A New Non-Functional Form of Milk Xanthine Oxidase Containing Stable Quinquevalent Molybdenum

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A new non-functional modified form of milk xanthine oxidase is described. This contains molybdenum in a quinquevalent state, which is resistant to both oxidation and reduction. The new species is derived from the native enzyme in a two-step process. The first step is the conversion into the desulpho form, via loss of the 'persulphide' sulphur, and the second involves reaction with ethylene glycol or other reagents. The species gives a characteristic Mo(V) electron-paramagnetic-resonance signal, without proton splittings, designated Resting II. This is virtually identical with signals reported previously from resting turkey liver xanthine dehydrogenase and rabbit liver aldehyde oxidase. The possibility is discussed that species Resting II, prepared with ethylene glycol, contains a $-\text{COCH}_2\text{OH}$ residue bound to a nitrogen ligand of molybdenum.

E.p.r. (electron-paramagnetic-resonance) spectroscopy is finding increasing application as a sensitive method for the detection of non-functional modified enzyme forms contaminating normal preparations of certain oxidative metalloenzymes. One example is provided by work on succinate dehydrogenase (Ruzicka & Beinert, 1974). Milk xanthine oxidase, unless it is prepared by affinity chromatography (Edmondson *et al.*, 1972), always contains one or more non-functional forms (Hart *et al.*, 1970) in addition to the active enzyme. Other Mo-containing hydroxylases, likewise, are often contaminated with several non-functional species (Dalton *et al.*, 1976; Barber *et al.*, 1976). The best characterized non-functional form of the milk enzyme, which is also the most regular contaminant of the active enzyme, has been given the name desulpho xanthine oxidase (Bray, 1976). In contrast with active xanthine oxidase, which on reduction gives the Rapid (or the Very Rapid) Mo(V) e.p.r. signal, the desulpho enzyme gives the Slow signal (Bray, 1976; Bray & Swann, 1972). We now report that a specific chemical modification of desulpho milk xanthine oxidase gives rise to a new enzyme species, which has characteristic properties.

Materials and Methods

Milk xanthine oxidase was prepared by the salicylate denaturation procedure of Hart *et al.* (1970). The desulpho form was prepared from it by treatment with cyanide, as described by Massey & Edmondson (1970). All experiments were performed in 50 mM-sodium pyrophosphate buffer containing 1 mM-

EDTA, adjusted to pH 8.2 with HCl. E.p.r. sample preparation, rapid freezing and e.p.r. spectroscopy and signal quantification were as described elsewhere (e.g. Dalton *et al.*, 1976; Barber *et al.*, 1976). Organic solvents and other reagents (Table 2) were generally of analytical-reagent grade, except for glycollic aldehyde (2-hydroxyacetaldehyde) (from Koch-Light Laboratories, Colnbrook, Bucks., U.K.). Ethylene glycol (ethane-1,2-diol) was usually of laboratory-reagent grade, but in one experiment analytical-reagent grade (from BDH Chemicals Ltd., Poole, Dorset, U.K.) gave similar results. Formaldehyde was diluted from the aqueous 37–40% (w/v) solution.

Results

During studies (D. J. Lowe, unpublished work) on the effect of chemical modifications of xanthine oxidase on its e.p.r. properties, it was found that treatment of preparations of the enzyme with ethylene glycol produced one unexpected and rather specific effect. This could be observed as follows: a sample of the enzyme was first extensively reduced with sodium dithionite (for example, by treatment with 20 mol of dithionite per mol of enzyme for 20 min); under these conditions the only Mo e.p.r. signal observed is the Slow signal (McGartoll *et al.*, 1970). If ethylene glycol (1.0–1.5 vol.) was now added to the sample, anaerobically, before it was frozen a new spectrum was obtained, similar to that in Fig. 1. On repeating this experiment, but with desulpho xanthine oxidase in place of the normal enzyme, the same spectrum was obtained. This indicates the species that gives the new signal is derived from the de-

Fig. 1. E.p.r. spectrum of the Resting II signal obtained by treating desulpho milk xanthine oxidase with ethylene glycol

In (a) and (b) the microwave frequency was 9.13 GHz and in (c) it was 35.3 GHz. The gain setting was increased in (b) to reveal the ^{95}Mo and ^{97}Mo hyperfine lines. Different enzyme samples were used for the three experiments; the xanthine oxidase (total active centres) concentration was approx. 0.3 mM. Recording conditions were: temperature, approx. 120 K; modulation, 1 G, 2.5 G and 5 G in (a), (b) and (c) respectively; power 1 mW and 5 mW in (a) and (b) and 10 decibels attenuation in (c). The bars represent a magnetic field of 10 mT (100 G).

sulpho, rather than from the active, enzyme. Examination of the e.p.r. spectrum, particularly at 35 GHz (Fig. 1c), suggests strongly that all its major features are due to a single chemical species having rhombic symmetry. Hyperfine structure at higher and lower fields than the main peaks and visible both at 9 GHz (Fig. 1b) and at 35 GHz (Fig. 1c) is clearly due to ^{95}Mo and ^{97}Mo . No hyperfine structure due to interaction with protons is apparent. Parameters of the signal are given in Table 1, which shows the close

similarities of the signal, both to the Resting II signal of turkey liver xanthine dehydrogenase (Barber *et al.*, 1976) and to the resting signal of rabbit liver aldehyde oxidase (Rajagopalan *et al.*, 1968a). By analogy with the turkey enzyme we shall therefore refer to the new signal from the milk enzyme as Resting II.

Ethylene glycol and excess of dithionite could be removed from samples showing the Resting II signal, e.g. by anaerobic gel filtration, without affecting the form or intensity of the signal substantially. Further,

the signal was stable on exposure to air, with its intensity not decreasing significantly even during 3 months' storage at 0–5°C.

In additional experiments, the concentration of ethylene glycol and the duration of exposure of the desulpho enzyme to it were varied. At high ethylene glycol concentrations, the Resting II signal appeared within a few minutes and continued to grow slowly in intensity, whereas the Slow signal decreased gradually to a low value. Maximum conversion into a form showing the Resting II signal was achieved after incubation for 3 days with approx. 9M-ethylene glycol. In one experiment under these conditions (after aerobic removal of ethylene glycol and unchanged dithionite), double integration of the Resting

II signal indicated that this corresponded to 53% of the total enzyme active-centre concentration (calculated from the E_{450}). Subsequent re-addition of dithionite to this sample caused reappearance of traces of the Slow signal (compare Figs. 2a and 2b). This indicates that conversion of the desulpho enzyme by the ethylene glycol into the new species was still incomplete. The dithionite re-addition did not, however, affect the intensity of signal Resting II itself. For example, in another experiment this signal decreased in intensity by less than 10%, 20 min after addition of 20 mol of dithionite per mol of enzyme. Thus the Mo that causes the Resting II species is resistant to reduction to the quadrivalent level. Additional evidence for resistance of the species that gives this signal

Table 1. Comparison of the parameters of the Resting II e.p.r. signal from milk xanthine oxidase with those of corresponding signals from related enzymes

Hyperfine splittings are given in gauss (10G = 1mT).							
Enzyme	Signal	g_1	g_2	g_3	$g_{av.}$	$ A_1 $ [^{95}Mo]	Reference
Milk xanthine oxidase	Resting II	1.980	1.973	1.967	1.973	62	Present work
Turkey liver xanthine dehydrogenase	Resting II	1.977	1.972	1.965	1.971	—	Barber <i>et al.</i> (1976)
Rabbit liver aldehyde oxidase	Resting	1.980	1.973	1.964	1.972	61	Rajagopalan <i>et al.</i> (1968a)*

* Calculated from data in the reference given.

Fig. 2. High- and low-temperature forms of the Resting II signal (at 9 GHz)

The recording temperature was about 120 K in (a) and (b) and 40 K in (c) and (d). (a) and (c) were obtained from a sample of ethylene glycol-treated desulpho enzyme that had been reduced with dithionite, then gel filtered anaerobically to remove excess of reducing agent. The sample gave strong Fe/S I and Fe/S II signals (not shown). In (b) and (d) the same enzyme sample was used, but after exposure to oxygen for 1.0 s in the rapid-freezing apparatus, which caused almost complete disappearance of the Fe/S signals. Characteristic low-temperature splitting of the Resting II signal is apparent in (c) but not in (d). Traces of Slow signal are discernable along with Resting II in (a), causing the slightly modified line-shape, compared with that of the normal signal in (b). E.p.r. conditions were: modulation 2 G; microwave power 5 mW in (a) and (b) and 0.1 mW in (c) and (d). The bar represents a magnetic field of 2 mT (20 G).

Table 2. *Changes in Mo(V) e.p.r. signal occurring on treating reduced desulpho xanthine oxidase with various reagents*

Samples of the desulpho enzyme were first reduced with dithionite, at pH 8.2, to give the Slow signal. Reagents, at the final concentrations indicated, were added anaerobically to these samples, which were frozen in e.p.r. sample tubes, after incubation (at about 20°C) for the periods indicated. E.p.r. spectra were recorded and examined qualitatively. Approximate relative intensities of the various signals are indicated by + and - signs. ++, Strong signal; +, signal clearly apparent; -, signal not detected.

Reagent added (concn.)	Incubation (h)	Mo(V) e.p.r. signals observed		
		Slow	Resting II (or similar)	Other
Ethylene glycol (9M)	0.5	trace	++	-
Ethylene glycol (0.5M)	3	++	+	-
Glycollic aldehyde (0.5M)	3	+	+	-
Glycerol (7M)	7	trace	+	-
Glycerol (0.5M)	3	++	-	-
1,4-Dioxan (7M)	0.1	++	-	-
Formaldehyde (0.5M)	3	++	-	+
Methanol (0.5M)	3	++	-	-

* Relatively small amounts of a signal, other than Slow or Resting II, was present.

to oxidation to Mo(VI) was provided by experiments with ferricyanide. The intensity of signal Resting II was not affected by treatment with this reagent at a concentration of 2.4 mM for 5 min.

As reported elsewhere (Bray *et al.*, 1976), the iron-sulphur centres of ethylene glycol-treated desulpho milk xanthine oxidase preparations may be reduced with dithionite and subsequently re-oxidized by oxygen, without affecting intensity of the Resting II signal. In these experiments (Bray *et al.*, 1976) integration of the Resting II signal was only 46% of the theoretical maximum value. However, direct evidence that in such experiments the iron-sulphur centres that are reduced and oxidized include those of individual molecules (or half-molecules) giving the Resting II signal is provided by data in Fig. 2. Like most Mo(V) signals from Mo-containing hydroxylases, signal Resting II shows a temperature-dependent splitting phenomenon (cf. Lowe *et al.*, 1972; D. J. Lowe, unpublished work). Splitting of the Mo signals depends on interaction between the Mo(V) and the reduced iron-sulphur unpaired electrons and is therefore observed only when these reduced species are both present, simultaneously, in the same enzyme molecule. Figs. 2(a) and 2(c) show the change in signal Resting II that occurs on lowering the recording temperature from 120 K to 40 K, for a sample reduced with dithionite. Figs. 2(b) and 2(d) show that, after re-oxidation with oxygen for 1 s, no such change in signal Resting II takes place on lowering the temperature. A reaction time of 1 s is expected for the normal enzyme (cf. Olson *et al.*, 1974), and was indeed found, for the ethylene glycol-modified form (Bray *et al.*, 1976), to give largely complete re-oxidation of the iron-sulphur centres. The splitting data are therefore in accordance with expectations.

Data concerning the specificity of the reaction of desulpho xanthine oxidase with ethylene glycol are summarized in Table 2. Other reagents that gave rise to signals similar to, or identical with, the signal Resting II produced by ethylene glycol included glycollic aldehyde and glycerol (propane-1,2,3-triol). The former reacted possibly rather faster than did ethylene glycol, whereas glycerol was slower. However, so far we have not attempted to characterize the products of these reactions fully, and their e.p.r. signals have been examined only in the presence of residual Slow signal. In additional experiments (Table 2), 1,4-dioxan and methanol failed to react with the desulpho enzyme, whereas formaldehyde gave, not Resting II, but instead small amounts of what appeared to be a new signal.

Discussion

It is clear that signal Resting II corresponds to a new non-functional species from milk xanthine oxidase with the Mo in a modified chemical environment. Apart from its characteristic e.p.r. spectrum, its resistance to oxidation and to reduction distinguishes it clearly from the native enzyme and from the desulpho form. Detection of this signal from the milk enzyme provides further evidence for the extreme similarity of the Mo sites in all the Mo-containing hydroxylases so far studied. Thus the corresponding signals from turkey liver xanthine dehydrogenase (Barber *et al.*, 1976) and from rabbit liver aldehyde oxidase (Rajagopalan *et al.*, 1968a,b) not only have very similar e.p.r. parameters but also show comparable resistance to oxidation or reduction. However, it is important to note that, whereas signal Resting II has been detected only in ordinary untreated

preparations of the turkey and rabbit enzymes, in contrast, for the milk enzyme, we have seen this signal only after treatment with ethylene glycol or related reagents.

Stability of signal Resting II in the milk enzyme to oxidation and reduction makes integration of the e.p.r. signal of particular interest. Ideally it should be possible to convert all the Mo in the enzyme into the signal-giving form. However, so far, we have obtained at best only half the theoretical yield. This is undoubtedly due, at least in part, to incompleteness of the reaction with ethylene glycol.

The precise nature of the changes in the desulpho enzyme that give rise to the new species are not certain, though understanding them is of interest in relation to the structure of the active centre. Under the conditions used, ethylene glycol is not expected to cause any extensive denaturation (see Tanford, 1968). Further, the data in Table 2 make it much more likely that some specific chemical modification of the enzyme in the vicinity of Mo is involved. To arrive at a plausible hypothesis for the origin of signal Resting II, we first consider another signal from milk xanthine oxidase, which corresponds to Mo(V) in a relatively stable state, namely the Inhibited signal. From work with deuterated reagents Pick *et al.* (1971) proposed that this signal arises when the normal enzyme is treated with methanol or formaldehyde, as a result of formylation of a group (perhaps $-\text{NH}-$ or $-\text{NH}_2$) in the active centre. With methanol the first step in this reaction was assumed to be its oxidation, while bound to the enzyme, to formaldehyde. It has further been suggested (Bray, 1976) that the group formylated may be a nitrogen ligand of Mo; in the functional enzyme it is this nitrogen atom that is capable of carrying the exchangeable proton 'seen' in the Rapid signal. The same nitrogen atom would be present in the desulpho enzyme and would carry the proton 'seen' in the Slow signal. To explain signal Resting II, we then merely have to postulate acylation (cf. Bray *et al.*, 1976) of the nitrogen atom in the desulpho enzyme, to give a structure of the type $-\text{Mo}-\text{N}(\text{R})-\text{COR}$. Presumably, either with ethylene glycol or glycolic aldehyde, R would be $-\text{CH}_2\text{OH}$. The indication (Table 2) that formaldehyde also reacts with the desulpho enzyme, giving a signal different from Resting II, tends to support this hypothesis. E.p.r. signals from acylated desulpho enzyme would be little dependent on the nature of R. Nevertheless, when $\text{R} = \text{H}$, it is possible (as has already been proposed for the Inhibited signal) that this atom is suitably located relative to the Mo for a proton-split Mo signal to be observed. However, further studies, particularly on reactions of both the desulpho and the native enzyme with aldehydes,

will be required to prove the hypothesis. It is interesting to speculate, nevertheless, that small amounts of form Resting II found in preparations of other Mo-containing hydroxylases may have arisen by reaction, perhaps during isolation, between the desulpho enzymes and some aldehyde that happened to be present.

Two possible future uses of the Resting II signal may be mentioned. Understanding of interactions between paramagnetic groups in macromolecules might be helped by theoretical work on the splitting of signal Resting II. The large, apparently nearly isotropic splitting of about 2.2 mT (22 G) (Fig. 2c), as well as the absence of proton splitting, should be particularly helpful here. In quite a different context, availability of a form of the enzyme in which, as can readily be demonstrated by e.p.r., the Mo is no longer redox-active, may facilitate studies on the relative roles of the other centres (that is flavin and iron-sulphur) in reactions of the enzyme.

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References

- Barber, M. J., Bray, R. C., Lowe, D. J. & Coughlan, M. P. (1976) *Biochem. J.* **153**, 297–307
Bray, R. C. (1976) *Enzymes*, 3rd edn., **12**, 299–419
Bray, R. C. & Swann, J. C. (1972) *Struct. Bonding (Berlin)* **11**, 107–144
Bray, R. C., Barber, M. J., Lowe, D. J., Fox, R. & Cammack, R. (1976) *FEBS Symp.* in the press
Dalton, H., Lowe, D. J., Pawlik, R. T. & Bray, R. C. (1976) *Biochem. J.* **153**, 287–295
Edmondson, D., Massey, V., Palmer, G., Beacham, L. M. & Elion, G. B. (1972) *J. Biol. Chem.* **247**, 1597–1604
Hart, L. I., McGartoll, M. A., Chapman, H. R. & Bray, R. C. (1970) *Biochem. J.* **116**, 851–864
Lowe, D. J., Lynden-Bell, R. & Bray, R. C. (1972) *Biochem. J.* **130**, 239–249
Massey, V. & Edmondson, D. (1970) *J. Biol. Chem.* **245**, 6595–6598
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. (1974) *J. Biol. Chem.* **249**, 4350–4362
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
Ruzicka, F. J. & Beinert, H. (1974) *Biochem. Biophys. Res. Commun.* **58**, 556–563
Tanford, C. (1968) *Adv. Protein Chem.* **23**, 121–282