Studies by Electron-Paramagnetic-Resonance Spectroscopy on the Mechanism of Action of Xanthine Dehydrogenase from *Veillonella alcalescens*

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E.p.r. (electron-paramagnetic-resonance) spectroscopy was used to compare the chemical environment and reactivity of molybdenum, flavin and iron-sulphur centres in the enzyme xanthine dehydrogenase from *Veillonella alcalescens* (*Micrococcus lactilyticus*) with those of the corresponding centres in milk xanthine oxidase. The dehydrogenase is frequently contaminated with small but variable amounts of a species resistant to oxidation and giving a new molybdenum(V) e.p.r. signal, 'Resting I'. There is also a 'desulpho' form of the enzyme giving a Slow Mo(V) signal, indistinguishable from that of the milk enzyme. Molybdenum of the active enzyme behaves in a manner analogous to that of the milk enzyme, giving a Rapid Mo(V) signal on partial reduction with substrates or dithionite. Detailed comparison shows that molybdenum in each enzyme must have the same ligand atoms arranged in the same manner. As with the milk enzyme, complex-formation between reduced dehydrogenase and purine substrate molecules, presumably interacting at the normal substrate-binding site, modifies the Rapid signal, confirming that such substrates interact near molybdenum. The dehydrogenase-flavin semiquinone signal is identical with that of the oxidase but, in contrast, there is only one iron-sulphur signal. The latter gives an e.p.r. spectrum similar to that of aldehyde oxidase.

The various xanthine oxidases and dehydrogenases, together with aldehyde oxidase, form an apparently closely related group of enzymes, all catalysing similar reactions and containing in their molecules molybdenum, flavin and iron-sulphur centres. The name 'molybdenum hydroxylases' has been proposed for these enzymes (Bray, 1975). The most extensively studied of them is milk xanthine oxidase whose mechanism of action is now beginning to be relatively well understood, largely as a result of studies by e.p.r. (electron-paramagnetic-resonance) spectroscopy and stopped-flow techniques (see Olson *et al.*, 1974b; Bray, 1975). The catalytic reaction of the milk enzyme is believed to involve transfer of two electrons from the reducing substrate molecule (for example, xanthine) to molybdenum of the enzyme, this metal being initially in the hexavalent state. This reaction is followed by a rapid distribution of the electrons amongst the various redox-active groups of the molecule. Up to three xanthine molecules can react sequentially with the enzyme (expressed per active-centre system, i.e., per half enzyme molecule). Reduced forms of the enzyme contain molybdenum in the quinquivalent and

the quadri-valent states, the former giving rise to highly characteristic e.p.r. signals. Reoxidation of the reduced enzyme molecule by molecular oxygen involves reaction of this at the flavin centre of the enzyme (Olson *et al.*, 1974a). It is less certain which groups of the reduced enzyme react with other oxidizing substrates (Bray, 1975).

Another enzyme of the group, xanthine dehydrogenase from *Veillonella alcalescens*, has been purified and studied by Smith *et al.* (1967). [N.B. This organism is also known as *Micrococcus lactilyticus*; see Dalton & Zubietta (1973).] Limited e.p.r. studies on this enzyme were reported by Aleman *et al.* (1966) and gave rise to the suggestion [Rajagopalan *et al.* (1968b); see also Handler (1971)] that the catalytic mechanism may not involve the cycling of molybdenum between oxidized and reduced states, the importance of which is now firmly established for the milk enzyme. We, therefore, thought it important to reinvestigate the properties of *Veillonella* xanthine dehydrogenase and particularly to carry out more detailed e.p.r. studies on it, with a view to establishing the extent to which it resembles the milk enzyme in its catalytic mechanism. Studies on another molybdenum hydroxylase, turkey liver xanthine dehydrogenase, are reported in the following paper (Barber *et al.*, 1976).

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Materials and Methods

Analytical methods and molecular-weight determination

Protein was determined by the Folin-Ciocalteu method (Lowry *et al.*, 1951) with dried bovine serum albumin as standard. Molybdenum, FAD and iron were determined as described by Hart *et al.* (1970). The molecular weight of xanthine dehydrogenase was estimated by gel filtration on Sephadex G-200 (Pharmacia, London W.5, U.K.) (cf. Andrews *et al.*, 1964). The following standards were employed: milk xanthine oxidase (275 000), catalase (240 000), β -amylase (215 000), γ -globulin (160 000), lactate dehydrogenase (130 000), bovine serum albumin (68 000), α -chymotrypsin (22 500), and myoglobin (17 800).

Assay of xanthine dehydrogenase activity

Assays were carried out spectrophotometrically in the presence of air, with xanthine as reducing substrate and ferricyanide as oxidizing substrate. Reaction was initiated by addition of the enzyme to a solution (total volume 3 ml) containing 1 mM- $K_3Fe(CN)_6$, 0.5 mM-xanthine, 0.5 mM-EDTA and 50 mM-potassium phosphate buffer, pH 7.8. The reaction, at 25°C, was followed at 420 nm, in a 1 cm light-path cuvette and with a reference cell containing the same components without enzyme. One unit of enzyme activity is defined (cf. Smith *et al.*, 1967) as the quantity which, when present in 1 ml of solution, causes a ΔE of 0.1/min, and specific activity is defined as the number of units/mg of protein. Though this assay is complicated by non-enzymic reaction between ferricyanide and uric acid (Fridovich & Handler, 1958; Komai *et al.*, 1969) it was, nevertheless, convenient and reproducible.

Maintenance and growth of Veillonella alcalescens

The organism was obtained from Dr. P. N. Hobson (Rowett Research Institute, Bucksburn, Aberdeen), and was maintained anaerobically in a solid agar-thioglycollate medium (Brewer, Oxoid Ltd., London S.E.1, U.K.), supplemented with 3% sodium lactate. The organism was subcultured into this medium, monthly. For large-scale cultivation, the organism was grown in 400-litre all-glass fermentation vessels, in a liquid medium, at 37°C. A 10% inoculum was used at each transfer. The growth medium was as given by Smith *et al.* (1967), except that the concentrations of peptone and of yeast extract were decreased fivefold and that thioglycollic acid (0.25 g per litre) replaced sodium dithionite. The supplement of trace elements was also increased tenfold. Cells were harvested after 8 h growth, and packed cells (about 1 kg wet weight from 400 litres of medium) were frozen and stored in liquid N_2 .

Extraction and purification of xanthine dehydrogenase

The purification procedure was closely related to that of Smith *et al.* (1967). Unless otherwise stated, all steps were performed at 4°C in the phosphate buffer solution containing EDTA, as used in the assay. Cells were suspended in this buffer and disrupted by means of a French press at 2°C. The extract was cooled immediately to 0°C, treated with bovine deoxyribonuclease (grade DN-100; Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K.) and centrifuged. Protamine sulphate (final concn. 8 g/l) was added to the supernatant solution and the mixture was centrifuged again. The enzyme remained in the supernatant; it was then chromatographed on DEAE-cellulose (Whatman DE 32; W. and R. Balston, Maidstone, Kent, U.K.). The sample was applied in the 50 mm buffer and eluted with a similar buffer at 150 mM. Fractions eluted from the column (64 mm $\times$ 200 mm) after the xanthine dehydrogenase contained rubredoxin, followed by ferredoxin (Dalton & Zubietta, 1973). Combined xanthine dehydrogenase fractions were purified by $(NH_4)_2SO_4$ fractionation (50–65% satn.), and then, after desalting by gel filtration, the enzyme was rechromatographed on DEAE-dextran gel (Sephadex A 50; Pharmacia, London W.5, U.K.), by using gradient elution with phosphate buffer, as before, from 0.05 to 0.4 M. In this process a red protein was eluted before the xanthine dehydrogenase. Investigation of the properties of the former, showed it to be methylmalonyl-CoA mutase (H. Dalton & K. N. Joblin, unpublished work). The peak xanthine dehydrogenase fractions were concentrated and submitted to two successive final purifications by gel filtration on Sephadex G-200. Up to 27% of the xanthine dehydrogenase activity present in the initial crude extract was recovered in the final product. Several samples of xanthine dehydrogenase, prepared by the above procedure, showed specific activities of about 800 units/mg. Though this value is twice that reported by Smith *et al.* (1967), lower activities were obtained with some preparations.

E.p.r. measurements on xanthine dehydrogenase samples

E.p.r. spectra were recorded on a Varian E9 spectrometer, as described by Lowe *et al.* (1972). Preparation of samples by manual mixing was as described by Swann & Bray (1972) and rapid freezing was by the method of Bray *et al.* (1973). Owing to the difficulty of preparing xanthine dehydrogenase in large quantity and at the highest purity, some e.p.r. studies had to be carried out on samples of relatively low specific activity (down to 42 units/mg). Nevertheless, the most crucial experiments used enzyme of the highest specific activity (e.g. Figs. 1a and 1c) and,

further, all the experiments described below have been repeated with more than one enzyme sample. Unless otherwise stated, e.p.r. samples were in phosphate buffer (as for the assays), with EDTA. Protein concentrations ranged from 8 to 50 mg/ml.

Purification of ferredoxin and rubredoxin

These proteins were obtained as by-products from the xanthine dehydrogenase purification. Ferredoxin was purified as described by Dalton & Zubieta (1973) and had $E_{390}/E_{280} = 0.82$. Rubredoxin was purified by rechromatography on DEAE-cellulose DE 32 and by $(\text{NH}_4)_2\text{SO}_4$ fractionation and had $E_{490}/E_{280} = 0.42$.

Results

Properties of xanthine dehydrogenase

The properties of our xanthine dehydrogenase were very similar to those reported by Smith *et al.* (1967). The spectrum of the enzyme in the visible range could not be distinguished from the one given by these workers. The shoulders to the main flavin peak, appearing in the regions of 420 and 470 nm respectively were accurately reproduced. The lowest value of the ratio E_{280}/E_{450} which we observed was 7. Two preparations of the enzyme gave Mo/FAD ratios close to 1. Two other preparations appeared to have rather lower values of this ratio, possibly indicating the presence of small amounts of the demolybdo enzyme. Gel filtration indicated the molecular weight of xanthine dehydrogenase to be possibly slightly lower than that of milk xanthine oxidase. A value of 220 000–270 000 was obtained.

Qualitatively, at least, the catalytic properties of the enzyme were as reported by Smith *et al.* (1967). However, relative to activity with ferredoxin as acceptor, oxidase activity of our preparations appeared somewhat higher than that of these workers (up to 29% instead of 12%). A new finding was that ferredoxin could be replaced as acceptor by rubredoxin. Not only rubredoxin from *Veillonella alcalescens* was active, but also those from *Clostridium pasteurianum* and from *Desulfovibrio desulfuricans* (both kindly provided by Professor J. R. Postgate, University of Sussex, U.K.). Whether this has any physiological significance is not clear. Maximum activities obtainable with ferredoxin and rubredoxin were roughly equal. In one experiment in the presence of xanthine and air, optimum activity at 295 nm was observed with rubredoxin at 20 μM or with ferredoxin at 40 μM . In both cases, there was some inhibition when the acceptor was present at higher concentrations. That the superoxide radical was not acting as a mediator in reduction of rubredoxin in this system

was shown by the lack of inhibition observed on adding superoxide dismutase (5 $\mu\text{g}/\text{ml}$; kindly supplied by Professor G. Rotilio, University of Rome, Italy).

Changes in the molybdenum(V) e.p.r. signals of xanthine dehydrogenase induced by reducing substrates

As shown in Figs. 1(a) and 1(b) we were able to reproduce accurately the e.p.r. spectra reported previously for *Veillonella* xanthine dehydrogenase by Aleman *et al.* (1966). Fig. 1(a) shows the spectrum

Fig. 1. Effect of reduction with purine on the e.p.r. spectrum of xanthine dehydrogenase

(a) Spectrum of untreated enzyme; (b), (c) and (d) spectra of the samples reduced with an excess of purine. The same enzyme sample was used in (a) as in (c). The purine concentration was 50 mM in (c) and the reaction time was extended to 30 min in (d). Spectra were recorded at 9.1 GHz and about 120 K, with the use of 0.0004 T (4 G) modulation and a microwave power of 5 mW [0.0005 T (5 G) and 10 mW for (b)]. A scale of approximate g values is shown.

from a sample of enzyme, as prepared, and Fig. 1(b) is the spectrum obtained after addition of purine. Neither of these spectra is closely similar to any of the reported e.p.r. signals from milk oxidase (Bray & Swann, 1972; Bray, 1975). Further, the milk enzyme as normally prepared is free from signals. As shown in Figs. 1(c) and 1(d), however, further treatment of *Veillonella* xanthine dehydrogenase with purine yielded e.p.r. signals which were clearly quite similar to known molybdenum(V) signals from the milk enzyme. Thus Fig. 1(c) is like the milk Rapid signal (a free radical signal is also present), whereas Fig. 1(d) is like the Slow signal.

It is now well established for the milk enzyme that the Rapid signal arises from the active enzyme, whereas the Slow signal comes from a non-functional degradation product, to which Bray (1975) gave the name 'desulpho xanthine oxidase'. The data in Fig. 1 suggested to us that the active *Veillonella* enzyme might be like the milk enzyme, but that its preparations might be contaminated, not by one, but by two non-functional species, each giving rise to a specific molybdenum(V) e.p.r. signal. To confirm this hypothesis, we carried out more detailed experiments on the enzyme, by reducing it with xanthine (Fig. 2) and with dithionite (Fig. 3).

Figs. 2(a)–2(e) show data obtained on a single sample of the enzyme during treatment with xanthine and reoxidation by molecular oxygen. The untreated enzyme showed the spectrum of Fig. 2(a). Although only slightly different from that of Fig. 1(a), Fig. 2(a) is more obviously, and more nearly completely, due to a single chemical species [presumably Mo(V)], giving a simple rhombic type of spectrum. The signal of this species will be referred to as Resting I [the I is added for comparative purposes: see Barber *et al.* (1976)]. The signal shows no indications of proton interaction and its *g* values are listed in Table 1. We found that Resting I signal was not given by all samples of the enzyme, nor did its intensity correlate, in any obviously negative or positive way, with the specific activity of the preparations. That it was due to a particularly stable form of molybdenum(V) was shown, apart from the obvious stability of the signal in the presence of air, by the finding that treatment of a sample (corresponding to that of Fig. 2a) with ferricyanide (1mM, for 1min) did not change the intensity or form of the signal. This is consistent with the Resting I signal being due to a new non-functional form of *Veillonella* xanthine dehydrogenase. However, this was never present in large amounts, integration of a rather strong Resting I signal indicating only 2% of the total molybdenum present to be in the signal-giving form.

The following signal changes occurred on adding xanthine to the enzyme sample of Fig. 2(a). After treatment with the substrate for 1 min (Fig. 2b),

Fig. 2. Effect of reduction with xanthine and reoxidation with oxygen on the e.p.r. spectrum of xanthine dehydrogenase

(a)–(e) Successive treatments of a single sample of xanthine dehydrogenase; (a) untreated enzyme; (b) after reduction with 1mM-xanthine anaerobically for 1 min; in (c) and (d) the reaction time was extended to 10 and to 40min respectively; in (e) the sample was shaken with air. Relative instrumental gain settings are indicated. A different enzyme sample (free from the Resting signal) was used in (f), and here the rapid-freezing method was used to give 450ms reaction time, anaerobically, with xanthine (final concentration 0.5mM). (The reason for the abnormally small linewidth of the free-radical signal in this experiment is not clear.) Spectra were recorded at 9.1 GHz and about 110K with 0.0005 T (5 G) modulation [0.0008 T (8 G) for (f)] and 5mW microwave power. A scale of approximate *g* values is shown.

Table 1. *Parameters of e.p.r. signals from xanthine dehydrogenase samples*

Molybdenum(V)	
Resting I*	g_1 2.010, g_2 1.979, g_3 1.964, g_{av} 1.983
Slow	g_1 1.972, g_2 1.968, g_3 1.958, g_{av} 1.966 $A[^1H]_{1,2,3}$ 0.0015–0.0017 T (15–17 G)
Rapid (purine)†	g_1 1.991, g_1 1.968, g_{av} 1.976 $A[^1H]_1$ 0.0011 T (11 G), $A[^1H]_2$ 0.0013 T (13 G)
FADH•	g 2.004 Width 0.0020 T (20 G)‡
Fe/S	g_1 2.026, g_2 1.939, g_3 1.925, g_{av} 1.963 Fully sharpened at 60–65K Power for 50% satn. at 12K is 0.5 mW Power for 50% satn. at 22K is 5 mW Power for 50% satn. at 37K is 50 mW

* This signal was not given by all samples of the enzyme.

† Provisional interpretation, only; g_1 was measured from the 35 GHz spectrum of Fig. 4 and g_1 from that at 9 GHz in Fig. 1(c). Additional experiments at 35 GHz indicated several species. Note, however, that at 9 GHz, resolution of g_x and g_y components was not apparent, even when the modulation was decreased to 0.00016 T (1.6 G) (pH 8.2).

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

in addition to a strong free radical signal (see below), features of the Rapid signal were clearly present. Some Resting I signal remained, however, which was observable from its g_2 feature, the height of which indicated that this signal was present at about 20% of its initial intensity. After more prolonged treatment with xanthine (Fig. 2c) the Rapid signal remained, whereas the Resting I signal had disappeared. After 60 min incubation (Fig. 2d) the Rapid signal had been replaced by the Slow signal, whereas after reoxidation by oxygen (Fig. 2e), the Resting I signal was restored to its initial intensity and the Rapid and Slow signals were no longer visible. A single rapid-freezing experiment is illustrated in Fig. 2(f) which used a sample of enzyme different from that used in Figs. 2(a)–2(e) and which did not give Resting I signal. After reduction with xanthine for 450 ms, a time presumably not very greatly in excess of the turnover time, a clear Rapid signal was detected (Fig. 2f).

Reduction with sodium dithionite was used for a more detailed comparison of the Rapid and Slow signals from xanthine dehydrogenase with those from milk xanthine oxidase. Reduction of the dehydrogen-

Fig. 3. *Rapid and Slow Mo(V) e.p.r. spectra, obtained with dithionite, from xanthine dehydrogenase (a) and (b) and from milk xanthine oxidase (c) and (d)*

(a) and (c) Rapid signal obtained after brief reduction; (b) and (d) Slow signal obtained on more complete reduction; a free-radical signal (presumably FADH•) is also present in the dehydrogenase samples. Reduction conditions were: (a) 1 min; (b) 40 min; (c) 2 s (rapid freezing); (d) 20 min; a low dithionite concentration was used in (a) and an excess in (b)–(d); cyanide-treated xanthine oxidase (Massey & Edmondson, 1970) was used in (d); all samples were in sodium pyrophosphate buffer (50 mM, pH 8.2). E.p.r. conditions were: 9.3 GHz; 120 K; 0.00025 T (2.5 G) modulation; 5 mW power. A scale of approximate g values is shown.

Fig. 4. Mo(V) e.p.r. spectrum of xanthine dehydrogenase recorded at 35 GHz

The enzyme sample was reduced for 60 min at pH 7.8 by an excess of dithionite in the presence of 50 mM-purine. Most of the features are due to the Slow signal but the g_1 doublet of the Rapid signal is also apparent at low field. Recording conditions were: 35.2 GHz; about 120 K; 0.00032 T (3.2 G) modulation; attenuation 10 decibels.

Fig. 5. Fe/S e.p.r. signals from xanthine dehydrogenase recorded at (a) 9.2 and (b) 35.4 GHz

The enzyme samples were reduced with 50 mM-purine for 1 min at pH 7.8. The stick diagrams show the positions of the principal g values of the Fe/S signal; additional features in the centres of the spectra are due to Mo(V) and, at 35 GHz only, also to free radicals. Recording conditions in (a) and (b) were respectively: temperature, 40 and approx. 25 K; modulation, 0.0005 T (5 G) and 0.002 T (20 G); power, 1 mW and 30 decibels attenuation.

ase by excess of dithionite gave a particularly well-defined Slow signal, illustrated in Fig. 3(b). The corresponding signal from the milk enzyme is shown in Fig. 3(d). Figs. 3(a) and 3(c) show the Rapid signals, obtained from the *Veillonella* and the milk enzymes respectively, by limited reduction with dithionite. A spectrum from the *Veillonella* enzyme recorded at 35 GHz is illustrated in Fig. 4, and parameters of the various e.p.r. signals are summarized in Table 1.

Low-temperature splitting of molybdenum signals due to interaction with reduced iron-sulphur centres (Lowe *et al.*, 1972) is observed for both the *Veillonella* and the turkey liver enzymes (Lowe, 1974, and unpublished work).

Free-radical and iron-sulphur signals from the enzyme

Reduction of *Veillonella* xanthine dehydrogenase gave rise to e.p.r. signals from the $\text{FADH}^\bullet$ semiquinone radical and from iron-sulphur centres. The free-radical signal was a major feature of the spectrum of enzyme, reduced with, for example, xanthine or purine (Figs. 1c and 2b-2d). Parameters of the signal are given in Table 1.

Signals from iron-sulphur centres in the *Veillonella* enzyme were readily detected at low temperatures, after reduction with xanthine, purine or dithionite. No difference was noted in the form of the signal, depending on the reducing agent. Spectra obtained with purine were recorded both at 9 and at 35 GHz (Fig. 5). The signal is quite different from that obtained with the milk enzyme (Lowe *et al.*, 1972). Only a single species was detectable, at least down to 8 K, this having almost axial parameters (Table 1). The signal is fully sharpened at a slightly higher temperature and saturates rather more readily than does the signal with $g_{\text{av.}} = 1.95$, from

Fe/S I, in milk xanthine oxidase. This indicates slower relaxation behaviour of Fe/S in the *Veillonella* enzyme. In one experiment, integration of the Fe/S signal corresponded to about 0.7 electron/mol of xanthine dehydrogenase. This sample had specific activity of 740 units/mg. However, the extent of contamination with non-functional forms which this activity represents is not known, so that definite conclusions cannot be drawn as to how many electrons the iron-sulphur system of the *Veillonella* enzyme is capable of accommodating.

Discussion

Preparation of xanthine dehydrogenase

Our method of preparation of xanthine dehydrogenase from *Veillonella alcalescens* may have some advantages over that of Smith *et al.* (1967). Further, it yielded a number of by-products which were of interest for other work. The catalytic properties of the enzyme prepared by the two methods are similar though our specific activities were usually higher. However, the value for the ratio E_{280}/E_{450} of 7, indicates that our enzyme is probably not completely pure, since other molybdenum hydroxylases give values close to 5 (Bray, 1975). [Smith *et al.* (1967) do not quote a value for this ratio.]

Environment of molybdenum in the enzyme

Comparison of molybdenum(V) e.p.r. signals for the *Veillonella* enzyme with those of the milk enzyme provides a means of comparing the environments of the metal atoms in the active centres of the two enzymes. Milk xanthine oxidase gives rise to four types of signals given the names Very Rapid, Rapid, Slow and Inhibited, and of these, only the first two are due to the functional enzyme (Bray & Swann, 1972; Bray, 1975). For the *Veillonella* enzyme we have observed two of these signals, Rapid and Slow, and additionally a new signal, Resting I.

Changes in the e.p.r. spectrum observed in the $g = 1.97$ region, occurring in the presence of substrates, are readily interpreted in terms of the

oxidation-reduction reactions shown in Scheme 1. In this scheme the active enzyme molecules give only the Rapid signal, which is detectable even at short reaction times. The Slow signal is shown arising from reduction of the non-functional desulpho form of the *Veillonella* enzyme, this reduction being brought about by interaction of the desulpho molecules with reduced active enzyme molecules. This is a reaction which is known to occur slowly in milk xanthine oxidase (Morell, 1952; Swann & Bray, 1972; Olson *et al.*, 1974b). For the milk enzyme, the desulpho form may be obtained by treatment with cyanide (Massey & Edmondson, 1970), but it also arises spontaneously, being present in all normal enzyme samples. Further, for milk xanthine oxidase, the Slow signal is known from pulse-radiolysis experiments (R. C. Bray & E. M. Fielden, unpublished work), to be due to molybdenum(V) of the desulpho enzyme, not molybdenum(III) as suggested by Massey and co-workers (Massey *et al.*, 1970; Olson *et al.*, 1974b). Although the *Veillonella* enzyme is relatively resistant to inhibition by cyanide (Smith *et al.*, 1967), our detection of the Slow signal provides unequivocal evidence for a form of this enzyme analogous to desulpho milk xanthine oxidase (see below for parameters of the Slow signal). This desulpho form presumably arises by loss of a sulphur ligand from molybdenum of the active dehydrogenase (cf. Bray, 1975).

As indicated in Scheme 1, there is no evidence that the new non-functional form which gives rise to the Resting I signal, can exist with its molybdenum in the hexavalent state. Relatively slow disappearance of Resting I signal in the presence of the substrate (Fig. 2) is assumed to be due to reduction to the quadrivalent level, by a process analogous to reduction of desulpho enzyme. The precise nature of the species giving rise to the Resting I signal is not clear. Its $g_{av.}$ (Table 1) is significantly different from that of the Slow or the Rapid signals, suggesting that the ligands of molybdenum may be identical with those of neither the active nor the desulpho enzyme. Though direct evidence is lacking, we suggest that

Scheme 1. Proposed oxidation-reduction reactions of molybdenum in active xanthine dehydrogenase and in non-functional forms of the enzyme

the species giving Resting I signal is another degradation product of active xanthine dehydrogenase. Some support is provided by the observation of a rather similar signal from turkey liver xanthine dehydrogenase (Barber *et al.*, 1976). The high stability of molybdenum(V) in Resting I signal is interesting. Stability may be even higher than that of this valence state of the metal in the Inhibited signal from milk xanthine oxidase (Pick *et al.*, 1971). However, the nature of reactions which might lead to production of the new species is uncertain.

Because of the relatively small amounts of enzyme available to us, we have not examined *Veillonella* xanthine dehydrogenase under conditions expected to give the Very Rapid or the Inhibited signals. These are given not only by the milk enzyme but also by turkey liver xanthine dehydrogenase (Barber *et al.*, 1976). Since the *Veillonella* enzyme has been reported by Smith *et al.*, (1967) to be particularly sensitive to inhibition by methanol it would presumably be expected to give an Inhibited signal.

From detailed examination of both the Slow and Rapid signals from the *Veillonella* enzyme, there seems to be every reason to conclude that molybdenum atoms in it and milk xanthine oxidase must have the same ligand atoms, arranged in the same manner. This conclusion is clearest for the desulpho forms of the enzymes. Each one of the e.p.r. parameters for the *Veillonella* Slow signal (measured both at 9 and at 35 GHz) is not significantly different from the corresponding values for the desulpho milk enzyme (Bray & Swann, 1972; Lowe, 1974; Bray, 1975).

The exact form in which the Rapid signal from the milk enzyme appears depends on the extent of complex-formation with substrate molecules (Pick & Bray, 1969). This situation also applies to the *Veillonella* enzyme. The Rapid signals (uncomplexed) of the milk and of the *Veillonella* active enzymes seem to be essentially the same (Figs. 3a and 3c), indicating that the close analogy in the environments of the molybdenum atoms is not limited to the desulpho forms. However, there seem to be a real difference in the signals obtained from the two enzymes when the Rapid signal is modified by the binding of purine molecules. Thus the spectrum of Fig. 1(c) is not quite identical with that of the milk enzyme (or that of the turkey enzyme) reduced with an excess of purine [cf. Fig. 4 of Barber *et al.* (1976)].

Similarities in the molybdenum environments in the enzymes is entirely consistent with the similarities of the reactions they catalyse. At the present time, however, there are no clues from the e.p.r. spectra as to why first, the milk enzyme oxidizes purine in the 6 position, whereas the *Veillonella* enzyme (Smith *et al.*, 1967) oxidizes it in the 8 position, and secondly, why only the latter enzyme causes rapid xanthine dismutation.

Flavin and iron-sulphur centres

Turning now to the flavin and iron-sulphur centres in the dehydrogenase, we note that the flavin semiquinone e.p.r. signal is indistinguishable from that from the milk enzyme and that the radical must therefore be of the 'blue' type (Palmer *et al.*, 1971). The iron-sulphur signal, in contrast with those from the milk enzyme, is reminiscent of the signal from aldehyde oxidase observed at 25K (Rajagopalan *et al.*, 1968a) and of the signal from adrenodoxin (Palmer *et al.*, 1967).

Mechanism of action of the enzyme

As noted in the introduction, the milk enzyme is believed to function by two-electron reduction of molybdenum, followed by very rapid intramolecular partitioning of these electrons on to molybdenum, iron-sulphur and flavin. Reoxidation by oxygen occurs principally by interaction of this acceptor with the flavin, in its fully reduced form, by a two-electron reaction.

There seems to be every reason to accept that reduction of the *Veillonella* xanthine dehydrogenase occurs in an analogous manner. The Resting I signal is clearly not due to the active functional enzyme. In earlier work (Aleman *et al.*, 1966; Rajagopalan *et al.*, 1968b), partial disappearance of the Resting signal in the presence of the substrate, as in our studies, must have been accompanied by the appearance of a smaller Rapid signal, so that the net intensity of the molybdenum(V) signal decreased rather than increased. This led to the erroneous suggestion that molybdenum might not be participating in the redox cycles of the enzyme.

The present work does not provide evidence as to why the *Veillonella* enzyme is primarily a dehydrogenase, rather than an oxidase. However, studies on turkey liver xanthine dehydrogenase (Barber *et al.*, 1976) showed that here the difference is related to the difficulty of reducing flavin below the semiquinone level. Only fully reduced flavin is expected to react at all readily with oxygen. Presumably a similar explanation applies in this case. Indeed, data in Fig. 3 certainly seem to indicate that the flavin of the *Veillonella* enzyme is difficult to reduce completely. Further, Smith *et al.* (1967) found in optical titrations, that the semiquinone-absorption band persisted in the presence of excess of substrate. They suggested, however, that the semiquinone might somehow be related to activity towards ferredoxin as electron acceptor, not connecting it with the lack of oxidase activity. The origin of activity towards ferredoxin in the *Veillonella* enzyme and the site at which this acceptor interacts remain uncertain. However, ferredoxin activity cannot be related to persistence of the flavin semiquinone, since this

feature is common to the turkey enzyme, which has no activity with this acceptor.

Occurrence in molybdenum hydroxylases of the type of iron-sulphur centre that is present in the *Veillonella* enzyme, does not correlate with any particular pattern of acceptor specificity, since similar centres are apparently present in aldehyde oxidase. To explain our observation that *Veillonella* xanthine dehydrogenase can utilize rubredoxin as well as ferredoxin, perhaps we should simply postulate that the crucial factor determining this reactivity among molybdenum hydroxylases concerns an unusual degree of accessibility to large molecules of an unspecified acceptor site in the enzyme.

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