Oxidation–Reduction Studies of the Mo–(2Fe–2S) Protein from *Desulfovibrio gigas*

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Potentiometric titration followed by e.p.r. measurements were used to determine the midpoint reduction potentials of the redox centres of a molybdenum-containing iron–sulphur protein previously isolated from *Desulfovibrio gigas*, a sulphate-reducing bacterium [Moura, Xavier, Bruschi, Le Gall, Hall & Cammack (1976) *Biochem. Biophys. Res. Commun.* **72**, 782–789; Moura, Xavier, Bruschi, Le Gall & Cabral (1977) *J. Less Common Metals* **54**, 555–562]. The iron–sulphur centres could readily be distinguished into three types by means of *g* values, temperature effect, oxidation–reduction potential values and reduction rates. The type-I Fe–S centres are observed at 77 K. They show mid-point potential values of –260 mV (Fe–S type IA) and –440 mV (Fe–S type IB). Centres of types IA and IB appear to have similar spectra at 77 K and 24 K. The Fe–S type-II centres are only observed below 65 K and have a midpoint potential of –285 mV. Long equilibration times (30 min) with dye mediators under reducing conditions were necessary to observe the very slow equilibrating molybdenum signals. The potential values associated with this signal were estimated to be approx. –415 mV for Mo(VI)/Mo(V) and –530 mV for Mo(V)/Mo(IV).

Different types of molybdenum-containing iron–sulphur proteins have been reviewed (for molybdenum-containing hydroxylases and sulphite oxidase see Bray, 1975; for nitrogenase see Orme-Johnson & Davis, 1977; for formate dehydrogenase see Thauer *et al.*, 1977). Examples of these redox proteins are milk xanthine oxidase (Bray, 1975) which contains (2Fe–2S) centres and the Fe–Mo-protein of nitrogenase (Smith & Lang, 1974), which contains (4Fe–4S) centres. In preliminary communications we have reported the purification, amino acid analysis and physicochemical characterization of a molybdenum-containing iron–sulphur protein isolated from the sulphate-reducing bacterium *Desulfovibrio gigas* (Moura *et al.*, 1976a,b, 1977b). It has a mol.wt. of 120 000 and contains one Mo atom per molecule, and no evidence was found for the presence of subunits.

The optical absorption and c.d. spectra and e.p.r. spectrum in the presence of dimethyl sulphoxide showed that the iron–sulphur centres are arranged in (2Fe–2S) clusters (Moura *et al.*, 1976a). Furthermore, temperature-dependence studies of the e.p.r. spectra showed the presence of at least two types of (2Fe–2S) centres (Moura *et al.*, 1976a), which somewhat resemble the type-I and type-II centres of milk

xanthine oxidase in their *g* values and temperature-dependence (Lowe *et al.*, 1972). Unlike xanthine oxidase, however, the *D. gigas* protein contains no flavin, and no enzyme activity has yet been detected (Moura *et al.*, 1976b).

In the present paper we report a more complete e.p.r. study of the different redox centres of the *D. gigas* protein, which gives evidence of at least three types of (2Fe–2S) centres, which differ not only in the temperature-dependence and *g* values, but also in midpoint-reduction-potential values and reduction rates. The molybdenum is shown to react relatively slowly with chemical oxidants and reductants, like the 'Slow' molybdenum of the desulpho form of xanthine oxidase (Bray, 1975).

Experimental

Purification scheme

The purification scheme of the molybdenum-containing protein from *D. gigas* was outlined by Moura *et al.* (1976a). A detailed description of the purification procedure is presented here. *D. gigas* (N.C.I.B. 9332) was grown in a lactate/sulphate medium and

a frozen paste of cells (about 4kg) was treated as previously described to obtain an acidic protein extract (Le Gall *et al.*, 1965). All the operations were carried out at 0–4°C unless otherwise stated. Tris/HCl and phosphate buffers, pH 7.6, of appropriate concentration were used.

Step I. The acidic protein extract was dialysed overnight against 20 litres of water and adsorbed on two DEAE-cellulose columns (40mm×300mm) (Whatman DE-52) previously treated according to the manufacturer's indications and equilibrated with 0.01M-Tris/HCl and eluted with a discontinuous gradient using 300ml of each concentration from 0.15 to 0.50M-Tris/HCl with intervals of 0.05M. The fractions eluted with 0.15–0.30M-Tris/HCl contain the Mo-protein together with flavoproteins, desulphoviridine (Lee & Peck, 1971), and some cytochrome c_3 (mol.wt. 26000) (Bruschi *et al.*, 1969), whereas rubredoxin (Le Gall & Dragoni, 1966), flavodoxin (Dubourdieu & Le Gall, 1970), and (4Fe–4S) ferredoxin (Le Gall & Dragoni, 1966; Bruschi *et al.*, 1976) are eluted later (we observe that a slightly different pattern of elution is obtained when the gradient elution is done with NaCl in 0.01M-Tris/HCl buffer; Moura *et al.*, 1977a). This fraction was collected in a volume of approx. 1000ml.

Step II. The fraction from step I was dialysed overnight against 20 litres of water and adsorbed in a second DEAE-cellulose column (40mm×300mm) equilibrated with 0.01M-Tris/HCl, and eluted with a discontinuous gradient using 300ml of each concentration from 0.15 to 0.50M-Tris/HCl with intervals of 0.05M. This gradient enables the separation of a band mainly containing the Mo-protein [with traces of cytochrome c_3 (mol.wt. 26000) and desulphoviridine] from the other components of the acidic extract, in a total volume of 200ml.

Step III. The Mo-protein fraction was diluted 3-fold with water and concentrated on a small DEAE-cellulose column (20mm×60mm) eluted with 0.4M-Tris/HCl, giving a volume of 30ml.

Step IV. The concentrated fraction was passed over a Sephadex G-50 column (50mm×1000mm) equilibrated with 0.01M-Tris/HCl. A fraction in a volume of 360ml with an A_{280}/A_{322} ratio of 8.0 was obtained.

Step V. The protein solution was adjusted to about 50% saturation with $(\text{NH}_4)_2\text{SO}_4$ and the precipitate was removed by centrifugation at about 10000 rev./min and discarded. An increase to 65% satn. made the Mo-protein precipitate. It was resuspended in 30ml of 0.4M-Tris/HCl and dialysed against 10 litres of 0.2M-Tris/HCl. An A_{280}/A_{322} ratio of 5.7 was obtained.

Step VI. At this stage the residual traces of desulphoviridine were finally removed by chromatography in silica (Baker, Deventer, Holland) and calcinated alumina (Laboratoire du Bois de Bologne, Paris, France). The fraction (in 0.2M-Tris/HCl) was applied

to a silica column (25mm×300mm) equilibrated with 0.2M-Tris/HCl and the fraction not bound contained the Mo-protein, with an A_{280}/A_{322} ratio of 4.8. This fraction was concentrated on an alumina column (25mm×100mm) equilibrated with 0.2M-Tris/HCl and eluted with 0.03M-phosphate buffer, in a final volume of 40ml and an A_{280}/A_{322} ratio of 4.5. The protein was judged homogeneous by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate (Moura *et al.*, 1976b) and electrofocusing, and a single N-terminal amino acid sequence was detected (Moura *et al.*, 1976a).

Other methods

The iron content was determined by atomic-absorption spectroscopy with a model SP.1900 spectrometer (Pye Unicam, Cambridge, U.K.).

Protein concentrations were determined by amino acid analysis and were based on a minimal mol.wt. of 120000 determined by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate (Summers *et al.*, 1965) and gel filtration on Sephadex G-150 (Moura *et al.*, 1976a,b, 1977b). Because glutamic acid, aspartic acid, alanine and glycine are stable to acid hydrolysis, they were used in the calculation of a mean value for the protein concentration. Molar absorption coefficients were calculated by using protein concentrations determined as described above. In Table 1, a maximum limit for the error is presented, taking into account the maximum deviations observed in the molecular weight [120000 ± 4000] was calculated by comparing the values determined at different polyacrylamide-gel concentrations (5–11%) and in the protein determination.

Optical spectra were recorded on a Cary 14 spectrometer (Varian Associates, Palo Alto, CA, U.S.A.).

E.p.r. spectra were recorded in a Varian E4 X-band spectrometer. Samples were cooled with He gas by using a flow cryostat (Oxford Instruments, Osney Mead, Oxford, U.K.). The signal intensities were plotted against the redox potentials and displayed on a Nicolet 1020A digital oscilloscope (Nicolet Instruments Corp., Madison, WI, U.S.A.) and fitted with the theoretical curves (for details see Cammack *et al.*, 1976).

Table 1. *Molar absorption coefficients of the D. gigas Mo–(2Fe–2S) protein*

The results are based on a mol.wt. of 120000 (cf. under 'Other methods').

λ (nm)	ϵ (litre·mol ⁻¹ ·cm ⁻¹)
320	101 000 ± 5000
415	57 000 ± 3000
462	58 000 ± 3000

Oxidation-reduction titrations of the molybdenum-containing iron-sulphur protein were carried out as previously described (Cammack *et al.*, 1976, 1977) by using an apparatus similar to that designed by Dutton (1971). The protein in 0.1M-Tris/HCl, pH 8.5, was poised at different potentials, and after a suitable equilibration time samples were taken anaerobically and frozen for e.p.r. spectroscopy. The mediators were as listed by Cammack *et al.* (1976) and were used at a concentration of 25 μ M. The protein concentration in the potentiometric titrations was 10 μ M; in the other e.p.r. measurements a concentration of 16 μ M was used. Concentrations were estimated from the absorption coefficients at visible wavelengths (see Table 1).

Experiments in Figs. 1, 2 and 3 were performed in the absence of dye mediators (dependence of the e.p.r. signals on the temperature and comparison of the e.p.r. spectra at different reduction times).

Results

Iron content and molar absorption coefficients of the protein

A content of 12–13 iron atoms per molecule was determined by atomic absorption spectroscopy and referred to protein concentrations determined by amino acid analysis. In a previous publication (Moura *et al.*, 1976a) 20 iron atoms per molecule were reported. We observed a lower value (12–13) after very long dialysis times; this value is reproducible from three different protein preparations.

Strong evidence has been presented for the presence of (2Fe–2S) clusters (Moura *et al.*, 1976a). It seems most likely that six clusters of this type exist in each molecule of the protein. The molar absorption coefficient at 462 nm calculated per cluster is 9700, in good agreement with the value observed for *Pseudomonas putida* (2Fe–2S) ferredoxin, ϵ_{455} = 9600 litre·mol⁻¹·cm⁻¹ (Yasunobu & Tanaka, 1973), which has a similar absorption spectrum.

Iron-sulphur centres Fe–S I and Fe–S II

Fig. 1 shows e.p.r. spectra of the protein, reduced with dithionite. The signals around $g=1.97$ are due to molybdenum and will be discussed below. At 71 K (Fig. 1a) a signal at $g_x=1.93$, $g_y=1.94$, $g_z=2.02$ was observed, corresponding to a type of iron-sulphur centre that we have termed Fe–S I (Moura *et al.*, 1976a; note that in Fig. 2 of that paper, the scales of g value and magnetic field were incorrectly labelled). As the temperature was decreased, further signals appeared at $g_x=1.90$, $g_z=2.06$, indicating the presence of a second type of centre Fe–S II.

As the signal of the centre Fe–S II appeared with decreased temperature, a splitting developed in the g_z

peak of centre Fe–S I, marked with an arrow in Fig. 1. This behaviour is consistent with a spin-spin interaction between the two iron-sulphur centres. In xanthine oxidase a splitting was observed in the signal of Mo(V) and was attributed to a spin-spin interaction with reduced centre Fe–S II (Lowe *et al.*, 1972).

Reduction by dithionite

The iron-sulphur centres were readily reduced by dithionite, but showed different kinetics of reduction.

Fig. 1. E.p.r. spectra of *D. gigas* Mo-(2Fe–2S) protein at various temperatures as shown

The sample was reduced with 5 mM-dithionite for 2 min before freezing. Other conditions of measurement were: microwave power, 1 mW; frequency 9.06 GHz; modulation amplitude 0.4 mT; frequency 100 KHz. (a) 71 K; (b) 67 K; (c) 62 K; (d) 47 K.

Fig. 2. Reduction of the protein by 5 mM-dithionite at 20°C. E.p.r. spectra are shown for (a) unreduced protein, (b) protein reduced for 1 min, (c) protein reduced for 10 min; (d) difference between traces (c) and (b) obtained with a Nicolet 1020A digital oscilloscope. Conditions of measurement were: temperature 24 K, microwave power 20 mW, frequency 9.25 GHz, modulation amplitude 1 mT.

After 1 min of reduction, centre Fe-S II was reduced virtually completely, as shown by the upward feature at $g=2.06$, but centre Fe-S I was only partly reduced, as shown by the features at $g=1.94, 2.02$ in Fig. 2(b). Complete reduction of centre Fe-S I was achieved in less than 10 min (Fig. 2c). Reduction of the Mo was much slower. No reduction was observed at 1 min, and only partial reduction at 10 min. By subtracting spectrum (b) from (c) we obtained the spectrum of those features reduced between 1 and 10 min, i.e. centres Fe-S I and Mo (Fig. 2d). The interpretation of these results is complicated by the

Fig. 3. Increase of e.p.r. signal amplitudes with reduction time for Fe-S centre I and Mo

The protein was poised at -594 mV in the presence of dye mediators as described in the Experimental section, and samples were collected at intervals. E.p.r. spectra were measured as for Fig. 1, at 77 K. ●, Mo (slow) signal; ■, centre Fe-S I.

observation (see below) that the Fe-S I signal arises from two components with different midpoint potentials.

Oxidation-reduction potential titration of the protein: centres Fe-S IA and Fe-S IB

Even in the presence of bipyridyl dye mediators, the reduction of the molybdenum was much slower than the reduction of iron-sulphur centres, as shown in Fig. 3. To obtain equilibration of molybdenum with the poisoning system, it was necessary to hold the potential constant for at least 30 min before taking each sample for e.p.r. measurements. In the titration shown in Fig. 4, the potential of the protein and mediators was first adjusted to the lowest value and maintained there for 1 h, then samples were taken at successively higher potentials. Similar results were observed by titration to successively lower potentials, but disappearance of the Mo(V) signal was less complete at the lowest potentials. If a shorter equilibration time, 2 min, was taken before removal of samples, the results for titration of the iron-sulphur centres were essentially the same, but the molybdenum showed extremely non-ideal behaviour, as seen with the 'Slow' molybdenum of desulpho-xanthine oxidase (Cammack *et al.*, 1976).

In Fig. 4 the intensities of the various signals are plotted as a function of applied redox potential. The signal at $g=2.06$ corresponding to centre Fe-S II measured at 24 K showed a single reduction process with midpoint potential (E_m) -285 mV. The signal at $g=1.94$ showed two stages of reduction, the first at -260 mV, the second at approx. -440 mV. Neither

Fig. 4. Redox titration curves

E.p.r. signal intensities are plotted versus redox potential for *D. gigas* Mo-(2Fe-S) protein in 60 mM-Tris/HCl, pH 8.0, at 25°C. (a) Fe-S centre I measured by the height of the $g=1.94$ signal; (b) the same feature, measured at 24 K; (c) Fe-S centre II measured by the height of the $g=2.06$ signal at 24 K; (d) Mo(V) signal, measured at 77 K.

of these processes could be due to centre Fe-S II, since they were observed at 77 K as well as 24 K. Since both of the reduction processes gave rise to signals of the type previously referred to as Fe-S I, we refer to them as Fe-S IA (E_m -260 mV) and Fe-S IB (E_m -440 mV). From the titration of the signal at $g=1.94$ (Fig. 4a), centres Fe-S IA and Fe-S IB contribute to the height of this signal in the ratio 1:2. These may represent the relative intensities of the two types of centres, if it is assumed that the

Fig. 5. Spectra around $g=1.97$ corresponding to molybdenum

(a) 'Resting' signal from the unreduced enzyme recorded at a gain setting of 5000; (b) 'Slow' signal from the protein reduced with 5 mM-dithionite for 10 min recorded at a gain setting of 1500. Other conditions of measurement were: temperature 77 K, microwave power 0.25 mW, frequency 9.25 GHz, modulation amplitude 1.0 mT.

signals of the two centres have similar line shapes. The g_x signal of centre Fe-S IB is obscured by the mediator signal around $g=2.00$. The integrated intensity of the Fe-S II centre could not be easily assessed, because the central g value was obscured by other signals. However, a correct estimation of the relative amount of the three types of iron-sulphur centres can only be done by integration by using spectra simulation.

E.p.r. signals from molybdenum

The protein as prepared showed a small signal at 77 K around $g=1.97$ (Fig. 5a). By analogy with the 'Resting' signal of xanthine dehydrogenase of *Veillonella alcalescens* (Dalton *et al.*, 1976), this may be due to a small amount of modified protein that contains a stable form of Mo(V). The integrated intensity of this signal indicated that it corresponded to only 11% of the total molybdenum. On reduction with dithionite (cf. Fig. 3) a more intense signal at

Table 2. Comparison of iron-sulphur centres in *D. gigas* protein and milk xanthine oxidase
Data for xanthine oxidase are from Lowe *et al.* (1972) and Cammack *et al.* (1976). Midpoint potentials are for the enzyme in Tris/HCl buffer, pH 8.2. Abbreviation: nm, not measured.

Centre	<i>g</i> values			<i>E_m</i> (mV)
	<i>g_x</i>	<i>g_y</i>	<i>g_z</i>	
<i>D. gigas</i> protein				
Fe-S IA	1.93	1.94	2.02	-260
Fe-S IB	1.93	1.93	2.02	-440
Fe-S II	1.89	nm	2.06	-285
Xanthine oxidase				
Fe-S I	1.899	1.935	2.022	-336
Fe-S II	1.97	2.07	2.12	-255

$g=1.97$ appeared (Fig. 5*b*). The reduction was slow, as noted above, even in the presence of mediator dyes. On prolonged reduction, the signal intensity began to decrease. On reoxidation, the signal at first reappeared and finally decreased (Fig. 4*d*). This behaviour is that expected for the conversions $\text{Mo(VI)} \rightleftharpoons \text{Mo(V)} \rightleftharpoons \text{Mo(IV)}$, since Mo(V) is paramagnetic and the other two valences are e.p.r.-silent. To calculate the two midpoint potentials of this reduction process it is necessary to determine the maximum signal intensity as a function of the total molybdenum (Clark, 1960). In the titration in Fig. 4, the maximum intensity was estimated to be 0.85 spin per protein molecule. From this, the midpoint potentials were estimated to be: Mo(VI)/Mo(V), -415 mV; Mo(V)/Mo(IV), -530 mV (see Table 2). However, the data showed considerable deviations from ideal behaviour, suggesting that there may be a heterogeneous population of molybdenum species.

Our original paper (Moura *et al.*, 1976*a*) reported an Mo signal slightly different from that shown in the present paper. We think this is due to the superposition of a 'Resting' signal present in freeze-dried samples.

The slow molybdenum signal of the reduced protein was much less readily saturated with microwave power at 77 K than was the 'Resting' signal of the unreduced protein. Since the former signal was only observed under conditions where the iron-sulphur centres were in the reduced state, this suggests that relaxation of Mo is enhanced by the paramagnetic centres in the protein. It is difficult to assess the distance between the centres from this type of data (however, see Bray, 1975). Also no additional features were detected when the molybdenum signal was observed at lower modulation amplitude (0.25 mT).

Discussion

The spectroscopic properties of the iron-sulphur centres and molybdenum in this protein resemble those of the hydroxylases such as xanthine oxidase

and aldehyde oxidase, that contain molybdenum, flavin and iron-sulphur centres (Bray, 1975). The optical spectrum (Moura *et al.*, 1976*b*, 1977*b*) is very similar to that of deflavo-xanthine oxidase (Komai *et al.*, 1969). The redox behaviour of the molybdenum resembles that of the 'Slow' molybdenum of desulpho-xanthine oxidase and desulpho-xanthine dehydrogenase. This is an inactive form produced by the loss of a persulphide group near the molybdenum (Massey & Edmondson, 1970). As isolated, these enzymes always contain a significant proportion of the desulpho form. It is thus possible that the protein obtained may have lost some of its prosthetic groups.

The midpoint potentials of molybdenum and iron-sulphur centres in the *D. gigas* protein are all fairly similar (within 100 mV) to the corresponding values for xanthine oxidase (Table 2), but are spread over a wider range. The potentials of the iron-sulphur centres in the *D. gigas* protein are within the range of redox potentials observed for ferredoxins (Hall *et al.*, 1974), and the Mo signal shows a more negative value than the corresponding one observed for xanthine oxidase.

The effects of buffers have been studied in detail in xanthine oxidase. Large differences between the potentials of the Fe-S centres (-336 mV and -255 mV) were measured when xanthine oxidase was buffered with Tris than with pyrophosphate, which might be explained by the effect of bound buffer ions (Cammack *et al.*, 1976). However, the differences between redox values of the iron-sulphur centres in Tris are even greater for the *D. gigas* protein (-440 mV and -285 mV).

The sequence of reduction of the centres by dithionite (Fig. 2) was: at 1 min, almost all the Fe-S II centres and part of the Fe-S I centre were reduced; at 10 min, centre Fe-S I was completely reduced and Mo was partly reduced to Mo(V). This might represent a sequence of electron transfer within the molecule. However, the results are also consistent with a mechanism in which the reduction of one of the centres (not molybdenum) by dithionite is slow,

and the reducing electrons are rapidly distributed among the centres. Then because of their midpoint potentials, centres Fe-S IA and Fe-S II would be reduced first, followed by centre Fe-S IB and finally Mo. The determination of the exact mechanism would require the various rate constants, and the site of reduction by dithionite, to be elucidated.

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