Studies by Electron-Paramagnetic-Resonance Spectroscopy and Stopped-Flow Spectrophotometry on the Mechanism of Action of Turkey Liver Xanthine Dehydrogenase

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Studies by e.p.r. (electron-paramagnetic-resonance) spectroscopy and by stopped-flow spectrophotometry on turkey liver xanthine dehydrogenase revealed strong similarities to as well as important differences from the *Veillonella alcalescens* xanthine dehydrogenase and milk xanthine oxidase. The turkey enzyme is contaminated by up to three non-functional forms, giving molybdenum e.p.r. signals designated Resting I, Resting II and Slow. Slow and to a lesser extent Resting I signals are like those from the *Veillonella* enzyme, whereas Resting II is very like a resting signal described by K. V. Rajagopalan, P. Handler, G. Palmer & H. Beinert (1968) (*J. Biol. Chem.* **243**, 3784–3796) for aldehyde oxidase. Another non-functional form that gives the Inhibited signal is produced on treatment of the enzyme with formaldehyde. Stopped-flow measurements at 450 nm show that, as for the milk enzyme, reduction by xanthine is rate-limiting in enzyme turnover. The active enzyme gives rise to Very Rapid and Rapid molybdenum(V) e.p.r. signals, as well as to an FADH• signal. That these signals are almost indistinguishable from those of the milk enzyme, confirms the similarities between the active sites. There are two types of iron-sulphur centres that give signals like those in the milk enzyme, though with slightly different parameters. Quantitative reduction titration of the functional enzyme with xanthine revealed two important differences between the turkey and the milk enzymes. First, the turkey enzyme FADH•/FADH₂ system has a redox potential sufficiently low that xanthine is incapable of reducing the flavin completely. This finding presumably explains the very low oxidase activity. Secondly, whereas the Fe/S II chromophore in the milk enzyme has a relatively high redox potential, for the turkey enzyme the value of this potential is lower and similar to that of its Fe/S I chromophore.

In the preceding paper (Dalton *et al.*, 1976) studies by e.p.r. on the enzyme xanthine dehydrogenase from *Veillonella alcalescens* were described. Comparison of the results with earlier ones on milk xanthine oxidase (Bray, 1975) showed, despite some specific differences, that the structures of the e.p.r. chromophores and the mechanisms of action of these two enzymes must be very similar. To date, extensive e.p.r. work has been reported on only one other molybdenum hydroxylase, namely, aldehyde oxidase (Rajagopalan *et al.*, 1968*a,b*). However, this work did not lead to any clear understanding of the mechanism of action and revealed a number of anomalies. Only some of these were cleared up by studies, not including e.p.r. spectroscopy, by Branzoli & Massey (1974*a,b*). We therefore thought it desirable that another molybdenum hydroxylase should be studied in detail by e.p.r. spectroscopy supplemented by stopped-flow work. For such studies we selected turkey liver xanthine dehydrogenase, which has been purified and extensively investigated (Cleere & Coughlan,

1974, 1975; Cleere *et al.*, 1974, 1975). This xanthine dehydrogenase is available in larger quantities than is the one from *Veillonella alcalescens*.

Materials and Methods

Enzymes

Fresh-frozen turkey livers were obtained from Castlemahon Co-operative Co., Newcastle West, County Limerick, Ireland, and stored at –20°C until required for use. Xanthine dehydrogenase was isolated from this source by a slight modification of the method of Cleere & Coughlan (1975) and was concentrated by pressure filtration in the presence of 4 mM-cysteine. The enzyme was stored as frozen beads at liquid-N₂ temperature. For some experiments, recovered enzyme was used. This was obtained by dialysis to remove substrates, followed by pressure filtration in the presence of cysteine. The enzyme samples used had E_{280}/E_{450} 8–10 and E_{550}/E_{450} 0.35–0.38. Xanthine–NAD⁺

oxidoreductase activity (EC 1.2.1.37) of the enzyme was generally measured at 30°C, by using 0.15 mM-xanthine and 0.5 mM-NAD⁺ in potassium phosphate buffer, pH 7.8 (50 mM containing 0.1 mM-EDTA). Activity was expressed as the activity/ E_{450} ratio (30°C, 340 nm), by dividing the $\Delta E_{340}/\text{min}$ by the E_{450} of the enzyme used in the assay. In connexion with the stopped-flow experiments (Fig. 8), activity in the standard assay was compared with that in pyrophosphate buffer (pH 8.2, 50 mM), other conditions remaining unchanged. Activity was found to be 2% higher than under standard conditions. The oxidase activity of a number of recovered samples of the enzyme was checked and found to be similar to that of the freshly prepared enzyme, that is about 3% of the dehydrogenase activity.

Milk xanthine oxidase was prepared as described by Hart *et al.* (1970), and the activity/ E_{450} ratio (23.5°C, 295 nm) determined.

Estimation of concentration and percentage functionality of the enzymes

Total concentration of turkey liver xanthine dehydrogenase was estimated from E_{450} , taking ϵ_{mM} as 73 litre·mmol⁻¹·cm⁻¹ (Cleere & Coughlan, 1975). Total active-site concentration (functional and non-functional) was taken as twice the enzyme concentration. The proportion of functional sites was estimated from the activity/ E_{450} (30°C, 340 nm), taking the limiting value for fully active enzyme as 325 (Cleere *et al.*, 1974). The corresponding values for the milk enzyme were taken as $\epsilon_{\text{mM}} = 72$ litre·mmol⁻¹·cm⁻¹ (Bray, 1975) and limiting activity (23.5°C, 295 nm)/ $E_{450} = 197$ (McGartoll *et al.*, 1970).

E.p.r. measurements on enzyme samples

Techniques were generally as used by Dalton *et al.* (1976). Titrations were carried out as described by Palmer *et al.* (1964) [see also Swann & Bray (1972)]. Xanthine [sodium salt, crystalline, grade III, from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.], was dried over P₂O₅ before use. When successive incubations were carried out on a single sample of enzyme, corrections for evaporation were applied as described by Swann & Bray (1972). The isopentane correction in rapid-freezing experiments was taken as $\times 1.7$. Unless otherwise stated, all experimental reactions were carried out in sodium pyrophosphate buffer (50 mM, pH 8.2, adjusted with HCl, containing 1.0 mM-EDTA), with xanthine dehydrogenase at concentrations between 25 and 100 μM (total enzyme) and between 23 and 42% functional. Immediately before the experiments, cysteine was removed by gel filtration on Sephadex G-25 (with the standard buffer). When this was not done, the enzyme was consistently

found to be significantly reduced, with iron-sulphur signals approx. 5% maximally developed and with traces of the Rapid signal.

Some samples of xanthine dehydrogenase were submitted, before use, to successive reduction with sodium dithionite and reoxidation with air. Treatment with 50 mM-sodium dithionite for a few minutes at about 20°C was followed by removal of the reducing agent by gel filtration and exposure of the enzyme to air until it was reoxidized (no further change in E_{450}).

Quantification of e.p.r. signals

Double integration of the e.p.r. signals was performed with copper-EDTA as standard. Corrections for the difference in g value between the standard and unknown were applied (Aasa & Vänngård, 1970). Corrections were also applied because outer hyperfine lines from the 25% of molybdenum isotopes with $I = 5/2$ were generally ignored and for saturation, where appropriate. The Rapid, Slow and FADH[•] signals were generally recorded at 120 K with 5 mW power. Under these conditions, the correction for saturation for Rapid and Slow signals was $\times 1.15$. Saturation of the FADH[•] signal depended on the extent of reduction of the enzyme, the correction factor ranging from about $\times 2.0$ at low extents of reduction to $\times 1.04$ at high extents. Fe/S I and II signals were measured together at 25 K and 1 mW power. The entire spectrum was integrated with appropriate correction for the FADH[•] and molybdenum present. There was little saturation of Fe/S signals under these conditions. When necessary, the g_1 feature of the Fe/S II signal was also examined at 100 mW power.

When it was necessary to quantify both Rapid and Resting II signals in the same sample (Fig. 9), this was done from measurement of the α and β peaks [nomenclature of Palmer *et al.* (1964)]. Resting II signal did not overlap the β feature. The amplitude of this peak could therefore be taken as a measure of the intensity of the Rapid signal. The intensity of Resting II signal was taken as proportional to $\alpha - 0.67\beta$.

Stopped-flow experiments

These were performed, anaerobically, in a modified American Instrument Co. stopped-flow apparatus (Thorneley, 1974).

Results

Types of e.p.r. signals

Under appropriate conditions we have obtained six different e.p.r. signals from molybdenum(V)

Fig. 1. Molybdenum e.p.r. spectra of non-functional forms of xanthine dehydrogenase

The spectra shown are: (a) Resting I signal obtained from an untreated sample of the enzyme (300 μ M total enzyme; a small amount of Resting II signal is also present as well as traces of Rapid signal); (b) Resting II signal obtained from a sample from another batch of the fully oxidized untreated enzyme; (c) Slow signal obtained on reduction of an enzyme sample with dithionite; (d) Inhibited signal obtained on inactivation with formaldehyde. All samples were in 50mM-pyrophosphate buffer, pH 8.2. Conditions for preparing samples (c) and (d) were respectively as follows: reaction with 0.8mM-dithionite for 20 min at 20°C; reaction with 150mM-formaldehyde for 15 min at 20°C. E.p.r. conditions were: temperature, 120K; modulation, 0.00025 T (2.5 G); microwave power, 5mW [2mW in (a)]; microwave frequency, 9.1–9.3GHz. A scale of approximate g values is given.

from turkey liver xanthine dehydrogenase, as well as two signals from iron-sulphur systems in the enzyme and an $\text{FADH}^\bullet$ free-radical signal. All of these signals, except for two of those from molybdenum (Resting I and Resting II), are closely analogous to corresponding signals from normal milk xanthine oxidase. The e.p.r. signals will now be described individually.

Molybdenum(V) signals from non-functional forms of xanthine dehydrogenase

Of the molybdenum(V) e.p.r. signals obtainable from turkey liver xanthine dehydrogenase, only two, the Very Rapid and the Rapid, appear within the turnover time on adding substrates to the enzyme (see under 'Molybdenum(V) signals from functional xanthine dehydrogenase') and are therefore to be attributed to reduction of the active enzyme. Fig. 1 shows the four signals we have obtained from non-functioning forms of the enzyme. Figs. 1(a) and 1(b) illustrate two signals which we observed, though in very variable amounts, in samples of untreated turkey liver xanthine dehydrogenase. We shall refer

Table 1. Parameters of e.p.r. signals for turkey liver xanthine dehydrogenase samples

Molybdenum(V) (non-functional forms)	
Resting I*	g_1 1.993, g_2 1.980, g_3 1.956, g_{av} 1.976
Resting II*	g_1 1.977, g_2 1.972, g_3 1.965, g_{av} 1.971
Slow	g_1 1.971, g_2 1.968, g_3 1.957, g_{av} 1.965 $A[\text{H}]_{1,2,3}$ 0.0015–0.0017 T (15–17 G)
Inhibited	g_1 1.990, g_2 1.978, g_3 1.952, g_{av} 1.973 $A[\text{H}]_1$ 0.00045 T (4.5 G), $A[\text{H}]_2$ 0.0004 T (4.0 G), $A[\text{H}]_3$ 0.00055 T (5.5 G)
Molybdenum(V) (functional forms)	
Very Rapid	g_1 2.023, g_2 1.954, g_3 1.949, g_{av} 1.975
Rapid†	g_1 1.991, g_2 1.970, g_3 1.966, g_{av} 1.976 $A[\text{H}]_1$ 0.0012 T (12 G), $A[\text{H}]_2$ 0.0015 T (15 G), $A[\text{H}]_3$ 0.0015 T (15 G)
$\text{FADH}^\bullet$	g 2.004 Width 0.0019 T (19 G)‡
Fe/S	
Fe/S I	g_1 2.017, g_2 1.932, g_3 1.906, g_{av} 1.95
Fe/S II	g_1 2.08, g_2 2.00, g_3 1.92, g_{av} 2.00

*These signals were not given by all untreated (i.e. resting) samples of the enzyme.

† Measured from the spectrum of Fig. 4(a); provisional values, since this is probably not a single species (cf. Bray & Vänngård, 1969).

‡ Measured as the peak-to-peak width on the first-derivative spectrum for samples reduced with xanthine.

to these signals as Resting I and Resting II respectively. Both signals have a rhombic form and show no indications of interaction with protons. g values are given in Table 1.

Evidence that Resting I and Resting II signals derive from small amounts of non-functional forms of turkey liver xanthine dehydrogenase is as follows. First, as already noted, the intensity of these signals in different samples of the enzyme was quite variable but always low. Maximum integrated intensity of either signal never exceeded about 1% of the total molybdenum present. Further, Resting I signal, when detectable, failed to change in intensity in rapid-freezing experiments when reducing substrates were added to the enzyme (see below; Fig. 2b). Similarly, Resting II signal failed to change in the presence of the substrate, even at longer reaction times (see below; Fig. 6b). Additional experiments with oxidizing or reducing agents revealed the following points. Neither signal changed significantly on treatment of the enzyme with 4mM-phenazine methosulphate or 2mM-cysteine, or on leaving it in air. Resting I signal disappeared on treatment with dithionite and did not readily return on re-exposure to oxygen. We were able to make use of this to eliminate Resting I signal and so simplify quantification of the other e.p.r. signals from some enzyme samples which initially showed large amounts of this signal (see the Materials and Methods section for details of the treatment). On the other hand, Resting II signal must derive from a highly stable form of molybdenum(V) in the enzyme, being resistant to brief treatment with either dithionite (50mM) or ferricyanide (1mM).

Fig. 1(c) shows the Slow signal from turkey liver xanthine dehydrogenase and Fig. 1(d) shows the Inhibited signal obtained on treating the enzyme with formaldehyde. Parameters of the Slow and Inhibited signals are given in Table 1.

Molybdenum(V) signals from functional xanthine dehydrogenase

Rapid signals were readily observable from xanthine dehydrogenase, on reduction with substrates, either in rapid-freezing experiments (Figs. 2b, 3a and 3b), or in manual-mixing experiments (Figs. 4a and 4b). As already mentioned, the Rapid signal developed without affecting the Resting I signal, when this was detectable. Thus the intensity of the latter signal was unchanged 60ms after addition of xanthine to the enzyme (Figs. 2a and 2b). The form of the signal changed on formation of complexes with substrate, when this was present in excess (Figs. 4a and 4b). The signals obtained from the turkey and milk enzymes with excess of purine are compared in Figs. 4(b) and 4(c).

The Very Rapid signal, obtained on reducing turkey

Fig. 2. Molybdenum e.p.r. signals obtained from xanthine dehydrogenase and milk xanthine oxidase in rapid-freezing experiments

The pH in (a) and (b) was 8.2; in (c) and (d) it was 9.9; in (a)–(c) xanthine dehydrogenase was used and in (d) milk xanthine oxidase. In (a) no substrate was added and the signal is mainly Resting I. In (b) xanthine (0.9mM; reaction time 60ms) was added to enzyme, at the same concentration and from the same batch as in (a); Rapid (as well as $\text{FADH}^{\bullet}$) signals are present, whereas Resting I signal remains unchanged. A different enzyme sample was employed in (c), which shows the Very Rapid signal; here, and with the milk enzyme in (d), the reaction time with xanthine (1.0mM) was 16ms; in (c) and (d) similar functional active-site concentrations of the two enzymes were used. Relative instrument-gain settings are shown on the Figure. E.p.r. conditions were: 120K; modulation 0.00025T (2.5G); microwave power, 5mW; microwave frequency, 9.1–9.3GHz. A scale of approximate g values is given.

liver xanthine dehydrogenase with xanthine, for the shortest reaction times, at high pH values, is shown in Fig. 2(c), and is compared with the corresponding signal from the milk enzyme, shown in Fig. 2(d). Parameters of the Rapid and Very Rapid signals are given in Table 1.

Fig. 3. Molybdenum and free-radical e.p.r. signals obtained on reduction of xanthine dehydrogenase and milk xanthine oxidase with xanthine in rapid-freezing experiments

(a) and (b) show xanthine dehydrogenase and (c) and (d) milk xanthine oxidase. Reaction times with xanthine (1.0mM) at 20°C and pH 8.2 were 180ms in (a) and (c) and 800ms in (b) and (d). All samples correspond to the same concentration of functional active sites (18 μ M). Relative instrument gain settings are shown on the Figure. E.p.r. conditions were: 120K; modulation 0.00025 T (2.5 G); microwave power, 5mW; microwave frequency, 9.1–9.3 GHz. A scale of approximate g values is given.

FADH• signal

The flavin semiquinone free-radical signal from turkey liver xanthine dehydrogenase was readily obtainable, both in rapid-freezing experiments (Figs. 2b, 3a and 3b) and in manual-mixing experiments (Figs. 4a and 4b). It had a linewidth of 0.0019T (19G) (Table 1), confirming the earlier conclusion from optical work (Cleere & Coughlan, 1975) that the radical is of the 'blue' type (Palmer *et al.*, 1971).

Signals from iron-sulphur systems

Iron-sulphur e.p.r. signals from the enzyme are shown in Fig. 5. Allopurinol was used here as reducing agent to suppress Rapid signals from molybdenum (cf. Massey *et al.*, 1970). However, there was no difference in the form of the Fe/S signals when xanthine or dithionite was substituted. As with the milk enzyme, two types of iron-sulphur centres could be distinguished and their signals will be referred to, by analogy, as Fe/S I and Fe/S II. The latter signal was detectable only at lower temperatures than were required to sharpen the former. Their parameters are given in Table 1;

g values for Fe/S II signal are to be regarded as provisional, in the absence of studies at 35 GHz.

Reductive titration with xanthine

Reductive titration of milk xanthine oxidase has given much important information on the enzyme. Though recent work has used dithionite as reducing agent (Olson *et al.*, 1974b), the earliest studies used xanthine (Bray *et al.*, 1961). We preferred a physiological substrate and therefore worked with xanthine, carrying out titrations side by side on the turkey and the milk enzymes (Figs. 6 and 7). Each titration point was obtained on a separate sample of enzyme, by using a comparatively short reaction time (1.5min). Under these conditions, at least for the milk enzyme, only functional enzyme is expected to be reduced (Swann & Bray, 1972). Fig. 6(b) confirms that this applies also to the turkey enzyme. Thus two samples, though they differed almost twofold in percentage contents of functional enzyme, nevertheless yielded data falling on the same titration curves.

Our results on the milk enzyme (Fig. 6a) are the most complete available with xanthine as reducing agent and are in full agreement with earlier work

Fig. 4. Effect of substrates on the form of Rapid e.p.r. signal

Xanthine dehydrogenase was used in (a) and (b); milk xanthine oxidase is shown for comparison in (c). (a) is the dehydrogenase reduced with limited amounts of xanthine (two electrons per functional active centre); (b) was obtained on reduction with a large excess of purine (23 mM); (c) shows milk xanthine oxidase also reduced with 23 mM-purine. In all cases reduction was for about 1 min at pH 8.2 and 20°C. E.p.r. conditions were: 120K; modulation 0.00016 T (1.6 G) [0.00025 T (2.5 G) in (a)]; microwave power, 5 mW; microwave frequency, 9.1–9.3 GHz. A scale of approximate *g* values is given.

(cf. Bray, 1975). Olson *et al.* (1974b) have shown how such titration curves may be explained from a consideration of the redox potentials of the various groups on the enzyme molecule. Results on the turkey liver enzyme (Fig. 6b), although generally similar, reveal a number of important differences, the most striking being in the behaviour of the $\text{FADH}\cdot$ free-radical signal. This signal was several-fold more intense for the turkey enzyme, maximum intensity corresponding to approx. 50% conversion of the flavin into the semiquinone, as compared with 15% for milk enzyme. Further, the signal did not disappear in the presence of excess of reducing agent. We attribute this (cf. Olson *et al.*, 1974b) to a low redox potential in the turkey enzyme for the system $\text{FADH}\cdot/\text{FADH}_2$. Comparable behaviour was observed in rapid-freezing experiments (see below); flavin was, however, fully reducible by dithionite (see Fig. 1c). Saturation behaviour of the $\text{FADH}\cdot$

Fig. 5. E.p.r. signals from iron-sulphur centres in xanthine dehydrogenase

The sample was reduced anaerobically with allopurinol (1.5 mM) for 1 min at pH 8.2. (a) shows the Fe/S I signal. Some free-radical signal is also apparent but little molybdenum(V) is detectable. (b) was recorded at a lower temperature, so that the signal from Fe/S II was developed and at a higher microwave power and so that the Fe/S I signal was largely saturated (the free-radical signal was also presumably saturated). Temperature, modulation and microwave powers in (a) and (b) were respectively: 51 and 25 K; 0.001 and 0.001 T (10 and 10 G); 20 and about 400 mW; microwave frequency, 9.1–9.3 GHz. A scale of approximate *g* values is given.

signal was highly dependent on the extent of reduction of the enzyme (see the Materials and Methods section).

Both samples of turkey enzyme, when used for titration, were free from Resting I signals and only one of them showed Resting II signals in significant amounts. As shown in Fig. 6(b) (and noted above), the intensity of the latter signal did not change during the titration.

Behaviour of the turkey Rapid signal was entirely analogous to that observed with the milk enzyme.

Fig. 6. Anaerobic titration of (a) milk xanthine oxidase and (b) turkey liver xanthine dehydrogenase with xanthine

The e.p.r. signals were quantified as described in the Materials and Methods section and their intensities expressed as electrons per functional active site. Concentrations of xanthine added are expressed in the same units. The symbols are: total iron-sulphur (Fe/S I plus Fe/S II) ($\circ$, $\bullet$); Mo(V) Rapid (Δ , $\blacktriangle$); FADH $^+$ ($\square$, $\blacksquare$); Mo(V) Resting II (∇). Each point on the graphs corresponds to the average value for two separate samples. At each xanthine concentration, a fresh enzyme sample was used; the reaction time (at about 20°C), before freezing was 1.5 min. For both (a) and (b), two separate experiments, each using a different batch of enzyme, are illustrated (full and open symbols). Percentage enzyme functionality values were as follows; milk enzyme: both 80%; turkey enzyme: 23% (full symbols) and 39% (open symbols).

This signal first increased in intensity, then decreased again, as the quantity of xanthine was increased, corresponding to successive reduction of molybdenum from the hexivalent to the quinquivalent and then to the quadrivalent states. Maximum

Fig. 7. Changes in the relative amounts of the Fe/S I and Fe/S II e.p.r. signals from milk xanthine oxidase and turkey liver xanthine dehydrogenase during titration with xanthine

The experiments are the ones illustrated in Fig. 6. Normalized amplitudes of the g_1 feature of the Fe/S II signal and the g_2 feature of the Fe/S I signal were measured. Their ratio is plotted as a function of the amount of xanthine added. Symbols are: milk enzyme (Δ , $\blacktriangle$); turkey enzyme ($\circ$, $\bullet$); open and full symbols correspond to separate experiments (as in Fig. 6).

conversion into the quinquivalent state corresponded to about 35% of the metal of the functional enzyme, for both the milk and the turkey enzymes (Fig. 6).

Apart from the fact that about 1% of the total iron in the enzyme (corresponding to 5% of that from the functional enzyme) was reduced in the starting material, the behaviour of the combined iron-sulphur signals from the turkey enzyme was analogous to that observed with the milk enzyme (Fig. 6). In both cases maximum integrated intensities of the signals corresponded to close to two electrons per functional active centre. For both enzymes, too, the end point of the titration, for maximum development of these signals, corresponded to six (or perhaps eight) electrons per functional centre. However, a pronounced difference between the enzymes with regard to Fe/S is illustrated in

Fig. 7. For the milk enzyme, Fe/S II has a higher redox potential than Fe/S I (Lowe *et al.*, 1972; Olson *et al.*, 1974b). Thus, at low xanthine concentrations, Fe/S II signal is preferentially developed, as is shown by the two- to three-fold decrease in the ratio of the amplitudes of the two signals during the course of the titration (Fig. 7). In contrast, for the turkey enzyme, no such behaviour is apparent, the ratio remaining essentially constant throughout (Fig. 7). We conclude that the two Fe/S centres in the turkey enzyme must have redox potentials very similar to one another.

Fast-kinetic studies

The difficulty of preparing turkey liver xanthine dehydrogenase in very large quantities precluded extensive rapid-freezing studies. However, some stopped-flow experiments were performed. As shown in Fig. 8 partial bleaching of the 450 nm absorption of the enzyme took place rapidly on adding either xanthine or NADH. Detailed studies on the extent of bleaching, as a function of the proportion of active enzyme were not carried out. However, the observed extent with xanthine (about 10% for 23% functional enzymes) was of the expected order of magnitude (cf. McGartoll *et al.*, 1970). The kinetics of reduction with NADH seemed complicated, whereas with xanthine no deviations from first-order were noted. The rate constant for the bleaching by xanthine, measured in three experiments on two different samples of the enzyme, was $33 \pm 1 \text{ s}^{-1}$. The turnover-rate constant for the enzyme under these conditions (pH 8.2, 30°C) is 32.5 s^{-1} [calculated from the extinction coefficient of Cleere & Coughlan (1975) and the limiting activity of Cleere *et al.* (1974), with correction for the difference in pH; see the Materials and Methods section]. It is therefore clear that, as for the milk enzyme (Massey *et al.*, 1969; Olson *et al.*, 1974a,b), reduction by the reducing substrate is rate-limiting in turnover.

As noted above, both the Rapid and Very Rapid molybdenum signals from turkey liver xanthine dehydrogenase were observed in rapid-freezing experiments at short reaction times. Iron-sulphur and radical signals could also be obtained. A difference in behaviour between the FADH• signals of the milk and turkey enzymes, at short reaction times, is illustrated in Figs. 3(a)–3(d). The radical signal of the milk enzyme was relatively weak at 180 ms and had declined virtually to nothing at 800 ms. By contrast, that of the turkey enzyme was much more intense at both reaction times and showed no evidence of decay. These findings are consistent with the titration results and confirm the relevance of the latter to the behaviour of the enzyme at short reaction times.

Fig. 8. Stopped-flow traces at 450 nm showing anaerobic reduction of turkey liver xanthine dehydrogenase by (a) xanthine and (b) NADH

Conditions were as follows: total enzyme concentration about $6 \mu\text{M}$; xanthine $80 \mu\text{M}$; NADH $30 \mu\text{M}$; pH 8.2; temperature 30°C.

Slow phases in the reduction of the enzyme

At reaction times of longer than 1–2 min, in the presence of substrates, desulpho enzyme in ordinary milk xanthine oxidase preparations is gradually reduced, as a result of its interaction with reduced functional enzyme molecules (Swann & Bray, 1972; Olson *et al.*, 1974b). Corresponding behaviour by the turkey enzyme is illustrated in Table 2. In the time range 1.5–10.5 min the Rapid signal gradually increased in intensity (phase III of Swann & Bray, 1972) and changed at longer reaction times to the Slow signal. The milk enzyme shows little FADH• signal in the presence of excess of xanthine at any reaction time (Swann & Bray, 1972), other than quite short ones (e.g. Fig. 3c). In contrast, for the turkey enzyme, FADH• increased steadily with time, from 1 s onwards, as flavin of the desulpho enzyme was reduced. (A relatively high rate of reduction of desulpho turkey enzyme is implied by the FADH• data in Table 2. Further, if a little Fe/S in this form of the enzyme was, in fact, reduced at 1.5 min reaction time, this would explain the observed Fe/S titration values of slightly greater than two electrons per active site in Fig. 6b.)

On reduction with dithionite for 20 min, the Slow signal was obtained and the flavin and iron-sulphur centres of both active and desulpho enzyme were fully reduced (Table 2). Thus here, Fe/S signal intensity approximated to two electrons per total active centre (functional plus non-functional).

Table 2. *Slow changes in intensity of e.p.r. signals from xanthine dehydrogenase*

Samples of turkey liver xanthine dehydrogenase containing 38–42% of functional enzyme were reduced anaerobically for the times indicated. E.p.r. signals were quantified as described in the Materials and Methods section. Intensities are expressed as electrons per functional active site (2 per mol); values in parentheses represent electrons per total (functional and non-functional) active site, this being more meaningful at the longer reaction times. The Mo(V) signal observed was the Rapid signal, unless otherwise stated. Reactions were carried out at pH 8.2 and 20–23°C, with the use of rapid freezing for the short reaction times. Substrate concentrations were: xanthine, 1.0 mM (up to 1 s) or 0.25 mM (from 1.5 min); dithionite, 0.8 mM.

Signal intensity (electrons per active centre)

Reducing agent	Reaction time	Mo(V)	FADH•	Fe/S (I+II)
Xanthine	0.2s	0.18	0.16	—
Xanthine	1.0s	0.14	0.15	—
Xanthine	1.5min	0.04	0.18	2.5
Xanthine	4.5min	0.07	0.27	—
Xanthine	10.5min	0.09* (0.03)*	0.41 (0.16)	—
Dithionite	20min	0.41† (0.16)†	<0.02	4.6 (1.9)

* The signal was a mixture of Rapid and Slow, with the former predominating.

† The signal was of the Slow type.

Saturation studies

Rajagopalan *et al.* (1968a) observed a signal very like Resting II signal in aldehyde oxidase. They studied changes in the saturation behaviour of this signal during reductive titration with substrates and found that, as substrate concentration increased, so the signal became progressively more difficult to saturate. Rajagopalan *et al.* (1968b) concluded that the molecules responsible for the signal from their resting enzyme were not catalytically inactive. As this conclusion differs from ours relating to the turkey enzyme, we thought it important to investigate saturation behaviour. By the procedure described in the Materials and Methods section, we followed changes in the saturation behaviour of both Resting II and Rapid signals in the titration experiments of Fig. 6(b). Results are presented in Fig. 9. In marked contrast with the findings of Rajagopalan *et al.* (1968a), the saturation behaviour of Resting II signal, on the sample of enzyme showing this signal, was quite independent of the presence or absence of xanthine (see upper curve in Fig. 9). This signal is much more readily saturated than is the Rapid signal (lower curve). The measurement procedure we used for separating the two overlapping signals is validated by the finding that the saturation behaviour of Rapid signal was the same in samples with and without Resting II signal (lower curve of Fig. 9).

Discussion

Origin of the e.p.r. signals

The behaviour of turkey liver xanthine dehydrogenase in the presence of substrates as observed

Fig. 9. *Saturation behaviour of the Rapid and Resting II molybdenum e.p.r. signals*

Signal amplitudes in arbitrary units (normalized by dividing by the instrument gain setting and the square root of the power in mW) are plotted against microwave power, on a double-logarithmic scale. The upper curve shows the behaviour of Resting II signal and the lower that of Rapid signal. ○, Obtained from a sample of untreated enzyme which showed Resting II signal only. After reduction of this sample for 1.5 min with excess of xanthine, both Rapid (▲) and Resting II (●) signals were measured separately, as described in the Materials and Methods section. Intensities of the two signals on this sample were such that (at low powers) Resting II signal amounted to about 19% of the total Mo(V) signal. Rapid signal was also measured (△) on another enzyme sample, not showing Resting II signal, after reduction with excess of xanthine. The observation temperature was 120K and the modulation 0.00025 T (2.5 G).

by e.p.r. spectroscopy is complicated. Nevertheless, it may readily be understood by reference to milk xanthine oxidase, providing proper account is taken of the presence of the various non-functional forms of the enzyme. On suitable reduction, molybdenum of the functional enzyme gives the Very Rapid and Rapid signals. In contrast, four forms with non-functional molybdenum centres give respectively the Resting I, Resting II, Slow and Inhibited signals. There is no evidence that the non-functional forms react with, e.g. purine substrates. However, the signals from them and the redox behaviour of their molybdenum centres are interesting in so far as they give information about the chemistry of the active centre of the enzyme. With respect to Fe/S and FADH• e.p.r. signals, there is no evidence that those from reduced non-functional molecules differ in any way from those from the functional enzyme.

Non-functional forms of turkey liver xanthine dehydrogenase

Ordinary turkey liver xanthine dehydrogenase preparations usually show three signals from non-functional forms, namely Slow, Resting I and Resting II signals. The occurrence of the Slow signal is expected, since Cleere *et al.* (1974) and Cleere & Coughlan (1974) have shown contamination of the enzyme with substantial amounts of the inactive desulpho form, which has lost an essential persulphide group and which is known for the milk enzyme to be responsible for this signal (McGartoll *et al.*, 1970).

No signal has been reported from milk xanthine oxidase which is analogous to Resting I signal. This signal is, however, rather like (but not identical with) a signal, also termed Resting I, observed in some preparations of *Veillonella* xanthine dehydrogenase by Dalton *et al.* (1976). These workers suggested that Resting I signal is due to a degradation product of the functional enzyme. Resting II signal seems extremely similar to both the resting signal of aldehyde oxidase (Rajagopalan *et al.*, 1968a) and a signal obtained on treating desulpho milk xanthine oxidase with ethylene glycol (D. J. Lowe, D. Sherwood & R. C. Bray, unpublished work). Oxidation-reduction behaviour of the two Resting signals was in most respects comparable with that of corresponding signals from other molybdenum hydroxylases.

Our saturation studies confirm that Resting II signal in turkey liver xanthine dehydrogenase is due to a non-functional species. It seems highly probable that for aldehyde oxidase, the corresponding signal also arises from non-functional molecules, whose iron-sulphur centres must have become indirectly reduced in the experiments of Rajagopalan *et al.* (1968a).

Finally, we note that a non-functional form of the turkey dehydrogenase giving the Inhibited signal is

obtained on treatment with formaldehyde. This signal is analogous to the corresponding one obtained on inactivation of milk xanthine oxidase either by formaldehyde or by methanol (Pick *et al.*, 1971). Its occurrence is consistent with inhibition of the turkey liver enzyme by methanol (Cleere & Coughlan, 1974).

Mechanism of action of turkey liver xanthine dehydrogenase

In most respects, our data confirm the close similarity of milk xanthine oxidase and turkey liver xanthine dehydrogenase. Each of the molybdenum signals, Very Rapid and Rapid as well as Slow and Inhibited, from the turkey enzyme is extremely similar to the corresponding signal for the milk enzyme. *g* values (Table 1) do not differ by more than about 0.002 from the milk enzyme values (Bray, 1974, 1975). Since in molybdenum compounds generally *g* values are quite sensitive to the nature of the ligands around the metal, we conclude that the ligands and their geometry are the same in the two enzymes. Detection of the Very Rapid signal from the turkey enzyme is particularly noteworthy. Previously, the milk enzyme was the only molybdenum hydroxylase known to give this signal, which is believed (Olson *et al.*, 1974b) to arise from an intermediate in which a xanthine residue is covalently bound to the enzyme.

Apart from the molybdenum signals, similarities of the FADH• and Fe/S signals in the milk and turkey enzymes are striking. Further, our pre-steady-state studies serve to emphasize the kinetic similarities between them. The main differences between the enzymes are ones of specificity. E.p.r. spectroscopy helps to explain some of these. As far as reducing substrates are concerned, specificity differences are fairly small (Cleere & Coughlan, 1975). In agreement with this, only minor differences between the substrate-binding regions of the enzymes are revealed by the molybdenum e.p.r. Differences include decreased intensity of Very Rapid signals in the turkey enzyme (compare amplitudes in Figs. 2c and 2d) as well as changes of form of Rapid signals on addition of purine (Fig. 4). The latter may reflect differences in the binding constants of substrate and product molecules, or both, between the two enzymes.

With respect to acceptor specificity, the milk and turkey enzymes differ more importantly (Cleere & Coughlan, 1975) and e.p.r. spectroscopy is more revealing. Comparison of e.p.r. signal types from iron-sulphur centres in molybdenum hydroxylases (cf. Dalton *et al.*, 1976) shows that milk xanthine oxidase and turkey liver xanthine dehydrogenase fall into one group, whereas aldehyde oxidase and *Veillonella* xanthine dehydrogenase fall into another. Clearly, therefore, types of iron-sulphur centres

do not correlate with oxidase or dehydrogenase behaviour. On the other hand there seems to be every reason to associate the increased concentrations and resistance to full reduction of the flavin semiquinone in the turkey enzyme with lack of reactivity to oxygen as oxidizing substrate. Olson *et al.* (1974b) have pointed out that in flavoproteins giving the 'blue' semiquinone, only the fully reduced form of the flavin reacts at all readily with oxygen, the semiquinone being more sluggish in its reaction. They provided evidence for milk xanthine oxidase that oxidase activity depends mainly on FADH₂ in the reduced enzyme reacting with oxygen. Our titrations show that, at all extents of reduction with xanthine, there is much more FADH[•], and therefore much less FADH₂, present in the turkey than in the milk enzyme. Lack of oxidase activity in the former is therefore in accordance with expectations. It should be added, however, that the nature of the difference in the reduced flavin site of molybdenum hydroxylases, which allows some of these enzymes but not others, to react readily with NAD⁺, remains quite uncertain. Finally, we note that the difference in the redox behaviour of the Fe/S II iron-sulphur centre of the turkey enzyme, relative to the milk enzyme, is marked. The relevance of this to the overall mechanism of action of the enzymes is, however, not at present certain.

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References

- Aasa, R. & Vänngård, T. (1970) *J. Chem. Phys.* **52**, 1612–1613
- Branzoli, U. & Massey, V. (1974a) *J. Biol. Chem.* **249**, 4339–4345
- Branzoli, U. & Massey, V. (1974b) *J. Biol. Chem.* **249**, 4346–4349
- Bray, R. C. (1974) *J. Less-Common Met.* **36**, 413–427
- Bray, R. C. (1975) *Enzymes*, 3rd edn., **12**, in the press
- Bray, R. C. & Vänngård, T. (1969) *Biochem. J.* **114**, 725–734
- Bray, R. C., Pettersson, R. & Ehrenberg, A. (1961) *Biochem. J.* **81**, 178–189
- Cleere, W. F. & Coughlan, M. P. (1974) *Biochem. J.* **143**, 331–340
- Cleere, W. F. & Coughlan, M. P. (1975) *Comp. Biochem. Physiol.* **B50**, 311–322
- Cleere, W. F., O'Regan, C. & Coughlan, M. P. (1974) *Biochem. J.* **143**, 465–468
- Cleere, W. F., Mulhern, J. F. & Coughlan, M. P. (1975) *Comp. Biochem. Physiol.* **B50**, 323–329
- Dalton, H., Lowe, D. J., Pawlik, R. T. & Bray, R. C. (1976) *Biochem. J.* **153**, 287–295
- Hart, L. I., McGartoll, M. A., Chapman, H. R. & Bray, R. C. (1970) *Biochem. J.* **116**, 851–864
- Lowe, D. J., Lynden-Bell, R. M. & Bray, R. C. (1972) *Biochem. J.* **130**, 239–249
- Massey, V., Brumby, P. E., Komai, H. & Palmer, G. (1969) *J. Biol. Chem.* **244**, 1682–1691
- Massey, V., Komai, H., Palmer, G. & Elion, G. B. (1970) *Vitam. Horm. (N.Y.)* **505**–531
- McGartoll, M. A., Pick, F. M., Swann, J. C. & Bray, R. C. (1970) *Biochim. Biophys. Acta* **212**, 523–526
- Olson, J. S., Ballou, D. P., Palmer, G. & Massey, V. (1974a) *J. Biol. Chem.* **249**, 4350–4362
- Olson, J. S., Ballou, D. P., Palmer, G. & Massey, V. (1974b) *J. Biol. Chem.* **249**, 4363–4382
- Palmer, G., Bray, R. C. & Beinert, H. (1964) *J. Biol. Chem.* **239**, 2657–2666
- Palmer, G., Müller, F. & Massey, V. (1971) in *Flavins and Flavoproteins* (Kamin, H., ed.), pp. 123–137, University Park Press, Baltimore
- Pick, F. M., McGartoll, M. A. & Bray, R. C. (1971) *Eur. J. Biochem.* **18**, 65–72
- Rajagopalan, K. V., Handler, P., Palmer, G. & Beinert, H. (1968a) *J. Biol. Chem.* **243**, 3784–3796
- Rajagopalan, K. V., Handler, P., Palmer, G. & Beinert, H. (1968b) *J. Biol. Chem.* **243**, 3797–3806
- Swann, J. C. & Bray, R. C. (1972) *Eur. J. Biochem.* **26**, 407–415
- Thorneley, R. N. F. (1974) *Biochim. Biophys. Acta* **333**, 487–496