The Molybdenum Centre of Native Xanthine Oxidase

EVIDENCE FOR PROTON TRANSFER FROM SUBSTRATES TO THE CENTRE AND FOR EXISTENCE OF AN ANION-BINDING SITE

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The observation by Bray & Knowles [*Proc. R. Soc. London Ser. A* (1968) **302**, 351–353] of direct transfer, during the catalytic reaction, of hydrogen atoms from substrate molecules to the enzyme xanthine oxidase was reinvestigated. The experimental phenomenon and its basic interpretation were confirmed and extended. In the reduced functional enzyme, molybdenum(V) interacts with two enzyme-bound protons, which are exchangeable with solvent protons. One of these is coupled to the metal with A_{av}^H 1.4 mT and the other with A_{av}^H 0.3 mT. The molecule also contains a site for the binding of anions, presumably as ligands of molybdenum. This is shown by effects of nitrate ions on the e.p.r. spectra. The spectra of the nitrate and 1-methylxanthine complexes of the reduced enzyme are very similar to one another, and are designated Rapid type-1 spectra. It is concluded that, in the Michaelis complex, the substrate molecule occupies the anion site, probably being bound to molybdenum via the nitrogen in its 9-position. During the turnover process, hydrogen from the substrate C-8 position, after transfer to the enzyme, appears as the proton more strongly coupled to molybdenum. This proton then exchanges with solvent deuterium with a rate constant of 27 s^{-1} , at pH 8.2 and 12°C . It has been confirmed that substrate molecules occupying the anion site do not interfere with observation of the transfer and exchange processes.

Proton-transfer reactions are thought to be ubiquitous in enzymic catalysis (Bender & Kézdy, 1975) and the possibility of their being intimately coupled to electron-transfer processes has been extensively discussed [Hamilton (1971); note, however, that specific points in this review relating to xanthine oxidase have been criticized by Bray (1975)]. It is thus likely that at least some enzymic carbon–hydrogen bond-cleavage reactions involve temporary transfer of the liberated hydrogen atom to a site in the enzyme molecule. Direct experimental observation of such processes has, however, been achieved in only a few instances. These include [see Popják (1970) and Sato *et al.* (1976)] NAD^+ - and B_{12} -dependent enzymes, for which the proton, after being accepted, is carbon-bound and non-exchangeable with solvent protons. A less well-authenticated instance (Bray & Knowles, 1968) involved the molybdenum-containing enzyme xanthine oxidase, but in this case, after transfer to the enzyme, the hydrogen apparently became exchangeable at quite a high rate.

Bray & Knowles (1968) presented preliminary evidence that hydrogen, derived from the position in the xanthine molecule at which it becomes hydroxylated in the xanthine oxidase reaction (the 8-position), is transferred temporarily during the catalytic cycle to a site in the vicinity of molybdenum in the enzyme molecule. In experiments using the rapid-freezing

procedure of Bray (1961), with xanthine deuterated specifically in the 8-position, they noted that the Rapid molybdenum(V) e.p.r. signal observed at reaction times less than the turnover time was of a different form from that observed at longer reaction times [see Bray (1975) for a review of e.p.r. signals from xanthine oxidase]. Whereas, at the longer times, the Rapid signal showed characteristic splitting due to interaction with protons (as originally reported by Bray & Meriwether, 1966), in contrast, at shorter times, the splitting was not present in a proportion of the molecules, presumably because deuterium was occupying the sites normally occupied by hydrogen.

Further work by Bray *et al.* (1969), Pick (1971), Bray (1971) and Edmondson *et al.* (1973) confirmed the reality of the phenomenon and extended it from deuterioxanthine to deutero-1-methylxanthine, as well as to the converse reaction with 1-methylxanthine in $^2\text{H}_2\text{O}$. In addition, some related kinetic information was obtained. However, none of the later work increased basic understanding of the phenomenon very greatly.

The observations were subsequently taken up by Stiefel (Stiefel, 1973; Stiefel *et al.*, 1977), in elaborating a general theory for the functioning of molybdenum-containing enzymes, involving the simultaneous transfer of electrons and protons. In support, he noted the tendency, in model compounds, of

ligands of molybdenum to protonate more readily when the metal was in the lower valency states than when in higher ones. He cited, as important evidence favouring his theory, the xanthine oxidase direct-hydrogen-transfer data, as discussed above.

Substantiation of the interpretation of the phenomenon thus assumes considerable importance, beyond its immediate interest with regard to xanthine oxidase. Nevertheless, there are problems. These arise from the complexity of the Rapid molybdenum e.p.r. signals (Bray & Vännegård, 1969; Pick & Bray, 1969; Bray *et al.*, 1978). These signals represent a number of different reduced forms of the enzyme, including complexes with substrate or product molecules. Furthermore, two different protons are involved in at least some of the signal-giving species. Unaware of such complications, Bray & Knowles (1968) concluded: 'since chemically diverse substrates give the (Rapid) signal ... the hydrogen (from the C-8 position of xanthine) must have been transferred to the enzyme when we see it in the signal'. In contrast, a situation might be envisaged in which an initially formed Rapid signal, corresponding not to free reduced enzyme, but to enzyme with the substrate molecule bound to it, gave way to a final signal corresponding to enzyme with a product molecule bound. If this were the case, disappearance of an initial deuterium effect on the signal might be observable without any transfer of hydrogen from the substrate to the enzyme. In order to exclude, rigorously, such a possibility, we thought it important to carry out more experiments. However, it was necessary to understand the Rapid spectra more fully before further studying the direct-hydrogen-transfer phenomenon.

The work described has depended particularly on the availability of computer facilities, for the production of e.p.r. difference spectra (Bray *et al.*, 1978) as well as for the simulation of e.p.r. spectra from their parameters (Lowe, 1978). It was also helped by improved rapid-freezing procedures (as described in the Materials and Methods section).

Materials and Methods

Substrates

1-Methylxanthine (Fluka A.G.) was from Fluorochem, Glossop, Derbyshire, U.K., and the 8-deutero substrate was prepared from it by heating in $^2\text{H}_2\text{O}$ for 16 h at 100°C in sealed vials. Replacement of protons from the C-8 position by deuterium was monitored initially with a Varian T60 n.m.r. spectrometer, but final purity was checked with an AEI MS30 double-beam mass spectrometer coupled to an AEI D550 data system. The substrate was run on a direct-insertion probe and the source set at 150°C ,

70 eV and $300\mu\text{A}$. The fragmentation pattern of the 8-deutero substrate after freeze-drying from acidic aqueous ($^1\text{H}_2\text{O}$) solution indicated isotopic purity better than 95%. Care was taken, when using the deuterated substrate, not to use elevated temperatures, to avoid back-exchange of the deuterium.

Xanthine oxidase samples

Milk xanthine oxidase was prepared by the salicylate denaturation procedure of Hart *et al.* (1970) and assayed as described by these workers. Functional active-centre concentrations were obtained from ϵ_{450} (per half-mol) = 36 and limiting activity/ A_{450} = 190 (Bray, 1975); final concentrations in different experiments ranged from 0.2 to 0.5 mM. The use of buffers causing large changes of apparent pH on freezing (Williams-Smith *et al.*, 1977) was avoided. Bicine [*NN*-bis-(2-hydroxyethyl)glycine] was used for all experiments with anions or 1-methylxanthine. With this buffer at a concentration of 10 mM we found by using indicators (Williams-Smith *et al.*, 1977) that the highest added anion concentration used (500 mM- NaNO_3) had no more than a marginal effect on apparent pH in the frozen state (decrease of about 0.2 pH unit, relative to a sample without nitrate, measured in the absence of protein).

For work with $^2\text{H}_2\text{O}$ as solvent, enzyme was dialysed against several changes of buffer in $^2\text{H}_2\text{O}$. The content of residual water was found to be 2% or less by n.m.r. pH values in $^2\text{H}_2\text{O}$ solution are expressed as pD_{app} values, i.e. the readings given by the pH-meter in the $^2\text{H}_2\text{O}$ buffer solutions. Procedures for mixing enzyme and substrate anaerobically were as described by Barber *et al.* (1976).

Rapid-freezing procedure

The method of Bray (1961), as modified by Bray *et al.* (1973), was used with a jet velocity of about 13 m/s. This gave optimum packing for xanthine oxidase, though higher velocities were advantageous when using myoglobin (Bray *et al.*, 1973). Work was facilitated by the use of a new ram-drive system for actuating the syringes. This consisted of a stepping motor ('Slo-Syn' Type MO93-FD14, from T. I. Supply, Slough SL1 4AD, Berks., U.K.) driving a ball-bearing screw assembly (thread lead 12.7 mm/turn; type 0500-0500 SRT from General Motors Power Products Europe, Wellingborough NN8 2DL, Northants., U.K.) This actuated the syringe plungers and was controlled by a Digidrive model CC4 control unit (from PKS Designs, Bournemouth BH4 9HJ, Dorset, U.K.). A specially constructed pulse generator enabled the syringe plungers to be moved through the required distance, at any velocity within the range 2–30 mm/s. Acceleration and deceleration

were virtually instantaneous. Up to three syringes with capacities of 0.25–5.0 ml could be accommodated simultaneously, allowing one- or two-stage mixing operations. Syringes, connecting tubes and mixing blocks were immersed in a water bath, if control of the reaction temperature was required. Facilities were available to maintain anaerobic conditions during operation (Bray *et al.*, 1973). Most of the experiments involved single-push operations, but double-push was also possible (Ballou & Palmer, 1974). In this, the first ram movement delivered solutions from the syringes into a reaction tube. After a pre-determined delay time, the mixture was then expelled via a jet into the cold isopentane.

E.p.r. and computer procedures

E.p.r. spectra were recorded on a Varian E9 instrument. Conditions routinely used were, at 9 GHz, power 10 mW, modulation 0.1 mT, temperature about 150 K and at 35 GHz, power attenuation 10 db, modulation 0.5 mT, temperature about 150 K. For the most precise *g*-value measurements at 9 GHz, the frequency was measured with a type 5256A and 5256L frequency meter (Hewlett Packard, Slough SL1 4DS, Berks., U.K.) and the field was standardized with the Mn standard described by Swartz *et al.* (1972), and with diphenylpicrylhydrazyl by using a dual-sample cavity. E.p.r. difference spectra were obtained with the help of a computer and visual-display system, by the procedures described by Bray *et al.* (1978). Simulations of e.p.r. spectra from their parameters on a PDP 11/10 computer (Digital Equipment Co., Galway, Ireland) used the procedure of Lowe (1978). In all cases, where a deuterium splitting in a simulated spectrum replaced a hydrogen splitting, we allowed for replacement of a doublet by a triplet, with splittings decreased by the factor 1/6.51. *g* values are considered accurate to ± 0.0005 .

N.m.r. procedures

Experiments were carried out on a Brüker 270 MHz n.m.r. spectrometer, at the National Institute for Medical Research, Mill Hill, London NW7 1AA, U.K., operated in the Fourier-transform mode. The resonance of the C-8 proton of free 1-methylxanthine was readily observable, without significant shift or broadening, in the presence of moderate concentrations of the enzyme, indicating a slow exchange situation for this substrate. Conditions used were as follows. Xanthine oxidase (14 μ M) was mixed with 1-methylxanthine (2.7 mM) in Bicine/²H₂O (100 mM), pD_{app} 8.2, under anaerobic conditions in an n.m.r. tube. Spectra were accumulated over a 3 min period at 12°C.

Results and Discussion

Types of Rapid molybdenum(V) spectra

Precise definition, for xanthine oxidase, of the term Rapid Mo(V) e.p.r. signals is not easy, but they may be regarded as signals derived from the functional enzyme, with *g*_{av.} about 1.975 and showing well-resolved splittings from exchangeable protons. Such spectra were given their name by Bray & Vänngård (1969), since they appear rapidly, i.e. within the turnover time of the enzyme, under most conditions when it is mixed with reducible substrates under anaerobic conditions. On the basis of observations and simulations with a number of different substrates, these workers (see also Pick & Bray, 1969) noted that, whereas reduction with dithionite or salicylaldehyde yielded a spectrum referred to as Rapid 'no complex detected', on the other hand purine substrates usually gave rather different spectra, which they called Rapid 'complex formed'. From work at 35 GHz, they distinguished subclasses 'type A' and 'type B' of the 'no complex detected' spectra, which always occurred together. More recent data on substrates and product complexes have been presented by Bray *et al.* (1978). The type-1 complex spectrum is well-defined for the substrates xanthine, 1-methylxanthine and purine, and is presumed to correspond to Michaelis complexes of partially reduced forms of the enzyme. All of the spectra discussed so far involve interaction of molybdenum with a strongly coupled proton, and in some cases interaction with a second more weakly coupled proton is detected also. The complex 'type-2' spectrum is, however, distinctly different and involves interaction with two equivalent protons. This spectrum has been reported, for xanthine, to represent a non-productive mode of binding of the substrate in the active centre. A complex type-2 spectrum is also given when uric acid is bound to the reduced enzyme.

Effect of anions on the Rapid spectrum

We first consider the reason for there being apparently more than one chemical species in the Rapid spectrum, when the signal is generated in the absence of purines. Bray & Vänngård (1969) suggested that the origin of their 'uncomplexed' A and B species might be the two molybdenum atoms of the enzyme molecule, in slightly different environments and therefore each giving its own, slightly different signal. Subsequent work on the dimeric nature of the enzyme, summarized by Bray (1975), has made this interpretation unlikely.

A second possibility was that the form of the Rapid spectrum depended on the number of electrons accepted by the enzyme half-molecule. To investigate this (Bray *et al.*, 1975) samples were frozen at different times after adding dithionite to the enzyme and the

Rapid spectra compared with one another. Though it could be shown that the number of electrons taken up at different times was different (e.g. two and four electrons per half molecule), nevertheless, the form of the Rapid spectrum at relatively high temperatures did not vary when observed either at 9 GHz (Bray *et al.*, 1975) or at 35 GHz (Barber, 1976).

The third possibility was that components of the buffers were binding to the enzyme and thereby influencing the form of the Rapid spectrum. It is known that many inorganic molybdenum(V) complexes contain a ligand site at which anions may be relatively weakly bound (Stiefel, 1977; Garner *et al.*, 1975). Furthermore, Vincent & Bray (1978) have shown that such complexes do indeed exist for another molybdenum-containing enzyme, nitrate reductase. For this, the anions nitrate and nitrite bind to the reduced enzyme to give well-defined molybdenum(V) e.p.r. spectra, different both from one another and from those obtained in the presence of other common anions.

That anion-binding is the true explanation of the variations in the xanthine oxidase Rapid signal, observed in the absence of purines, is demonstrated by the experiments of Figs. 1 and 2. We first studied the effect of nitrate by measurements in $^2\text{H}_2\text{O}$ at 35 GHz (Fig. 1), reducing the enzyme with a relatively low concentration of dithionite, in dilute Bicine buffer. In the absence of nitrate (Fig. 1a), more than one chemical species of Mo(V) is clearly involved. This follows from the structure in the g_2 region around 1270 mT and is in agreement with Bray & Vänngård (1969). In contrast, in the presence of high concentrations of nitrate ions (Fig. 1c), the spectrum assumes an apparently ideal rhombic form, characteristic of a single chemical species. Here, the second g_2 feature has vanished. Further, the g_1 feature (which in the absence of nitrate was broadened, presumably because of the presence of more than one species) has not only shifted, but also sharpened considerably. At low concentrations of nitrate, two peaks were obtained in the g_1 region (Fig. 1b). From this experiment and additional studies over a range of nitrate concentrations, we conclude that the dissociation constant for the enzyme–nitrate complex is about 5 mM. The experiment of Fig. 1(c) is the first in which a spectrum corresponding to a single chemical species has been reported for any form of the Rapid spectrum. In the light of this work and of analogous and more fully investigated anion effects on the Slow spectrum (S. Gutteridge, S. J. Tanner & R. C. Bray, unpublished observations), we conclude that the type-A and type-B spectra of Bray & Vänngård (1969) must have corresponded (though not necessarily respectively) to reduced enzyme species, such as a pyrophosphate or chloride complex and an aquo complex.

We now turn to the spectrum at 9 GHz of the

Fig. 1. Effect of nitrate ions on the 'uncomplexed' Rapid spectrum in $^2\text{H}_2\text{O}$ at 35 GHz

In the absence of nitrate ions (a), xanthine oxidase was reduced with dithionite under anaerobic conditions, producing the 'uncomplexed' Rapid spectrum. In the presence of 5 mM- and 500 mM- NaNO_3 , the enzyme produced the spectra shown in (b) and (c) respectively, after reduction by dithionite. For all the experiments, xanthine oxidase was in 10 mM-Bicine, pD_{app} 8.2, and reduced anaerobically with 2.5 mM-dithionite for 20 s at 20°C. See the Materials and Methods section for details of the e.p.r. conditions. The field scale corresponds to 35.0 GHz.

nitrate complex in $^1\text{H}_2\text{O}$ (Fig. 2a). This was obtained by reduction with dithionite in the presence of 200 mM-nitrate, in Bicine buffer at pH 8.2. For comparison, Fig. 2(b) shows the 'uncomplexed' Rapid spectrum, as reported by Bray & Vänngård (1969) and by Bray *et al.* (1978). Clearly, nitrate has a marked effect, under these conditions of observation, and interestingly, in the presence of this anion, though no purine is present, the spectrum seems to be of the Rapid type-1 form. This is apparent from the decrease in the overall linewidth of the main 'downward' features in the g_2 and g_3 region. Further, and in agreement with the 35 GHz spectra, there has been a shift of the g_1 feature to higher field.

A still more important feature of Fig. 2(a) is the structure in the g_1 region. This peak is split by an

Fig. 2. Effect of nitrate ions on the 'uncomplexed' Rapid spectrum in $^1\text{H}_2\text{O}$ at 9 GHz

Formation of the Rapid spectrum with dithionite in the presence of 200 mM- NaNO_3 (a) in $^1\text{H}_2\text{O}$ produces significant changes in features of the spectrum compared with that obtained on reduction without nitrate ions (b). The arrows indicate the splitting of the g_1 features due to a second weakly coupled proton. Reduction conditions were as in Fig. 1. The field scale corresponds to 9.3 GHz.

exchangeable proton giving A_1 about 1.3 mT (Figs. 2a and 2b). In addition, in the presence of nitrate ions, there is clearly a further partly resolved splitting of about 0.3–0.4 mT (see arrows in Fig. 2a). Although Bray & Vännård (1969) distinguished such a secondary splitting in the presence of the purine or xanthine type-1 spectrum, they were not able to distinguish it unequivocally in their 'uncomplexed' Rapid spectra. We can now see that this was because of the presence of more than one species, with spectra at 9 GHz overlapping in the g_1 region. We are therefore able to conclude, for the first time, that in the absence of substrate or product molecules, molybdenum in the reduced enzyme interacts with not one but two protons.

Nitrate is an oxidizing substrate for xanthine oxidase. Therefore, in the presence of it and dithionite, enzyme turnover to yield nitrite is expected, though at a relatively low rate (S. P. Vincent, personal communication). In view of possible complication of the spectra from the presence of a nitrite as well as a nitrate complex, we did not analyse the nitrate Rapid spectrum further, or attempt to carry out simulations.

Complex type-1 spectrum from 1-methylxanthine

We next studied the type-1 Rapid spectra obtained by reduction of the enzyme with 1-methylxanthine,

working with this substrate present at a high concentration. Bray *et al.* (1978) presented the spectrum in $^1\text{H}_2\text{O}$ at 9 GHz. We have now extended this to measurements in $^2\text{H}_2\text{O}$ also, and at 35 GHz. Results are shown in Fig. 3. Experimental spectra (Figs. 3a, 3b, 3d, 3f, 3g, 3i, 3j), obtained under a variety of reaction conditions, are compared with computer simulations (Figs. 3c, 3e, 3h and 3k). In general, under all conditions, agreement between the simulated and experimental spectra is good and provides quite rigorous evidence that only a single chemical species of Mo(V) is present. The one region where systematic deviations exist is in the 35 GHz $^2\text{H}_2\text{O}$ spectra (Figs. 3i–3k), at the 'bottom' of the g_2 peak. This is probably due to the presence of a little of the product complex (Bray *et al.*, 1978), but we suggest that probably not more than about 10% of this is likely to be present.

A point of particular interest is the great similarity of the spectra from the 1-methylxanthine and the nitrate complexes (compare Fig. 1c with Fig. 3j and Fig. 2a with Fig. 3a). Both show a pair of non-equivalent protons and the g values and proton splittings are all quite similar, e.g. the value of g_1 (which could be readily and precisely measured, without the help of simulations) was 1.9893 for 1-methylxanthine and 1.9890 for nitrate. It is therefore reasonable to assume a structural similarity between the two signal-giving species, and to conclude that the substrate is bound in the complex with very much the same geometry and type of bonding as is the nitrate. We suggest, therefore, that in the xanthine and 1-methylxanthine complexes giving the type-1 signal, for which productive binding of the substrate to the active centre is involved (Bray *et al.*, 1978), the purine molecule is bound directly to the molybdenum. The substrate must be in the mono-anion state (Fridovich, 1965) and the binding presumably occurs via the nitrogen in its 9-position (cf. Bergmann & Levene, 1976). Though charge distribution in this substrate species is uncertain (Christensen *et al.*, 1970), it is reasonable to postulate a negative charge in the 9-position of the bound molecule. The substrate held in the anion site would presumably interact with additional groups in the protein to account for tight binding in this Michaelis complex.

A further important point, which follows from the similarity of the 1-methylxanthine and nitrate Rapid spectra, is that, in the substrate complex, the two protons must be located on the enzyme molecule and not on the bound substrate molecule.

In view of the above considerations, we propose to modify nomenclature for Rapid signal types. We shall abandon terms such as 'uncomplexed' (no complex detected) and 'complexed' (complex formed). Presumably the co-ordination position on the molybdenum is always occupied, if only by water. If in doubt as to whether water, or some ion affecting the spectrum only slightly, is bound, we shall describe it

Fig. 3. 1-Methylxanthine Rapid type-1 spectrum: experimental and simulated spectra

The solvent used for the experimental spectra (a), (b), (f) and (g) was $^1\text{H}_2\text{O}$ and for (d), (i) and (j) it was $^2\text{H}_2\text{O}$. (c), (e), (h) and (k) are simulated spectra. For (a)–(e), the field scale corresponds to 9.3 GHz, and for (f)–(k), to 35.0 GHz. In all cases the enzyme was reduced anaerobically with 5 mM-1-methylxanthine in 50 mM-Bicine, pH 8.2 (or pD_{app} 8.2). In (d) and (j) substrate substituted with deuterium in the 8-position was used instead of the normal substrate. Reaction times (at 12°C) were for (a) 260 ms, (b) and (d) 2000 ms, (f) 56 ms, (g), (i) and (j) 2000 ms. Simulated e.p.r. spectra (see the Materials and Methods section) were based on the following parameters: g_1 1.9893; g_2 1.9703; g_3 1.9657; A_1^{H} 1.30, 0.40; A_2^{H} 1.39, 0.30; A_3^{H} 1.40, 0.20; half-linewidths, Δ_1 0.18; Δ_2 0.15; Δ_3 0.15. (Hyperfine splittings and linewidths are given in mT.) For spectra at 35 GHz, all linewidths were increased by 0.30 mT.

as an aquo complex. On the other hand, the distinction between the type-1 and type-2 complexes remains real and important. We shall continue to refer to spectra showing two non-equivalent protons as being of 'type 1' and to those showing two approximately equivalent protons as being 'type 2'. Thus we shall use names, such as 'xanthine Rapid type 1', 'xanthine Rapid type 2', '1-methylxanthine Rapid type 1', 'nitrate Rapid type 1' etc. (In naming the last two, the 'type 1' could, of course, be omitted, since the corresponding type-2 spectra have not been reported.)

Does the form of the Rapid spectrum obtained with 1-methylxanthine change during the reduction process?

The direct-hydrogen-transfer phenomenon involves changes in the form of the Rapid signal, attributed (as noted in the introduction, and as will be considered further below) to the transfer of hydrogen from the substrate to the enzyme and to its subsequent exchange with protons from the medium. If for any other reason the form of the Rapid signal was changing simultaneously with this, during the reduction time course, then observation of the

hydrogen-transfer phenomenon would be complicated. It was important, therefore, before working with isotopically labelled substrates, to investigate the Rapid signal when using unlabelled substrates in $^1\text{H}_2\text{O}$, studying the form of the signal as a function of time.

Bray *et al.* (1978) mentioned that with low concen-

trations of 1-methylxanthine the signal at early reaction times was of the substrate Rapid type-1 type, but that after longer times it had the product Rapid form. This is highly undesirable for studies on the direct-hydrogen-transfer phenomenon. We therefore increased the substrate concentration to the one used in Fig. 3, namely 5 mM. Happily at this concentration the Rapid signal form was independent of time, in the range of interest (compare Fig. 3a with 3b and 3f with 3g). We conclude that throughout the observed reduction process under such conditions, molybdenum(V) is present essentially exclusively as the 1-methylxanthine Rapid type-1 spectrum.

Absolute and difference e.p.r. spectra from reaction with ordinary 1-methylxanthine in $^2\text{H}_2\text{O}$

Spectra obtained at different reaction times, illustrating the direct-hydrogen-transfer phenomenon, are presented in Fig. 4. Figs. 4(d)–(f) show spectra obtained at increasing times after mixing xanthine oxidase with 1-methylxanthine in $^2\text{H}_2\text{O}$ solution. Preliminary inspection of these spectra is certainly consistent with the original concept that the proton giving the large splitting is present partly as hydrogen and partly as deuterium at short reaction times, with the proportion of deuterium increasing steadily with time.

To study the phenomenon in a more quantitative way, we obtained difference spectra. By subtracting the spectra obtained at longer reaction times from those obtained at shorter ones and vice versa, using the procedures of Bray *et al.* (1978), we were able to show that all spectra were made up from two species

Fig. 4. *E.p.r. spectra illustrating direct transfer of hydrogen from 1-methylxanthine to xanthine oxidase*

Rapid spectra recorded at different reaction times during reduction of the enzyme by ordinary 1-methylxanthine in $^2\text{H}_2\text{O}$ are shown in (d)–(f); (b), (c) and (g) are difference spectra from this series; (h) is a difference spectrum in the converse experiment in $^1\text{H}_2\text{O}$ using substrate labelled in the C-8 position with ^2H ; (a) is a simulation of (b) and (c), and (i) a simulation of (h). The reaction was carried out in 50 mM-Bicine buffer, pD_{app} 8.2, at 12°C . Reaction times in (d)–(f) were respectively 32, 56 and 145 ms. (b), (c) and (g) are difference spectra obtained by appropriate subtraction of spectra for the following reaction times (in ms) 32–56, 56–145 and 56–32 respectively; (h) is the converse difference spectrum, 145–56; (a), (b) and (c) correspond to the form Hd; (g) corresponds to form Dd (cf. Fig. 3d) and (h) and (i) to form Hh (see the text for the meanings of these symbols). Parameters used for the simulations were as in Fig. 3. The g values and ^1H hyperfine splittings are shown as stick diagrams for the Hd and Hh spectra at the top and bottom respectively.

only. These are illustrated respectively in Figs. 4(b) and 4(c) for the short-time spectrum, and Fig. 4(g) for the long-time spectrum. The latter is clearly the 1-methylxanthine Rapid signal, with deuterium in both sites (compare Fig. 4g with Figs. 3d and 3e). The other species (Figs. 4b and 4c), present initially in about 65% of the signal-giving molecules, decreased in relative intensity steadily with time and was distinct in form from anything that we have observed previously. The sharpness of the g_1 doublet in this spectrum is particularly apparent, as are the well-resolved shoulders at the bottoms of the main 'downward' features in the g_2, g_3 region. In fact, the spectrum corresponds to Rapid with hydrogen in the site giving the larger splitting and with deuterium in the site giving the smaller one. We shall refer to this as 'form Hd,' and shall use the analogous symbols Hh, Dd and Dh to correspond respectively to signals with hydrogen or deuterium atoms in the two sites interacting with the molybdenum atom. We simulated the spectrum of form Hd (Fig. 4a), using the same basic parameters as were used in Fig. 3. Agreement between the simulated spectrum and the experimental difference spectra (Figs. 4b and 4c) establishes that the latter are due to form Hd.

Since all the spectra obtained during reduction of the enzyme by 1-methylxanthine are made up from forms Hd and Dd, we have thus established that the proton giving the smaller splitting was in the deuterium form throughout the reaction, whereas the proton giving the larger splitting was initially hydrogen, changing gradually to deuterium. We established earlier that these two protons are bound to the enzyme, not to the substrate, so we conclude that the one giving the large splitting has been transferred from the substrate to the enzyme and gradually exchanged out with deuterium from the medium.

It should be mentioned that the parameters used to simulate the spectrum of form Hh (Fig. 3c, Fig. 4i), reproducing the features observed experimentally, assumed that the second proton not only broadened and split the main g_1 doublet, but also broadened the g_2 and g_3 features. Hence, as already noted, the marked differences between Figs. 4(b) and 4(c) on the one hand and Fig. 3(a) (and also Fig. 4h) on the other, are attributable entirely to the effects of the second proton. In additional experiments (not illustrated) we found that the direct-hydrogen-transfer phenomenon was observable at 35 GHz. At this frequency, however, the weakly coupled proton is completely lost in the linewidth.

The kinetics of the signal development and $^1\text{H}^+/^2\text{H}^+$ exchange processes are illustrated in Fig. 5. Integrated intensity of the signals from species Hd and Dd are plotted, as a function of time, for an experiment of the type shown in Figs. 4(d)–4(f), and already discussed. The theoretical lines through the experimental points adequately fit the data and were

Fig. 5. Time course of direct transfer of hydrogen from 1-methylxanthine to xanthine oxidase in $^2\text{H}_2\text{O}$

Integrated intensities of forms Hd (●) and Dd (▲) of the Rapid signal are plotted as a function of time (see the text for meanings of these symbols; intensities are expressed as electrons per functional molybdenum atom). The curves through the experimental points were calculated by using an analogue computer, in accordance with Scheme 1 with the following values of the rate constants: $k_a = 5.5\text{ s}^{-1}$, $k_e = 27\text{ s}^{-1}$, $k_d = 1.3\text{ s}^{-1}$.

Scheme 1. Reaction scheme for interpreting the data of Fig. 5

Forms Hd and Dd are the e.p.r.-detectable species; forms E and F are not detectable by e.p.r. k_a is the rate constant for signal appearance, k_d is that for signal disappearance [due to reduction to Mo(IV)] and k_e that for the $^1\text{H}^+/^2\text{H}^+$ exchange process. [Note that in applying the scheme it is necessary to allow for incomplete conversion to Mo(V) because of equilibrium with Mo(VI) and Mo(IV) (cf. Barber *et al.*, 1977). This was done by assuming the maximum possible conversion to Mo(V), achieved in equilibrium, but not in kinetic experiments, to be 35%.]

obtained according to Scheme 1. We conclude from Fig. 5 that the rate constant for the $^1\text{H}^+/^2\text{H}^+$ exchange process is 27 s^{-1} , at 12°C , under the conditions of the experiment.

Since the second proton, the one giving the smaller splitting, is not transferred from the substrate, our

results provide no information about the rate at which it exchanges with protons of the medium.

Similar experiments were performed with xanthine as substrate instead of 1-methylxanthine. Unfortunately, the spectra obtained under identical conditions were not simple to interpret, probably because they were composed of more than two signal forms.

Experiments with 1-methyl[8-²H]xanthine

An experiment, which was the converse of those just described, using 1-methyl[8-²H]xanthine in ¹H₂O is illustrated in Fig. 4(h). We would predict spectra in such an experiment to be made up from forms Dh and Hh. The difference spectrum illustrated in Fig. 4(h) is clearly of form Hh, as expected and as is shown by comparison with the simulation of Fig. 4(i). The other difference spectrum, for form Dh, is not illustrated, but was obtained and showed, relative to form Dd (Fig. 3e), a broadening rather than a splitting in the *g*₁ region, in agreement with simulation results. This experiment demonstrates that the hydrogen (in this case ²H) transferred from the substrate to the enzyme is derived specifically from the 8-position of the substrate molecule.

In all the direct-hydrogen-transfer experiments described in this and the previous section, we believe that the first substrate molecule has reacted at the active site and the product molecule has dissociated. The product has then been replaced by a second substrate molecule, occupying the anion site. The rapid signal observed is therefore taken (Bray *et al.*, 1978) to represent the Michaelis complex for the conversion of 2-electron-reduced enzyme to the 4-electron-reduced form (or alternatively that for the 4-electron to 6-electron conversion). We would have preferred to work with an ion such as nitrate bound to the enzyme, in place of a substrate or a product molecule, but tight binding of the purines precluded this. It might therefore be queried whether the hydrogen of the bound substrate molecule occupying the anion site does not complicate the observations described and possibly even invalidate the interpretation given above.

To remove this objection, it is necessary to show that labelled and unlabelled substrate molecules, when bound to the enzyme in the anion site, give signals indistinguishable from one another. This was achieved by comparing the signals from 1-methyl[8-¹H]xanthine and 1-methyl[8-²H]xanthine with one another, while working in ²H₂O with long reaction times, so that hydrogen transferred to the enzyme had exchanged out again completely. Identity of the spectra obtained at 35 GHz under these conditions (Figs. 3i and 3j) confirms that the isotope in the substrate had no more than a negligible effect on the signal. (In fact in similar experiments at 9 GHz, we observed a slight

narrowing of the 'downward' feature in the *g*₂, *g*₃ region when the ²H-labelled substrate was used in place of the ¹H form. However, this amounted to only about 0.05 mT, indicating very weak coupling indeed for this proton.) Parallel n.m.r. experiments (see the Materials and Methods section) showed that the enzyme did not catalyse proton exchange in the unoxidized substrate. Thus at least 80% of 1-methylxanthine molecules, exposed anaerobically to a moderate concentration of enzyme for as long as 15 min, retained the proton in the C-8 position. We conclude that the substrate molecule in the anion site in no way interferes with observation of the direct-hydrogen-transfer phenomenon.

Our results will be important in elucidating the catalytic mechanism of xanthine oxidase. They imply that the substrate is first bound to molybdenum in the Michaelis complex and that it then transfers its C-8 proton to the enzyme. The nature of the proton-accepting group in the enzyme and further details of the mechanism are considered elsewhere (Gutteridge *et al.*, 1978).

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