Biochem. J. (1991) 280, 817–820 (Printed in Great Britain)

Information from e.p.r. spectroscopy on the iron–sulphur centres of the iron–molybdenum protein (aldehyde oxidoreductase) of *Desulfovibrio gigas*

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E.p.r. spectra of reduced iron–sulphur centres of the aldehyde oxidoreductase (iron–molybdenum protein) of *Desulfovibrio gigas* were recorded at X-band and Q-band frequencies and simulated. Results are consistent with the view that only two types of [2Fe-2S] clusters are present, as in eukaryotic molybdenum-containing hydroxylases. The data indicate the Fe/SI centre to be very similar, and the Fe/SII centre somewhat similar, to these centres in the eukaryotic enzymes.

INTRODUCTION

The classification of molybdenum-containing enzymes into families on the basis of amino acid sequence data and spectroscopic properties of molybdenum has been reviewed by Wootton *et al.* (1991). Molybdenum-containing hydroxylases (Bray, 1975, 1988; Hille & Massey, 1985) are a major family that includes xanthine oxidases, xanthine dehydrogenases and aldehyde oxidases. These enzymes were assigned (Wootton *et al.*, 1991) to a single family, primarily on the basis of the unique spectroscopic properties (especially e.p.r. spectra) of their molybdenum centres. However, the best characterized among them, especially from eukaryotic sources, are also in all other ways remarkably similar to one another. In general, they are dimers of two identical subunits, each of M_r about 150000 and containing one molybdenum atom as the pterin molybdenum cofactor, one FAD molecule and two non-identical [2Fe-2S] iron–sulphur clusters, designated Fe/SI and Fe/SII. [A recent addition to this category is xanthine dehydrogenase from *Drosophila melanogaster* (R. Hughes, B. Bennett & R. C. Bray, unpublished work).] For these enzymes reducing substrates act at the molybdenum centre, whereas many oxidizing substrates, including O_2 and NAD^+, act at the flavin site. The latter, unlike the molybdenum centre and the iron–sulphur clusters, may be removed reversibly from some of the enzymes. In all members of the family where this has been specifically investigated, the pterin molybdenum cofactor is present (Rajagopalan, 1991*a*,*b*) as the simple molecule, not as a dinucleotide derivative (Johnson *et al.*, 1990*a*,*b*).

Uniformity in the properties of eukaryotic molybdenum-containing hydroxylases contrasts with a greater diversity among related prokaryotic enzymes. Unfortunately, few of these have as yet been extensively characterized. Thus spectroscopic information is not available, e.g. on xanthine dehydrogenase from *Pseudomonas putida* (Koenig & Andreesen, 1990), so it is not clear whether or not this enzyme, containing cytochrome *b* rather than flavin, should be treated as a true member of the family. Although for two other bacterial xanthine dehydrogenases (Dalton *et al.*, 1976; Wagner *et al.*, 1984) the molybdenum centres are 'normal' (Wootton *et al.*, 1991), in both cases there are anomalies relating to the iron–sulphur centres. Thus in one case [enzyme from *Clostridium acidiurici* (Wagner *et al.*, 1984)] centre Fe/SII has unusually low *g*-values, and in the other [enzyme from *Veillonella alcalescens* (Dalton *et al.*, 1976)] this centre may be missing altogether from the enzyme. E.p.r. data are not yet available on the iron–sulphur centres of the enzyme from *Pseudomonas aeruginosa* (Johnson *et al.*, 1991).

To obtain further information on relationships between eukaryotic and prokaryotic molybdenum-containing hydroxylases we have carried out new e.p.r. studies, both at X-band and at Q-band frequencies, on another bacterial enzyme, the aldehyde oxidoreductase of *Desulfovibrio gigas* (Turner *et al.*, 1987; note that these workers, somewhat anomalously since it does not react with O_2, referred to this enzyme as 'a form of aldehyde oxidase'). E.p.r. properties of molybdenum in this enzyme (Turner *et al.*, 1987) permit its unambiguous classification as a molybdenum-containing hydroxylase (Wootton *et al.*, 1991). However, the enzyme differs from the eukaryotic enzymes, as well as from most of the bacterial ones, in lacking FAD and in consisting of a single subunit of M_r about 120000 (Moura *et al.*, 1976, 1978). There were also indications in this early work that the enzyme, which was originally isolated from *Desulfovibrio gigas* as an iron–molybdenum protein of unknown function, might have a higher content of iron–sulphur centres than do the eukaryotic enzymes.

From our results presented here we conclude that there are only two types of iron–sulphur clusters present in *Desulfovibrio gigas* aldehyde dehydrogenase, and that these are generally similar to those in eukaryotic molybdenum-containing hydroxylases. Results of analytical work and of studies by Mössbauer spectroscopy support these conclusions (B. A. S. Barata, J. J. G. Moura, I. Moura, J. Le Gall & V. Huynh, unpublished work).

MATERIALS AND METHODS

Preparation of *Desulfovibrio gigas* aldehyde oxidoreductase

The protein was prepared by a modification (B. A. S. Barata, J. Le Gall & J. J. G. Moura, unpublished work) of the method of Moura *et al.* (1978). Such preparations have the following properties and composition: Fe (mol)/Mo (mol) 6.9; $A^{1cm}_{1\%\,protein}$ (462 nm) 1.7 (based on Folin protein determination); ϵ (462 nm)/4Fe 25 $mM^{-1} \cdot cm^{-1}$ (B. A. S. Barata, J. J. G. Moura, I. Moura, J. Le Gall & V. Huynh, unpublished work). Samples of the protein were dialysed against 50 mM-Bicine buffer, pH 8.2

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(adjusted with NaOH), and for e.p.r. were reduced anaerobically with an excess of $Na_2S_2O_4$ before being frozen in liquid N_2.

E.p.r. spectroscopy

E.p.r. techniques were as described in previous papers, e.g. Lowe *et al.* (1972) and Barber *et al.* (1976), and computer processing and simulation were as outlined by Bray & George (1985).

RESULTS AND DISCUSSION

E.p.r. spectra for the *Desulfovibrio gigas* protein, recorded at X-band and Q-band frequencies, are shown in Figs. 1 and 2 respectively, recorded under various conditions of microwave power and sample temperature and are compared with computer simulations. In agreement with Moura *et al.* (1978), experimental spectra show contributions from molybdenum(V) and from reduced iron–sulphur centres. Signals from the former are relatively sharp, and are readily distinguished from those from the latter by their being detectable at 120 K (Fig. 1*a*), at which temperature the iron–sulphur signals are too broad to be observable.

That the iron–sulphur signals, illustrated for X-band in Fig. 1(*b*) and for Q-band in Fig. 2(*b*), are due to two signal-giving species only, present in approximately equal amounts (and designated by analogy with xanthine oxidase as Fe/SI and Fe/SII), is shown by comparison with the computer simulations in Figs. 1(*c*) and 2(*c*) respectively [compare also Fig. 2(*f*) with Fig. 2(*g*)]. Agreement of experimental spectra recorded at two frequencies with simulations obtained by using a single set of parameters (as given in Table 1; note that linewidths, as is usual, are greater at Q-band than at X-band) provides a stringent test for the correctness of the above interpretation. Interestingly, the g_1 feature of Fe/SII in the *Desulfovibrio gigas* protein shifts to lower fields [compare Figs. 2(*b*) and 2(*a*); in this case from a *g*-value of about 2.057 to 2.069] as the signal becomes saturated on decreasing the temperature (or on increasing the microwave power). As pointed out by Hille *et al.* (1985), there are analogous apparent *g*-value shifts for xanthine oxidase. [This is apparent in the original data of Lowe *et al.* (1972); failure of these workers to take account of it led them to conclude, almost certainly erroneously, that for xanthine oxidase Fe/SII has two *g*-values greater than the free electron value.]

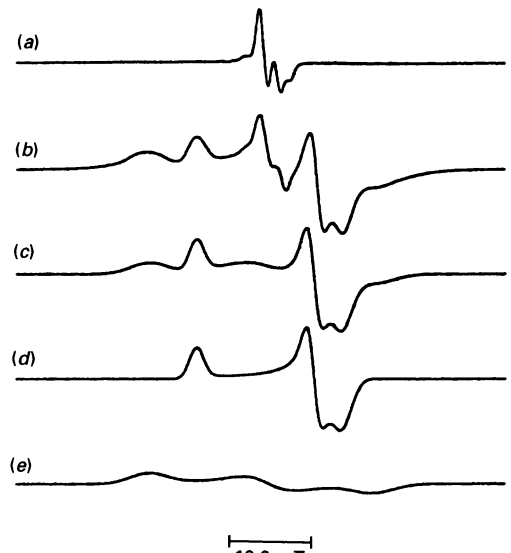

Fig. 1. X-band spectra from the reduced *Desulfovibrio gigas* enzyme

Experimental spectra are shown in (*a*) and (*b*), and computer simulations in (*c*) to (*e*). (*a*) was recorded at approx. 120 K, to show the Mo(V) signal, and (*b*) at approx. 45 K, to show this and the iron–sulphur signals Fe/SI and Fe/SII. In both cases the microwave power was 2 mW, the modulation amplitude 1 mT and the microwave frequency 9.33 GHz. Simulations were carried out by using the parameters given in Table 1. (*d*) corresponds to Fe/SI and (*e*) to Fe/SII at the same integrated intensity as Fe/SI. (*c*) is a simulation of the iron–sulphur contribution to (*b*), being the sum of spectra (*d*) and (*e*). The sample, in Bicine/NaOH buffer, pH 8.2, was reduced with an excess of $Na_2S_2O_4$.

Further support for the conclusion that there are two iron–sulphur centres in the *Desulfovibrio gigas* enzyme present in a stoichiometric ratio is provided by double-integration measurements of signal intensities performed over a range of microwave powers and sample temperatures. For xanthine oxidase (Bray, 1975) iron–sulphur signals are not detectable above about 120 K, whereas Fe/SI is fully sharpened at 40 K or below, the corresponding temperature for Fe/SII being 25 K. Analogous behaviour was reported by Moura *et al.* (1978) for the *Desulfovibrio gigas* protein, though they indicated that two types rather than one type of Fe/SI centre were present. In agreement with these workers, we found (results not shown) that the Fe/SI signal from the *Desulfovibrio gigas* protein was essentially fully sharpened at approx. 70 K and that Fe/SII, though scarcely detectable at 70 K, was fully sharpened at approx. 45 K. Additionally, we found that the normalized integrated intensity for the entire e.p.r. spectrum [Mo(V) plus Fe/SI plus Fe/SII] was constant, within experimental error, over the approximate temperature range 20–45 K and over the microwave power range 0.07–15 mW. Taking the integrated intensity under these conditions as 1.00, the corresponding values over the same power range at approx. 70 K and 120 K were 0.58 ± 0.04 and 0.08 ± 0.01 respectively (errors are S.D. for four measurements). These data are thus (after allowance for the molybdenum signals) in good agreement with the simulations in indicating a 1:1 ratio for Fe/SI to Fe/SII in the *Desulfovibrio gigas* enzyme.

Our conclusion that there are only two iron–sulphur centres, of the [2Fe-2S] type, in aldehyde oxidoreductase from *Desulfovibrio gigas* means that the enzyme is comparable in this respect with all six eukaryotic molybdenum-containing hydroxylases for which data are available (Lowe *et al.*, 1972; Hille *et al.*, 1985; Barber *et al.*, 1976, 1980, 1982; Coughlan *et al.*, 1984; R. K. Hughes, B. Bennett & R. C. Bray, unpublished work). Such a conclusion is fully compatible with the analytical data on the *Desulfovibrio gigas* enzyme sample used for the present work (see the Materials and methods section). The value of the molar absorption coefficient, ϵ_{462}, calculated per 4 mol of iron ($25\ mM^{-1} \cdot cm^{-1}$), agrees with the corresponding value ($24\ mM^{-1} \cdot cm^{-1}$) for deflavo xanthine oxidase (calculated from the data of Komai *et al.*, 1969). Both the absorption spectrum and the c.d. spectrum (Moura *et al.*, 1976) of the *Desulfovibrio gigas* protein are quite similar to those of the latter enzyme form. Existence of demolybdo xanthine oxidase and of demolybdo forms of other molybdenum enzymes is well established (Ventom *et al.*, 1988). Thus we conclude that the presence of the demolybdo form of the *Desulfovibrio gigas* enzyme in substantial amounts explains the Mo/Fe ratio of about 1:7 for the present preparations.

It remains to comment on apparent discrepanices between the present work and that of Moura *et al.* (1978). Regarding the e.p.r. spectra themselves, our results differ little from those of

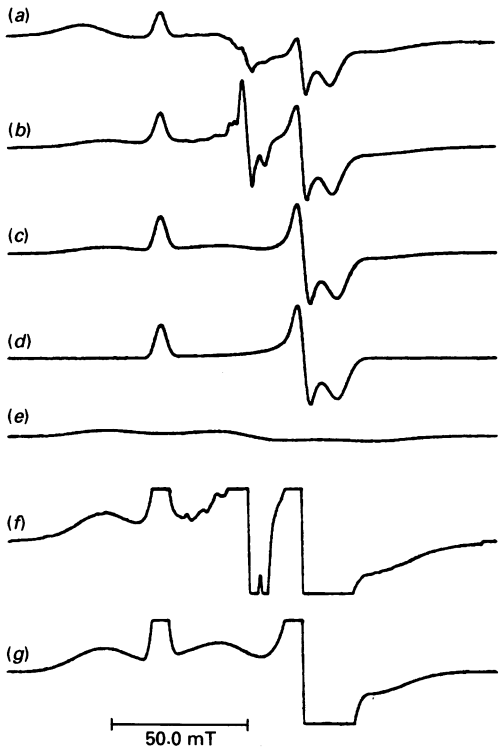

Fig. 2. Q-band e.p.r. spectra from the reduced *Desulfovibrio gigas* enzyme

Experimental spectra are shown in (*a*), (*b*) and (*f*), and computer simulations in (*c*) to (*e*) and (*g*). In (*a*) and (*b*) the attenuation of microwave power was 2 db, the modulation amplitude 1 mT and the microwave frequency 35.29 GHz; (*b*) was recorded at approx. 45 K and (*a*) at approx. 30 K. In (*a*) there is considerable saturation particularly of Fe/SI, with the result that the contribution of Fe/SII is emphasized. Simulations were carried out by using the parameters in Table 1. (*d*) corresponds to Fe/SI and (*e*) to Fe/SII at the same integrated signal intensity as Fe/SI. (*c*) is a simulation of the iron–sulphur contribution to spectrum (*b*) and is the sum of the spectra (*d*) and (*e*); (*f*) and (*g*) correspond to (*b*) and (*c*) respectively, with the amplification increased 4-fold so as to show the Fe/SII contribution more clearly. The sample was similar to that used in Fig. 1.

these workers. However, their preparations were reported to contain 12–13 iron atoms per molecule (perhaps related to a higher content of the demolybo enzyme than that in the current work). Thus Moura *et al.* (1978) expected more than two types of iron–sulphur centres to be present. Their main e.p.r. evidence for such additional centres came from plots, during potentiometric titration experiments, of the amplitude of the Fe/SI signal as a function of redox potential. There were indications of more than one component in such titrations. They also observed variations in linewidth in the g_1 feature of Fe/SI. The width of this peak increased as the recording temperature was decreased, the peak becoming clearly resolved into two features under some conditions. We did not carry out potentiometric titration studies, nor is the splitting of the Fe/SI peak apparent in Fig. 1 (though it is readily detectable for some samples of the enzyme under some recording conditions). Titration curves of the form obtained by Moura *et al.* (1978) could be explained by magnetic coupling between two different types of paramagnetic centres in the molecule. Interaction between Fe/SI and Fe/SII was invoked by these workers to explain the splitting of the g_1 feature of Fe/SI, but not the titration data. Perhaps coupling of molybdenum(V) to Fe/SI is involved in both phenomena. For xanthine oxidase,

Table 1. E.p.r. parameters for the Fe/S centres of the *Desulfovibrio gigas* enzyme: comparison with data from the literature

The parameters for *Desulfovibrio gigas* aldehyde oxidoreductase are the ones used in the simulations illustrated in Figs. 1 and 2; a single set of *g*-values was used at the two frequencies; linewidths in mT are given in parentheses (X-band value followed by Q-band). *g*-values for the centres are compared with corresponding data from the literature on other molybdenum-containing hydroxylases. For enzymes from six different eukaryotic sources, the values given are the means ± S.D., with data taken from seven investigations (Lowe *et al.*, 1972; Hille *et al.*, 1985; Barber *et al.*, 1976, 1980, 1982; Coughlan *et al.*, 1984; R. K. Hughes, B. Bennett & R. C. Bray, unpublished work). Data on two bacterial xanthine dehydrogenases, from *Veillonella alcalescens* (Dalton *et al.*, 1976) and *Clostridium acidiurici* (Wagner *et al.*, 1984), are also given.

Enzyme	*g*-value								
	Fe/SI				Fe/SII				
	1	2	3	Average	1	2	3	Average	
Desulfovibrio gigas	2.021 (0.85, 2.00)	1.938 (0.72, 1.80)	1.919 (1.17, 3.60)	1.959	2.057 (2.10, 10.0)	1.970 (2.50, 10.0)	1.900 (2.50, 12.0)	1.976	
Eukaryotic average	2.021 ± 0.004	1.933 ± 0.005	1.907 ± 0.010	1.954 ± 0.005	2.103 ± 0.015	2.002 ± 0.006	1.912 ± 0.010	2.005* ± 0.004	
Veillonella alcalescens	2.026	1.939	1.925	1.963		Not detected†			
Clostridium acidiurici	2.034	1.945	1.918	1.966	2.075	1.924	1.871	1.957	

* Data averaged almost certainly include some where spectra were partially saturated, leading to spectral distortions and to small apparent *g*-value shifts (see the text).

† Dalton *et al.* (1976) reported that this signal was not detectable down to 8 K. However, it may the desirable for the work to be repeated, since Smith *et al.* (1967) reported an iron content of the enzyme comparable with that of the eukaryotic enzymes.

broadening, also of the g_1 feature of Fe/SI, was observed by Lowe & Bray (1978) and attributed to magnetic interaction of this centre with molybdenum(V). These workers also studied, in some detail, the converse phemonemon, i.e. splitting of molybdenum(V) signals by Fe/SI, finding that the extent of this splitting varied from one molybdenum signal to another and also from one molybdenum-containing hydroxylase to another. Clearly, magnetic interactions among the centres may be complex and further work is needed, but the data are in no way inconsistent with the presence of only two iron–sulphur centres in the *Desulfovibrio gigas* enzyme.

In Table 1 *g*-values of the *Desulfovibrio gigas* enzyme determined in the present work are compared with data from the literature on six eukaryotic molybdenum-containing hydroxylases. The literature data are presented in the form of averages and standard deviations of *g*-values reported for the individual enzymes. The relatively small values of the standard deviations (which will of course depend both on experimental errors and on genuine differences among the enzymes) indicate that the eukaryotic molybdenum-containing hydroxylases are a very homogeneous group with regard to the nature of both of their iron–sulphur centres. For the *Desulfovibrio gigas* enzyme the Fe/SI *g*-values are clearly indistinguishable statistically from those of the eukaryotic group, since they deviate from the average values by only about 1 standard deviation. This implies similar structures. On the other hand, for Fe/SII the *g*-value discprepancy is larger, amounting to some 5 standard deviations, implying a greater structural difference. A somewhat comparable pattern is apparent in relation to iron–sulphur centres of the other bacterial molybdenum-containing hydroxylases for which data are available (Table 1), with Fe/SI *g*-values being in closer agreement with those of the eukaryotic group that is the case for Fe/SII.

Amino acid sequence data are now becoming available on some of the eukaryotic enzymes (Keith *et al.*, 1987; Lee *et al.*, 1987; Amaya *et al.*, 1990; Wootton *et al.*, 1991). The sequence associated with Fe/SI has been identified with some certainty (R. K. Hughes, W. A. Doyle, A. Chovnick, J. R. S. Whittle, J. F. Burke & R. C. Bray, unpublished work: cf. Wootton *et al.*, 1991) by analogy with two-iron ferredoxins (e.g. Rypniewski *et al.*, 1991) and with succinate dehydrogenase (e.g. Phillips *et al.*, 1987). According to the present e.p.r. data, an analogous Fe/SI sequence motif is clearly to be expected in the bacterial enzymes when sequences of these become available. On the other hand, candidates for the sequence associated with Fe/SII are less well defined in the eukaryotic enzymes, and in any case, from the e.p.r., less similarity is predicted to the bacterial enzymes in this region. Clearly, there is a need for sequence information on bacterial molybdenum-containing hydroxylases.

The work was supported by grants from the Science and Engineering Research Council, the British Council, Instituto Nacional de Investigacao Cientifica, Junta Nacional de Investigacao Cientifica e Tecnologica and National Institutes of Health, Grant GM 41482 (to J. J. G. M. and J. Le G.).

Received 5 September 1991; accepted 17 October 1991

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