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Studies by e.p.r. spectroscopy of carbon monoxide oxidases from *Pseudomonas carboxydovorans* and *Pseudomonas carboxydohydrogena*

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E.p.r. spectra were obtained at 8–120 K for carbon monoxide oxidases isolated from the carboxydotrophic bacteria *Pseudomonas carboxydovorans* and *Pseudomonas carboxydohydrogena*. Spectra from the two enzymes are extremely similar to one another. Under appropriate conditions each enzyme shows signals from Mo(V) atoms in two different chemical environments, as well as showing signals from two distinct iron–sulphur centres, presumed to be [2Fe-2S] clusters, and weak $FADH^{\bullet}$ free-radical signals. Parameters of most of the signals were measured, and they show considerable similarities to those of the corresponding signals from xanthine oxidase and related enzymes. Though the signals from carbon monoxide oxidases appear and disappear under reducing and oxidizing conditions, we have so far failed to demonstrate the kinetic competence of any of them. It seems likely that this was due to the presence in the enzyme preparation examined of high amounts of desulpho carbon monoxide oxidase together with another non-functional form of the enzyme giving a stable 'Resting' Mo(V) e.p.r. signal.

Carbon monoxide oxidase (carbon monoxide:acceptor oxidoreductase) is the key enzyme in energy metabolism of the aerobic carbon monoxide-oxidizing bacteria (carboxydotrophic bacteria) (Meyer & Schlegel, 1978, 1979, 1980; Cypionka *et al.*, 1980). The enzyme catalyses the oxidation of CO to CO_2 in the presence of unphysiological electron acceptors such as Methylene Blue (MB):

$$CO + H_2O + MB_{ox.} \rightarrow CO_2 + MB_{red.}$$

(Meyer & Schlegel, 1979, 1980). *In vivo* the electrons are fed into the respiratory chain for energy generation, enabling the bacteria to grow with CO as the sole energy and carbon source. Carbon monoxide oxidase has been characterized as purified preparations or in extracts from a number of carboxydotrophic bacteria: *Pseudomonas carboxydovorans* (Meyer, 1982), *Pseudomonas carboxydohydrogena* (Cypionka *et al.*, 1980; Kim & Hegeman, 1981), *Pseudomonas carboxydoflava*, *Achromobacter carboxydus*, *Comamonas compransoris* and the so-far-unidentified strains OM2, OM3 and OM4 (Cypionka *et al.*, 1980). Of these enzymes, the one from CO-grown *Ps. carboxydovorans* has been characterized most fully. Chemical analysis indicated this to be an iron–sulphur-containing molybdoflavoprotein (Meyer, 1982), and in chemical composition, absorption spectra and relative molecular mass it is quite like xanthine oxidase and other molybdenum-containing hydroxylases (cf. Bray, 1975).

Since e.p.r. spectroscopy has provided a wealth of otherwise unobtainable information on iron–sulphur-containing molybdoflavoproteins and other molybdenum-containing enzymes (Bray, 1975, 1980, 1982), a study by e.p.r. spectroscopy of carbon monoxide oxidase is obviously desirable. We now present results of such work on the enzyme from *Ps. carboxydovorans* and from *Ps. carboxydohydrogena*, in both the oxidized state and the reduced state.

Materials and methods

Carbon monoxide oxidase

The enzyme was prepared from cells of *Ps. carboxydovorans* or *Ps. carboxydohydrogena* grown with CO as carbon and energy source and with NH_4Cl as nitrogen source as described previously (Meyer, 1982), with the alteration that polyacrylamide-gel electrophoresis was included as an addi-

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tional final purification step. Samples (about 15 ml, containing about 100 mg) of enzyme were submitted to electrophoresis on preparative gels (size 180 mm × 120 mm × 16 mm) containing 7.5% (w/v) acrylamide for 10 h at 75 mA and 140 V. The brownish enzyme band was cut out and eluted from the gel into 50 mM-phosphate buffer (KH_2PO_4/NaOH), pH 7.0. Purity and specific activity were similar to those reported by Meyer (1982), at the time of preparation of the enzyme. However, some of the e.p.r. work was performed on recovered enzyme of possibly lower activity.

The molybdenum content of carbon monoxide oxidase samples was determined colorimetrically by the dithiol procedure, as used by Hart *et al.* (1970).

Enzyme samples for e.p.r. spectroscopy

The enzyme was concentrated by ultrafiltration (Minicon B concentrators from Amicon Ltd.) and filled into e.p.r. tubes. Where anaerobic conditions were required these were achieved as described by Swann & Bray (1972). Additions to the tubes were made with syringes with long needles. Samples were frozen in liquid N_2 before the spectra were recorded. Final enzyme concentrations were in the range 50–100 μM-Mo, in 0.1 M potassium phosphate buffer, pH 7.0. ^{13}C-enriched CO (91.8 atom %) was purchased from British Oxygen Co. Prochem. Ltd., London SW19 3UF, U.K.

E.p.r. spectroscopy

Spectra were recorded on a Varian E9 spectrometer linked to a computer system (Bray *et al.*, 1978). This was operated either with liquid-N_2 or with liquid-He (Lowe *et al.*, 1972) cooling. For absolute quantification of signal intensities, a Cu^{2+}–EDTA standard was employed; double integrations were performed, on spectra recorded under non-saturating conditions with the computer and corrections were applied for tube diameters, for transition probability (Aasa & Vänngård, 1975) and where appropriate for ^{95}Mo lines not included in the integration.

Results

General description

Under appropriate conditions e.p.r. signals from Mo(V), from reduced iron–sulphur centres and from a free radical were observed. It is convenient to refer to the individual signals by names, and these have been assigned by analogy with the signals from xanthine oxidase and other molybdenum-containing hydroxylases (Bray, 1975, 1980). Thus we refer to the signals as Mo(V) Resting, Mo(V) Reduced, Fe-S 1, Fe-S 2 and FADH$^{\bullet}$. These signals are illustrated in Figs. 1–3, and their parameters are summarized in Table 1. The signals are considered individually in greater detail below.

Resting Mo(V) signal

In the absence of any substrate or other reductant, the samples of the two enzymes that were examined displayed at about 123 K nearly identical rhombic e.p.r. spectra without superhyperfine structure, as shown in Figs. 1(*a*) and 1(*b*). These spectra are those of an Mo(V) compound with $g_{av.} = 1.9751$. The *g*-values are listed in Table 1. These come close to those of the Resting I and the Inhibited (formaldehyde) signals of xanthine dehydrogenase (Dalton *et al.*, 1976) and xanthine oxidase (Bray, 1980) respectively, which are both due to Mo(V). Additional confirmation for the origin of these spectra are the ^{95}Mo and ^{97}Mo hyperfine lines ($I = 5/2$), which are detected at lower and higher field (the gain was increased 10-fold). (Molybdenum occurs naturally as a mixture of 75% non-magnetic isotopes and 25% magnetic isotopes, ^{95}Mo and ^{97}Mo.)

For the enzymes from *Ps. carboxydovorans* and *Ps. carboxydohydrogena*, molybdenum present in the Mo(V) state, determined by double integration of the e.p.r. spectra of the Resting signal (as described in the Materials and methods section), was 20% and 40% respectively of the molybdenum present according to colorimetric analysis.

On incubation with formaldehyde, the g_1 and g_3 features of the signal appear to be broadened (Fig. 1*d*), the latter feature showing signs of being split [compare with Fig. 1(*c*), which corresponds to the spectrum of Fig. 1(*a*) on an expanded field scale]. A very similar effect was obtained by incubation of the enzyme with methanol (2 M). These effects were reversible by gel filtration.

Overall structural information can be obtained from studies of the spin–spin interaction of Mo(V) with Fe-S 1 (Lowe *et al.*, 1972; Lowe & Bray, 1978; Bray, 1982). Fig. 1(*e*) shows the effect of such interaction as a splitting of the Mo(V) signal at 38 K. In the present case, the anisotropic contribution to the splitting is unusually large. This permitted calculation (G. N. George, unpublished work) of the distance between the centres as between 0.8 and 1.5 nm.

The Resting signal was quite stable towards oxidizing agents such as O_2, ferricyanide or Methylene Blue and did not vanish rapidly on treatment with reducing agents. Thus it disappeared within a few minutes on anaerobic incubation with dithionite (2–3 mM) and more gradually (approx. 90% decrease in 1 h) in the presence of CO and Methylene Blue (5 mM). When the enzyme was reduced by CO only, in the absence of Methylene Blue, the Resting Mo(V) signal stayed intact, but slowly a free-radical signal appeared (apparent after 1 h, maximum attained after 16 h of incubation), as shown in Fig.

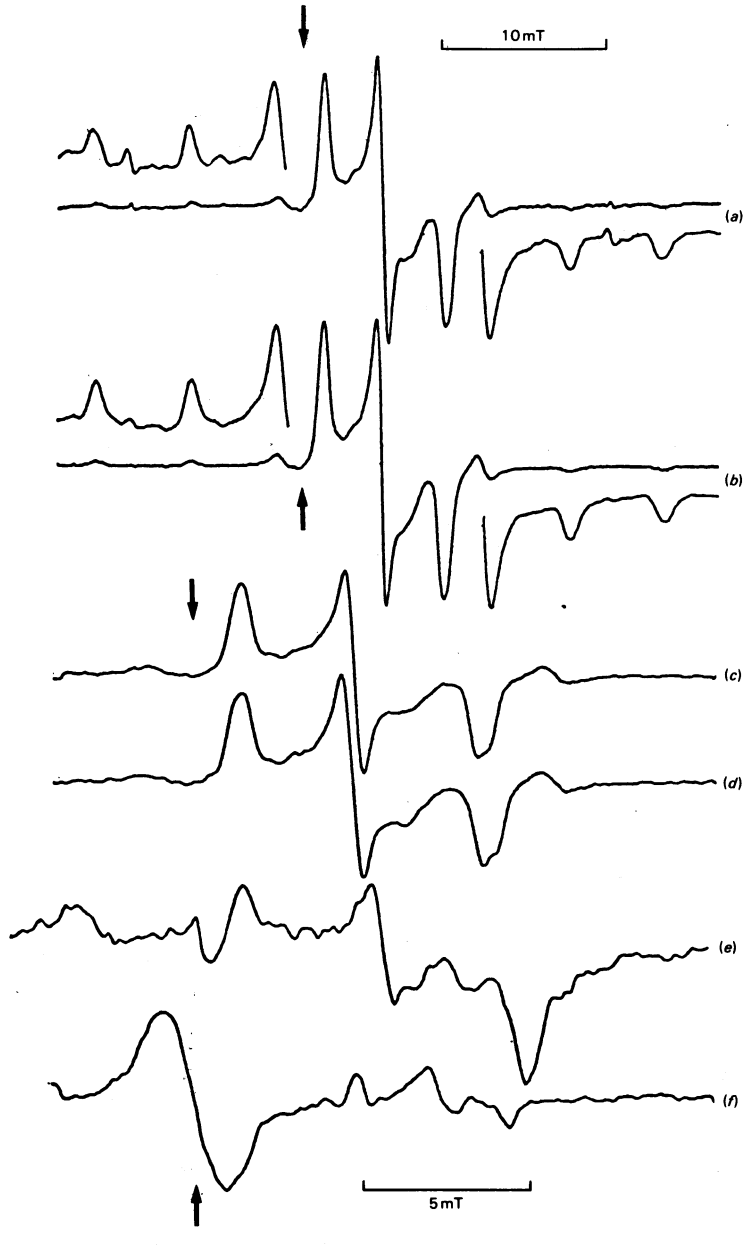

Fig. 1. *Resting Mo(V) signal from carbon monoxide oxidase*

(*a*) and (*b*) are e.p.r. spectra of untreated enzyme from *Ps. carboxydovorans* and *Ps. carboxydohydrogena* respectively. The parts of the spectra at high field and at low field are shown with the vertical scale increased 10-fold, to show the molybdenum hyperfine structure. (*c*) and (*d*) show on an expanded field scale the signal from the *Ps. carboxydovorans* alone and after treatment with formaldehyde (200mM for 15min) respectively. (*e*) shows a low-temperature form of the spectrum, from which Fe-S signals have been subtracted, and was recorded at 24.5K on the sample of (*c*) after reduction of the Fe-S centres with dithionite (2mM) for 1min. (*f*) is the FADH· free-radical signal; it was obtained by subtracting the spectrum of (*c*) from that of a similar sample treated with CO for 16h. The two field span markers apply to, and the two pairs of arrows indicate $g = 2.0037$ on, (*a*) and (*b*) and (*c*)–(*f*) respectively. The spectra were run at 123K, 9.3GHz, 0.16mT modulation amplitude and 10mW microwave power, except for (*e*), which was run at 24.5K with 0.01mW microwave power.

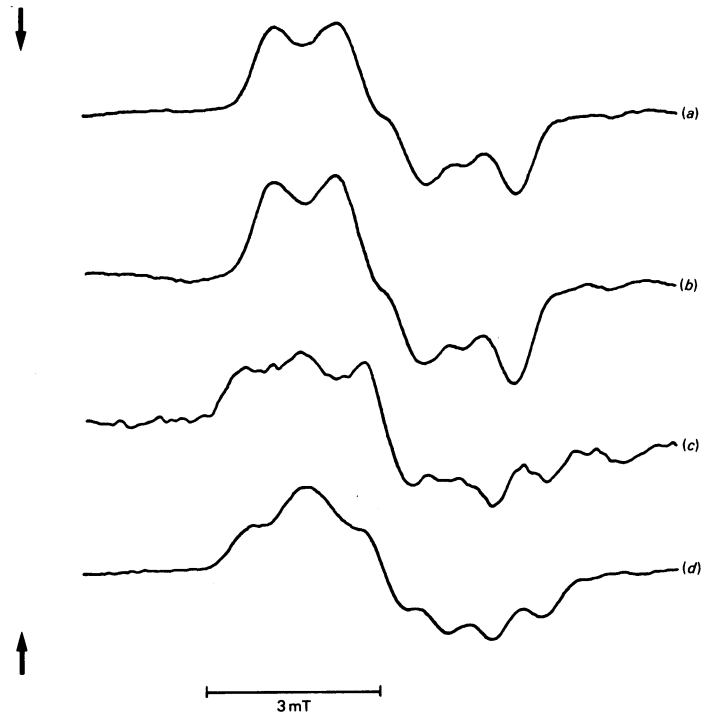

Fig. 2. *Reduced Mo(V) signal from carbon monoxide oxidase*

The signal was generated by reducing the enzyme with dithionite (2mM for 1h). (*a*) shows the spectrum of the enzyme from *Ps. carboxyhydrogena* and (*b*) that of the enzyme from *Ps. carboxydovorans*. These two spectra were recorded at 120K and 10mW. (*c*) is the modified form that the spectrum took under conditions where a splitting due to a coupling of Mo and Fe-S 1 centres was observable. It was recorded on the sample of (*b*) at 24.5K and 0.01mW. (*d*) is a simulation of spectrum (*c*), obtained from spectrum (*b*) by displacement and addition, and corresponds to an isotropic splitting due to the Fe-S centre of 0.8mT.

1(*f*), equivalent to about 15.6% of the Mo(V) signal intensity, i.e. about $0.03\,e^-$/(total Mo). This signal appeared also on enzyme reduction by dithionite. Its *g*-value and linewidth (Table 1) are indistinguishable from those of $FADH^\bullet$ in xanthine oxidase.

In an attempt to obtain direct evidence for CO binding to the molybdenum centre, we replaced ^{12}CO by ^{13}CO. The ^{13}CO was added anaerobically to the enzyme with a syringe in the absence and in the presence of dithionite or Methylene Blue, and finally the enzyme was re-oxidized by O_2. The enzyme was active with ^{13}CO (as indicated by Methylene Blue decolorization), but no isotope effect on the Resting or other Mo(V) signals could be detected.

Reduced Mo(V) signal

On reduction of the enzyme by dithionite or by CO in the presence of Methylene Blue, and roughly concomitantly with the decrease of the Resting Mo(V) signal, a new signal appeared, presumed also to be from Mo(V). Signals obtained with the two reductants could not be distinguished from one another. Figs. 2(*a*) and 2(*b*) show the Reduced Mo(V) signals of the enzymes from *Ps. carboxydovorans* and *Ps. carboxydohydrogena* respectively, which were obtained by anaerobic reduction by 2mM-dithionite for 1h. Again, the signals arising from the two enzymes are very similar. The line shape is complex, possibly owing to hyperfine coupling to protons, and we did not attempt an interpretation or to measure its *g*-values. Unlike the Resting Mo(V) signal, the Reduced Mo(V) signal does not seem readily comparable with other known signals from molybdoproteins. The maximum integrated intensity of the signal corresponded to about 30% of the molybdenum in the Mo(V) state. At lower temperatures, where the Fe-S 1 signal (see below) is fully developed, the Reduced Mo(V) signal becomes isotropically split owing to coupling of the Mo(V) with the Fe-S 1 centre (coupling constant = 0.8mT), as shown in Figs. 2(*c*) and 2(*d*) (cf. Lowe & Bray, 1978).

The Reduced Mo(V) signal and also the free-

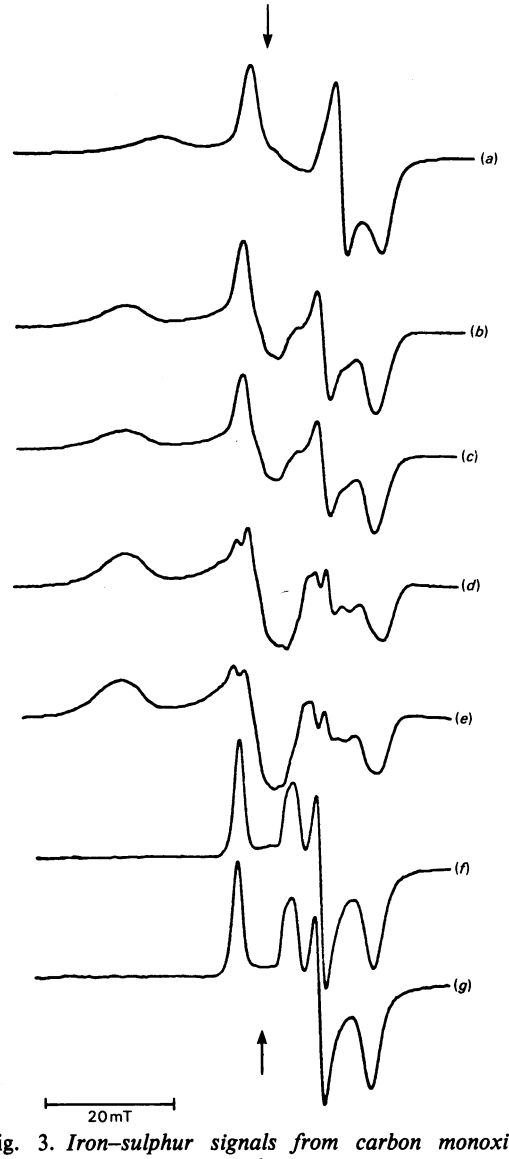

Fig. 3. *Iron–sulphur signals from carbon monoxide oxidase*

(*a*) shows for comparison the signals from milk xanthine oxidase recorded under non-saturating conditions (22K, 10mW). (*b*) and (*c*) show the composite of Fe-S 1 and Fe-S 2 signals, from the enzymes from *Ps. carboxydovorans* and *Ps. carboxydohydrogena* respectively, generated as in Fig. 2 and recorded under conditions of partial saturation for the Fe-S 1 centre (16K, 200mW). (*f*) and (*g*) also correspond to the *Ps. carboxydovorans* and *Ps. carboxydohydrogena* enzymes respectively, but were recorded at high temperature (49K, 10mW), so that only the Fe-S 1 signal was developed. (*d*) and (*e*) correspond to difference spectra obtained by subtracting spectrum (*f*) from spectrum (*b*) and spectrum (*g*) from spectrum (*c*) respectively, and approximate to the pure Fe-S 2 spectra. Some features due to Mo(V) are also present in all the spectra.

radical signal disappeared completely on further reduction with dithionite. Both signals were replaced by the Resting Mo(V) signal on bubbling with O_2 for about 30s.

Iron–sulphur centres

In the temperature range 8.5–80K both enzymes, after reduction by dithionite or by CO in the presence of Methylene Blue, display e.p.r. spectra due to the Fe-S 1 and Fe-S 2 centres. Figs. 3(*b*) and 3(*c*) show a mixture of Fe-S 1 and Fe-S 2 signals for the enzymes of *Ps. carboxydovorans* and *Ps. carboxydohydrogena* respectively. At high temperatures, only the Fe-S 1 signal was developed, which is shown for both enzymes in Figs. 3(*f*) and 3(*g*). By subtracting various amounts of Figs. 3(*f*) and 3(*g*) respectively from Figs. 3(*b*) and 3(*c*), approximately pure Fe-S 2 spectra of Figs. 3(*d*) and 3(*e*) were obtained. These signals are quite similar to those of the Fe-S 1 and Fe-S 2 centres of xanthine oxidase, as shown in Fig. 3(*a*), and as compared by their *g*-values in Table 1. The *g*-values of the two enzymes are nearly identical, but a little different from those of xanthine oxidase.

By recording the iron–sulphur spectra at different temperatures and powers, we established the saturation curves for Fe-S 1 and Fe-S 2 centres (Figs. 4*a* and 4*b*). The experimental values fitted well the theoretical curves, calculated from the semi-empirical equation of Beinert & Orme-Johnson (1967):

$$S = a[P/(1 + P/P_{\frac{1}{2}})^b]^{\frac{1}{2}}$$

where S is the normalized signal intensity, P is the microwave power incident on the samples, $P_{\frac{1}{2}}$ is the power at half saturation, and a and b are factors depending on the chemical species that is involved. Since the values of these parameters so obtained are similar to those encountered in the saturation curves of the Fe-S 1 and Fe-S 2 centres of xanthine oxidase (Lowe, 1974), this further confirms that the structures and the chemical environment of the iron–sulphur centres are similar to those of xanthine oxidase.

The Fe-S contents of the enzymes were determined by integration of the e.p.r. signals. Because of the overlap of the two signals, values for the combined content of the Fe-S 1 and Fe-S 2 signals are more reliable than are values for the content of either signal on its own. Relative to colorimetrically determined Mo, we found Fe-S 1 + Fe-S 2 signals corresponded to 1.8 unpaired electrons for the enzyme from *Ps. carboxydovorans* and to 2.1 electrons for that from *Ps. carboxydohydrogena*. The Fe-S 1/Fe-S 2 signal ratio was obtained by comparing integrations of spectra run under non-saturating conditions at high temperatures (where only the Fe-S 1 signal is developed) and at low

Table 1. *g-values of carbon monoxide oxidases and related iron–sulphur molybdoproteins*

Signals from carbon monoxide oxidase were generated as described in the text. *g*-values are believed to be accurate to ±0.0004 for Mo(V) and for FADH•, and to ±0.001 for Fe-S. Some values from the literature for molybdenum-containing hydroxylases are listed for comparison.

Enzyme	Signal	1	2	3	Average	Reference
Carbon monoxide oxidase (*Ps. carboxydovorans*)	Fe-S 1	2.023	1.946	1.899	1.956	Present work
Carbon monoxide oxidase (*Ps. carboxydohydrogena*)	Fe-S 1	2.025	1.947	1.901	1.958	Present work
Xanthine oxidase	Fe-S 1	2.022	1.935	1.899	1.952	Bray (1975)
Carbon monoxide oxidase (*Ps. carboxydovorans*)	Fe-S 2	2.137*	2.009	1.900	2.015	Present work
Carbon monoxide oxidase (*Ps. carboxydohydrogena*)	Fe-S 2	2.137*	2.010	1.896	2.014	Present work
Xanthine oxidase	Fe-S 2	2.12*	2.007	1.91	2.01	Lowe *et al.* (1972)
Carbon monoxide oxidase (both sources)	Mo(V) Resting	1.9964	1.9758	1.9531	1.9751	Present work
Xanthine oxidase	Mo(V) Inhibited (formaldehyde)	1.9911	1.9772	1.9513	1.9732	Tanner *et al.* (1978)
Xanthine dehydrogenase (*Veillonella alcalescens*)	Mo(V) Resting 1	2.010	1.979	1.964	1.983	Dalton *et al.* (1976)
Carbon monoxide oxidase (both sources)	FADH•	—	—	—	2.0039†	Present work
Xanthine oxidase	FADH•	—	—	—	2.0035‡	Bray (1975)

* The position of the g_1 feature of the Fe-S 2 signal was observed to shift with microwave power. Values given are those at low power; at high power (e.g. 200 mW) values were approx. 0.012 higher. This phenomenon is also apparent in the work of Lowe *et al.* (1972) on xanthine oxidase.

† Linewidth (peak-to-peak) 2.0 mT.

‡ Linewidth (peak-to-peak) 1.9 mT.

temperatures (where both the Fe-S 1 and the Fe-S 2 signals are developed). The value of the ratio so obtained was 0.8.

Discussion

The above results show that the carbon monoxide oxidases from *Ps. carboxydovorans* and from *Ps. carboxydohydrogena* contain molybdenum, Fe-S 1 and Fe-S 2 centres in the proportions approximately 1:1:1 (which is in accord with the previous chemical analysis). The Resting Mo(V) signal as well as the Fe-S and FADH• signals are very similar to e.p.r. signals observed with other iron–sulphur molybdoflavoproteins such as xanthine oxidase or xanthine dehydrogenase. Similarly the saturation behaviour of the Fe-S signals and the calculated distance from the Fe-S 1 centre to the molybdenum atom are also quite comparable with those of xanthine oxidase. It is therefore likely that the active-site structures of these different enzymes, though from very different sources and with different functions, are similar.

Nevertheless, the work leaves a number of questions unanswered. It is of course quite anomalous that an enzyme, for which a turnover time of about 135 ms can be calculated from published data (Meyer, 1982), should be reduced by the substrate only in minutes (with CO and Methylene Blue) or in hours (with CO only), as judged by the change in e.p.r. signals. It may therefore be questioned whether the observed e.p.r. signals have any catalytic relevance.

At present, two explanations for this problem may be offered. The extremely slow change of e.p.r. signals with CO alone might possibly be explained by the enzymic reaction requiring a concerted action of the substrate CO and an electron acceptor such as Methylene Blue, with the Resting Mo(V) signal-giving species being kinetically predominant. On the other hand, it seems more likely that our investigations have been made on an enzyme that, in the samples we measured, was mostly in a catalytically inactive form. This is despite the fact that the published catalytic-centre activity (Meyer, 1982) is similar to that of other iron–sulphur molybdoproteins.

Clearly much further work is required. However, it has recently been established (O. Meyer, unpublished work) that samples of carbon monoxide oxidase similar to those used in the present work can contain up to 70% of an inactive form of the enzyme (presumably analogous to desulpho xanthine oxidase). Whether this form is produced *in vivo* along

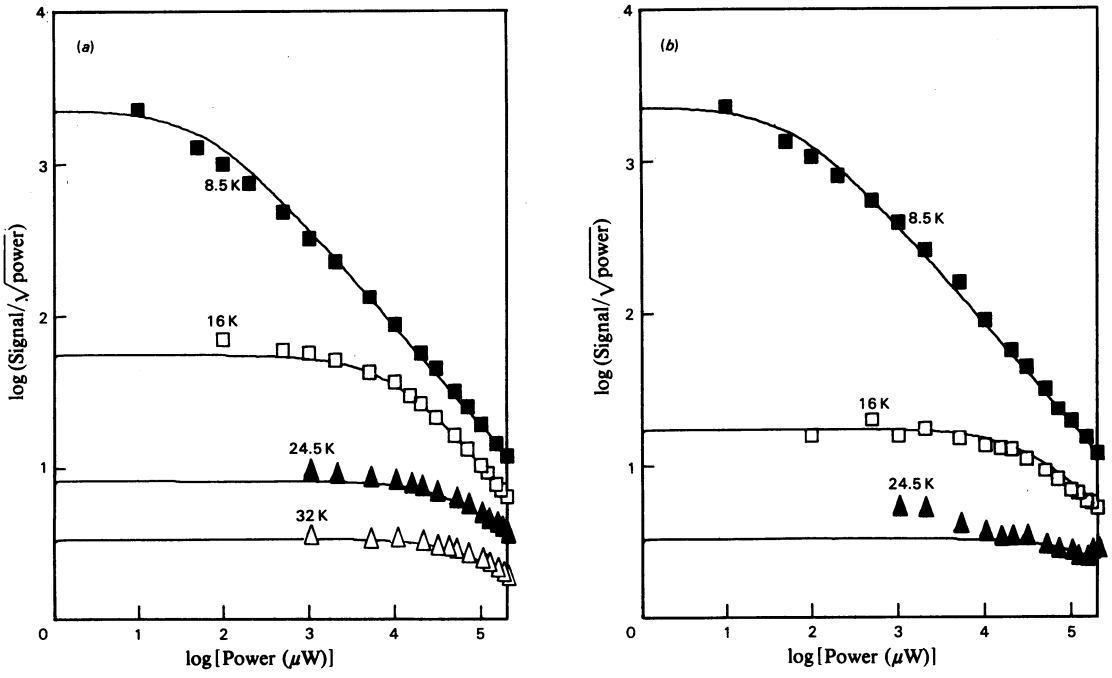

Fig. 4. *Saturation curves at various temperatures for (a) the Fe-S 1 and (b) the Fe-S 2 signals from carbon monoxide oxidase from Ps. carboxydovorans*

Normalized signal intensity divided by the square root of microwave power is plotted on a logarithmic scale (arbitrary zero) against microwave power. Sample temperatures are indicated on the graphs. The lines through the experimental points are theoretical curves (see the text). $P_{\frac{1}{2}}$ values and b values, given in order of increasing temperature, were: in (*a*) 0.05, 0.17; 10.5, 1.45; 85, 1.45; 150, 1.45; in (*b*) 0.06, 1.30; 40, 1.40; 350, 1.40.

with complete carbon monoxide oxidase is not known. High proportions of the inactive desulpho form of xanthine dehydrogenase have been reported to occur in *Drosophila melanogaster* ma-1 mutants (Wahl *et al.*, 1982). In any case, it seems likely that the presence of large amounts both of this and of the species giving the Resting Mo(V) signal can account for the slowness of the e.p.r. signal changes. The extent to which the enzyme is converted into the inactive desulpho form under the conditions of our experiments has yet to be established. However, in recent experiments it was found (O. Meyer, unpublished work) that under some conditions the enzyme loses activity rapidly when exposed to dithionite and air. Conversely, the inactive desulpho carbon monoxide oxidase could be re-activated by anaerobic incubation for about 100 min with 1 mM-sulphide plus 1 mM-dithionite, dithionite alone, or sulphide in the presence of CO and Methylene Blue.

In view of the similarity of their e.p.r. parameters, it is reasonable to assume a structural analogy between the species giving the Resting Mo(V) signal of carbon monoxide oxidase and the Inhibited signal of xanthine oxidase. The Inhibited signal arises as a result of a side reaction in the turnover of aldehyde substrates by active xanthine oxidase (Pick *et al.*, 1971; Bray, 1980; F. Morpeth & R. C. Bray, unpublished work). The signal-giving species is relatively inert and is believed (Bray & Gutteridge, 1982) to have a cyclic structure in which the substrate carbon atom is linked to molybdenum through two bridging oxygen atoms. This carbon atom shows strong hyperfine coupling to molybdenum (Tanner *et al.*, 1978). It might have been expected that the carbon atom of CO would be bound in a manner analogous to that of the aldehydes in the Inhibited species. However, failure to observe a ^{13}C effect, with labelled CO, on the Resting signal of carbon monoxide oxidase presumably indicates that the signal-giving species is unreactive to CO under the conditions of our experiments. What sort of a molecule may have reacted with active carbon monoxide oxidase to produce this stable signal is thus far from clear. Conditions for regenerating active xanthine oxidase from Mo(IV) and Mo(V) states of the Inhibited signal-giving species have recently been investigated (F. Morpeth & R. C. Bray, unpublished work).

Analogous work on the carbon monoxide oxidase Resting signal would be required to confirm the nature of the species.

Further work should establish whether the Reduced Mo(V) signal is due to the active or the desulpho form of carbon monoxide oxidase.

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