal at the highest possible intensity, free from contamination by Rapid. During the course of this work we found changes in the form of Very Rapid when it was developed at pH values greater than about 10.5. Fig. 1(a) shows the normal Very Rapid, as described by earlier workers. Figs. 1(b) and 1(c) show the high-pH forms of this signal (see also Tanner, 1978). We have not investigated the high-pH Very Rapid signal in detail and have not attempted simulations. However, the main difference from the normal Very Rapid (cf. Table 1) appears to be a decrease in the value of g_3 , by about 0.0045 unit. In addition g_1 and g_2 have shifted in the same direction, by about half of this amount. Additional experiments (not illustrated) indicated that the pK value for interconversion of the two forms of Very Rapid was about 11.4 ± 0.2 {Ches

[2-(cyclohexylamino)ethanesulphonic acid]/NaOH buffer}. It may or may not be fortuitous that this value is comparable with the pK of 11.84 for interconversion of singly and doubly ionized forms of xanthine (Christensen *et al.*, 1970).

For studies of the normal Very Rapid signal we finally selected a pH value of 10.2 ± 0.2 and a reaction time of 11 ms at 19–23°C. Strong signals were obtained under these conditions, with minimal contamination by Rapid or by the high-pH Very Rapid. Despite changes in apparent pH on freezing (Williams-Smith *et al.*, 1977), to which carbonate buffers seem particularly susceptible (Orii & Morita, 1977), the form and parameters of the normal Very Rapid signal in Ches buffer was not significantly affected by the presence or absence of carbonate buffer in the system.

The Very Rapid signal from xanthine oxidase in ^{17}O -enriched water

Fig. 2 illustrates the effect of ^{17}O -enriched water on the normal Very Rapid signal. Fig. 2(a) shows the spectrum in unenriched water, and 2(b) illustrates the corresponding simulation (parameters from Table 1). When enzyme in ^{17}O -enriched water was treated with xanthine, which could be dissolved in either enriched or ordinary water, a modified spectrum was observed, as illustrated in Fig. 2(c). Parts of this spectrum are shown at a higher spectrometer gain setting in Fig. 2(e). The spectrum shows clearly features expected for strong and essentially isotropic coupling of one oxygen atom to molybdenum. Thus each of the g_1 , g_2 and g_3 features is expected to have six weak satellite lines centred on it. Despite the limitations of signal/noise ratios and minor problems of contamination by other signal-giving species present in small amounts, virtually all these expected lines are observed. This interpretation of the signal is confirmed by the computer simulation illustrated in Figs. 2(d) and 2(f). This corresponds to simulated ^{17}O and ^{16}O spectra, added together in the proportions corresponding to the enrichment. (Somewhat arbitrarily, the ^{17}O enrichment was taken as 50.4% rather than 52.7% to allow for the freeze-dried enzyme containing a significant percentage of water.) Parameters used in the simulations (see Materials and methods section) are summarized in Table 1. g Values and linewidths are those reported by Tanner *et al.* (1978). The A values used (Fig. 2 and Table 1) were those that gave the best fit; no significant change was observed with the more sophisticated simulation program of Lowe (1978), including second-order terms.

From the quality of the fit of the simulated and experimental spectra, we conclude that a single oxygen atom only interacts with molybdenum in the Very Rapid signal. Splittings (Table 1) are indeed close to isotropic. The fit also suggests that the axes

Fig. 1. Effect of very high pH on the form of the Very Rapid signal

(a) shows the Very Rapid signal as normally observed, (b) shows the form obtained when the signal was developed at a pH value of about 11.0, (c) shows the pure high-pH form of Very Rapid obtained by subtracting from (b) the normal Very Rapid signal together with small amounts of the Rapid type 2 signal, until features due to these just disappeared. Reaction conditions with xanthine (10 mM) were 10 ms at 25°C in both cases. The buffer was 25 mM-Ches adjusted to the appropriate pH with NaOH.

Fig. 2. *Effect of ^{17}O enrichment on the Very Rapid signal: experimental and simulated spectra* (a) shows the experimental spectrum in ordinary water and (b) is the computer simulation of this (see Table 1 for parameters and the Materials and methods section for details). (c) Illustrates the experimental spectrum, for enzyme in water enriched with ^{17}O to approx. 50 atom%. In (e) the spectrum of (c) is shown with an expanded vertical scale. (d) and (f) correspond to computer simulations of (c) and (e) respectively, and are based on 50.4% enrichment and the parameters of Table 1. (g) Is a simulation corresponding to 100% enrichment with ^{17}O , shown for comparison with (f). The enzyme sample for (c) and (e) had been dissolved in ^{17}O -containing water for approx. 1 h before the Very Rapid signal was elicited by reaction with xanthine in enriched water (see the Materials and methods section). The arrows in (e) show the feature that was measured in quantifying the proportion of ^{17}O in samples.

of the g and A tensors are nearly if not exactly coincident.

Use of the Very Rapid signal to monitor oxygen exchange in oxidized xanthine oxidase

In principle, with an experimental system such as the present one, three types of experiment are possible, in which exchange of the coupled oxygen of the enzyme with solvent water is studied. Firstly, exchange in the oxidized enzyme could be studied. After suitable periods of such exchange, the Very Rapid signal would be developed and examination of the signal should then reveal the extent to which the exchange process had proceeded. Since a very short reaction time is required to develop Very Rapid, this signal is particularly suited to studies of this type.

In the second type of experiment, oxygen exchange in reduced states of xanthine oxidase could be followed. Here, since all the various partly reduced states of the enzyme give Mo(V) e.p.r. signals, it would probably be obligatory to reoxidize the samples after the exchange period was complete, before again developing the Very Rapid signal to measure the extent of the exchange. Finally, in principle one might study oxygen exchange in the signal-giving species itself. However, because of the transient nature of the Very Rapid signal, it is clearly the least suitable of all the molybdenum signals from xanthine oxidase for studies of this type. In the present subsection we describe exchange experiments of the first type. Experiments of the second type will be described in the following subsection.

Preliminary studies indicated that freeze-dried xanthine oxidase dissolved in ^{17}O -enriched water and left for periods of about 1 h, or more, before treatment with xanthine to give the Very Rapid signal, gave maximum development of ^{17}O hyperfine structure at close to the theoretical intensity (as illustrated in Fig. 2). To study the exchange rate in the oxidized enzyme, of the oxygen which is seen to be coupled in the Very Rapid signal, we prepared concentrated solutions of the freeze-dried enzyme in ^{17}O -enriched water. We then diluted the samples with ordinary water buffered at different pH values, and after appropriate intervals, added xanthine to

develop the Very Rapid signal. The reaction procedure is illustrated in Scheme 1. The apparatus and procedure are described in the Materials and methods section, together with the method of measuring the spectra to estimate the percentage exchange. The feature used to determine the ^{17}O content is low in amplitude and susceptible to error in measurement. Therefore two operators measured the spectra independently. The mean of the two sets

Fig. 3. Time course of oxygen exchange in oxidized xanthine oxidase at various pH values

Exchange of ^{17}O out of xanthine oxidase was monitored by observing the Very Rapid signal by the procedure illustrated in Scheme 1 (see also the Materials and methods section). The vertical scale is the percentage of ^{17}O remaining, estimated from the amount of high-field hyperfine structure left in each signal after exchange, relative to the signal recorded at the shortest time (200 ms). The points are the mean of two sets of measurements made independently by two operators (see the Materials and methods section and the text for details). Differences between the two sets of results were less than 10%. pH values during the exchange process (at 20–23 °C) were: 5.9 (●), 6.8 (■), or 7.8 (▲). Buffers were respectively as follows: 100 mM-Mes (4-morpholine-ethanesulphonic acid), pH 5.8, 100 mM-Pipes (1,4-piperazinediethanesulphonic acid), pH 6.8, and 100 mM-Bicine, pH 8.2, and these changed to the appropriate pH values when mixed with the enzyme solution. In all cases 0.5 mM-Bicine was present in the enzyme solution. The arrow shows the theoretical ^{17}O value expected at infinite time.

Scheme 1. Diagram representing successive addition of solutions to xanthine oxidase, in ^{17}O -enriched water, for the oxygen-exchange experiments of Fig. 3

The rapid-freezing procedure was used (see the Materials and methods section). Time is represented horizontally. Abbreviation used: XO, xanthine oxidase.

of results for ^{17}O exchange out of the oxidized enzyme at three different pH values are presented in Fig. 3. Of all the hyperfine features, the one at highest field was least affected by other signals and yielded the smallest variation, at most 10%, between the two sets of results. Because of the high cost of ^{17}O -enriched water, we did not obtain sufficient data for precise estimation of exchange rate constants. However, values for this appeared to be about 2 h^{-1} at both pH 5.9 and 6.8, and about 4 h^{-1} at pH 7.8. On the other hand, a single further measurement at the still higher pH value of 8.8 {Caps [3-(cyclohexylamino)propanesulphonic acid] buffer}, indicated little exchange after 2 h reaction time, suggesting, under these conditions, an exchange-rate constant of less than 0.2 h^{-1} . It is thus conceivable that complex-formation (*cf.* Malthouse *et al.*, 1980) between xanthine oxidase and Caps inhibits the exchange process.

Use of the Very Rapid signal to monitor oxygen exchange in xanthine oxidase during turnover of a substrate

The general procedure for these experiments was similar to that in the previous subsection, but the flow system illustrated in Scheme 2 was used. We selected salicylaldehyde as the reducing agent, since purine substrates and products tend to form complexes with the reduced enzyme (Bray *et al.*, 1978). Salicylaldehyde is one of the fastest reacting aldehyde substrates of the enzyme and appears to have the lowest apparent K_m (Booth, 1938*a,b*). The system permitted dilution of ^{17}O -enriched enzyme solution with ordinary water and simultaneous addition of the aldehyde. Since the quantity of salicylaldehyde was rather higher than that of the enzyme on a molar basis, with an excess of oxygen present, this procedure permitted observation of oxygen exchange taking place during some three catalytic cycles of the enzyme only.

Figs. 4(a) and 4(b) illustrate experiments in which essentially complete oxygen exchange took place, as

Fig. 4. Spectra for the Very Rapid signal illustrating oxygen exchange in xanthine oxidase in the presence of a reducing substrate

Only the high field part of the spectrum is illustrated (approx. $g = 1.955$ and below). Exchange of ^{17}O out of xanthine oxidase was monitored by observing the Very Rapid signal by the procedure illustrated in Scheme 2 (see also Materials and methods section). (a) and (b) correspond to samples initially in ^{17}O -enriched water treated briefly with ordinary water in the presence of salicylaldehyde before development of the Very Rapid signal. In (a) the treatment was for 10 s and in (b) this was extended to 2.5 min. In both cases features due to ^{17}O are scarcely, if at all, detectable in the spectra. (c) Is a control in which the salicylaldehyde solution was replaced by buffer. With a reaction time of 2.5 min, there was essentially no loss of ^{17}O from the enzyme in this sample, as expected from Fig. 3. The substrate was in 100 mM-Bicine buffer, pH 8.2.

Scheme 2. Diagram representing successive additions of solutions to xanthine oxidase in ^{17}O -enriched water, for the oxygen-exchange experiments of Fig. 4

The rapid-freezing procedure was used (see the Materials and methods section). Time is represented horizontally. Abbreviation used: XO, xanthine oxidase.

is shown by the virtual absence of high-field ^{17}O hyperfine lines. The total time interval from dilution with ordinary water to addition of xanthine was either 10 s or 2.5 min. However, the enzyme would probably remain in the reduced state for only 1–2 s. The lack of ^{17}O hyperfine lines in Figs. 4(a) and 4(b) may be contrasted with a control (Fig. 4c), in which the same system was employed but with buffer in place of salicylaldehyde so that no reduction took place. Here, there was virtually no exchange at 2.5 min reaction time, as is shown by the strong hyperfine lines, in agreement with data for exchange in the oxidized enzyme, as discussed in the previous subsection.

We conclude that exchange of the oxygen atom, which appears as the coupled oxygen atom in the Very Rapid signal, takes place rapidly during turnover of the enzyme, exchange actually occurring either whilst the enzyme is in the reduced state or, alternatively, taking place as a part of the catalytic cycle.

Discussion

For metal centres in enzymes in which the nature of the ligand atoms and the co-ordination geometry is unknown, deduction of structures from e.p.r. parameters is not easy. It now appears from e.x.a.f.s. spectroscopy (Bordas, *et al.*, 1980) that the molybdenum atom of functional xanthine oxidase in the oxidized state has as ligands a terminal sulphur atom and a terminal oxygen atom, together with three further sulphur ligands, two of these probably being from cysteine and one from methionine. However, this information is perhaps only moderately helpful in explaining the present results.

The magnitude of the oxygen splitting ($A_{\text{av.}} = 1.37 \text{ mT}$) is comparable with the preliminary value for this constant reported for the Rapid signal by Gutteridge *et al.* (1979), and it seems certain that the oxygen atom 'seen' in the Very Rapid signal must be a ligand of the metal. The size of the coupling is much different from smaller values (0.1–0.2 mT) estimated for oxygen bound axially to molybdenum in one model compound (Cramer *et al.*, 1979), suggesting oxygen is co-ordinated in a planar position in the enzyme.

The almost complete lack of anisotropy in the oxygen splitting of Very Rapid is remarkable (ΔA 0.06 mT). The anisotropic dipolar contribution of an oxygen atom at the typical distance of 0.21 nm (2.1 Å) from molybdenum would be about 0.12 mT. Thus there must be some cancellation of anisotropic exchange and dipolar terms, and it seems likely that the exchange contribution is of the order of 0.06 to 0.12 mT. This small anisotropic exchange coupling contrasts with the isotropic coupling of about 1.4 mT. For equal contributions of *s* and *p* orbitals

(*sp* hybridization), data tabulated by Goodman & Raynor (1970) indicate $A_{\text{iso.}}/A_{\text{aniso.}}$ would be about 16. For the oxygen of Very Rapid, the value of this ratio seems to be of this order. Since data on the anisotropy of oxygen coupled to molybdenum in suitable model compounds seem to be lacking, we may instead compare our results with those on copper complexes. Getz & Silver (1974) described ^{17}O splittings from water atoms co-ordinated to a cupric ion in the planar position. Here, the anisotropic contribution to the splitting from the *p* orbital was much larger, with $A_{\text{iso.}}/A_{\text{aniso.}}$ about 4.

In attempting to interpret the small anisotropy in Very Rapid we have to consider several types of possible structures. The coupled oxygen atom could be a terminal oxygen ligand, or it could be a carbon-bound oxygen atom liganded to the metal. Further possibilities, such as a hydroxy group or a water ligand, should presumably be dismissed immediately, because of the lack of proton splittings in the Very Rapid signal. From the e.x.a.f.s. information on the oxidized hexavalent state of the enzyme, we must also consider very seriously the possibility of a terminal oxygen atom in the signal-giving species. However, two points argue against the coupled oxygen atom of Very Rapid being in this form. Firstly, the π -donor character of O^{2-} as a ligand would be expected to lead to enhanced *p*-orbital character (Phillips & Williams, 1966). Furthermore, the relatively short bond distance for such a group [0.17 nm (1.7 Å)] would lead to a larger dipolar term than we allowed for. Thus, although the evidence is not conclusive, the small anisotropy of the splitting is nevertheless probably incompatible with a terminal oxygen atom and therefore, conversely, favours Mo–O–C.

The question of the origin of the carbon atom in an Mo–O–C group at once arises. An ionized group of the protein might be considered, e.g. serine or tyrosine. On the other hand, the carbon might be in the substrate. In keeping with this, Tanner *et al.* (1978) reported that when the Very Rapid signal is generated by using xanthine labelled specifically with ^{13}C in the C-8 position, then the signal so produced shows weak coupling of ^{13}C to molybdenum, with $A_{\text{av.}}(^{13}\text{C})$ 0.32 mT (see Table 1). Thus the relative magnitude of the ^{13}C and ^{17}O splittings are such as would appear to be consistent with the atoms being those of a single Mo–O–C system, though of course the data cannot prove this point rigorously.

Terminal oxygen is known to be present in oxidized xanthine oxidase from e.x.a.f.s. data (Bordas *et al.*, 1980). In our proposed mechanism (Scheme 3), this group acts as a nucleophile, reacting with the hypothetical carbonium ion, R^+ , derived from a substrate, RH, such as xanthine. Concerted with this, according to Bray *et al.* (1979), the proton of the displaced hybide is accepted by

Table 1. *E.p.r. parameters of the Very Rapid signal*

Conditions for developing the signal by reaction of xanthine with xanthine oxidase are given in the Materials and methods section. The values given (apart from the ^{13}C splittings) are those used in the computer simulations of Fig. 2. Hyperfine splittings, A , and half-linewidths, Δ are in mT.

Parameter	1	2	3	Av.	Reference
g	2.0252	1.9550	1.9494	1.9765	Tanner <i>et al.</i> (1978)
$A(^{17}\text{O})$	1.34	1.40	1.36	1.37	Present work
$A(^{13}\text{C})^*$	0.31	0.37	0.29	0.32	Tanner <i>et al.</i> (1978)
Δ	0.19	0.16	0.19	0.18	Tanner <i>et al.</i> (1978)

* Splittings due to the C-8 carbon atom of xanthine.

Scheme 3. *Proposed involvement of the Very Rapid signal-giving species in xanthine oxidase turnover*

Conversion of oxidized xanthine oxidase, by a substrate molecule, RH, to the Very Rapid signal-giving species is depicted. In the hydrolytic reaction step leading, after re-oxidation of Mo(V), to regeneration of the free enzyme, fission of the Mo-O bond would take place, so accounting for rapid exchange of ^{17}O in the presence of substrate. The Scheme is related to that of Bray *et al.* (1979), with the nucleophile identified as a terminal oxygen ligand but with the proton acceptor not specified. Electrons would be transferred to iron-sulphur or flavin (see the text).

the terminal sulphur atom while the two electrons reduce the molybdenum. We have not depicted the proton acceptor in Scheme 3 in view of recent experiments (J. P. G. Malthouse & R. C. Bray, unpublished work) using xanthine oxidase containing ^{33}S in the cyanide-labile site. These indicate that the proposed role of the sulphur atom as a proton acceptor may have to be revised. Nevertheless, the mechanism of Scheme 3 is still essentially that of Bray *et al.* (1979), with the proton acceptor not specified but with the nucleophile now identified as the terminal oxygen ligand. Correct preliminary orientation of the substrate in the active site in the Michaelis complex would of course be important.

We now turn to our data on the kinetics of exchange, with solvent water, of the oxygen atom 'seen' in the Very Rapid signal. Oxygen exchange in

oxidized xanthine oxidase is some 500 times slower than is that in molybdate (von Felten *et al.*, 1978). Nevertheless, according to the e.x.a.f.s. information (and as is indicated in Scheme 3), it seems that the oxygen atom of the enzyme involved is indeed a terminal ligand of molybdenum. Limited data only are available (Landis *et al.*, 1979) on oxygen exchange in low-molecular-weight complexes of the metal bearing a single terminal oxygen ligand. Thus it may be premature to comment on the difference between the enzyme and molybdate. For molybdate, there is both a pH-independent and a base-catalysed exchange mechanism, the latter becoming important at pH values above about 12 (von Felten *et al.*, 1978). For the enzyme, on the other hand, we found roughly a 2-fold increase in exchange rate on raising the pH from 5.9 to 7.8, the mechanistic significance of this being unclear.

The fact that the oxygen atom of the enzyme exchanges rapidly during turnover of the substrate salicylaldehyde is indeed noteworthy. Our finding may be accommodated in Scheme 3 by assuming that, in the final hydrolytic step leading to product release, the attacking water molecule donates a hydroxyl ion to the enzyme and a proton to the product molecule. Thus, according to this mechanism, the coupled oxygen atom of the Very Rapid signal would end up in the product molecule. In support of this, Murray *et al.* (1966) found that ^{18}O from nicotinamide *N*-oxide appears in uric acid during xanthine oxidase-catalysed oxidation of xanthine, when the *N*-oxide is utilized as electron acceptor. The usual acceptor, oxygen, reacts at the flavin site of the enzyme (Komai *et al.*, 1969). In contrast, presumably nicotinamide *N*-oxide must interact with the molybdenum site, thus making possible transfer of the isotope from acceptor to product by a mechanism that is at least of the same type as, if not identical with, the one we outline. Oxygen-transfer mechanisms have been postulated, though not established, for other molybdenum-containing enzymes (see Durrant *et al.*, 1977; Yamanouchi & Enemark, 1979). However, for xanthine oxidase, further work will be necessary, for

example, with ^{18}O , to establish such a mechanism unequivocally.

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