

## Mössbauer study of the native, reduced and substrate-reacted *Desulfovibrio gigas* aldehyde oxido-reductase

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(Received September 27/November 15, 1991) – EJB 91 1290

The *Desulfovibrio gigas* aldehyde oxido-reductase contains molybdenum and iron-sulfur clusters. Mössbauer spectroscopy was used to characterize the iron-sulfur clusters. Spectra of the enzyme in its oxidized, partially reduced and benzaldehyde-reacted states were recorded at different temperatures and applied magnetic fields. All the iron atoms in *D. gigas* aldehyde oxido-reductase are organized as [2Fe-2S] clusters. In the oxidized enzyme, the clusters are diamagnetic and exhibit a single quadrupole doublet with parameters ( $\Delta E_Q = 0.62 \pm 0.02$  mm/s and  $\delta = 0.27 \pm 0.01$  mm/s) typical for the [2Fe-2S]<sup>2+</sup> state. Mössbauer spectra of the reduced clusters also show the characteristics of a [2Fe-2S]<sup>1+</sup> cluster and can be explained by a spin-coupling model proposed for the [2Fe-2S] cluster where a high-spin ferrous ion ( $S = 2$ ) is antiferromagnetically coupled to a high-spin ferric ion ( $S = 5/2$ ) to form a  $S = 1/2$  system. Two ferrous sites with different  $\Delta E_Q$  values (3.42 mm/s and 2.93 mm/s at 85 K) are observed for the reduced enzyme, indicating the presence of two types of [2Fe-2S] clusters in the *D. gigas* enzyme. Taking this observation together with the re-evaluated value of iron content ( $3.5 \pm 0.1$  Fe/molecule), it is concluded that, similar to other Mo-hydroxylases, the *D. gigas* aldehyde oxido-reductase also contains two spectroscopically distinguishable [2Fe-2S] clusters.

Molybdenum hydroxylases are an important group of proteins within the molybdenum-containing enzymes [1–3]. In this class of enzymes, the molybdenum is found in a pterin cofactor (termed Mo-co), which contains no other transition metal besides molybdenum [4]. A different situation is found in the case of another group of molybdenum-containing enzymes, namely nitrogenase, where the cofactor is a spin-coupled cluster of molybdenum and iron atoms (termed the FeMo-co) [5, 6]. Another difference, with respect to the redox centers of these two classes of enzymes, is that nitrogenase contains four putative [4Fe-4S] clusters (termed the P-clusters) [7, 8], while the molybdenum hydroxylases, such as xanthine oxidase, xanthine dehydrogenase and aldehyde oxidase, contain two spectroscopically distinguishable [2Fe-2S] clusters (termed Fe/S I and Fe/S II) [2, 9].

A molybdenum [iron-sulfur] protein was isolated from *Desulfovibrio gigas* by Moura et al. [10, 11]. Previous studies have focused on the molybdenum site. Extended X-ray-absorption fine structure (EXAFS) spectra of this protein indicated a molybdenum environment similar to that in the desulfo form of xanthine oxidase [12]. Detailed investigations using the EPR technique revealed that extended reduction of the protein by dithionite yielded a molybdenum signal similar to that of the inactive desulfo form of various molybdenum-containing hydroxylases, while brief reduction of the protein generated an additional molybdenum signal similar to that of the active form of those enzymes [13]. Activity measurements

of the *D. gigas* protein showed aldehyde: 2,6-dichlorophenol indophenol oxido-reductase activity, indicating that it is a molybdenum-containing hydroxylase [4, 13] (B. A. S. Barata, J. LeGall, and J. J. G. Moura, unpublished results). Similar to other molybdenum hydroxylases, the absorbance and EPR spectra of the *D. gigas* protein show the presence of [2Fe-2S] clusters [11]. However, more than two [2Fe-2S] clusters/molecule were suggested from these earlier studies.

In this manuscript, we present a re-determination of the metal content of this *D. gigas* molybdenum [iron-sulfur] protein (termed aldehyde oxido-reductase) and report a detailed Mössbauer characterization of its iron-sulfur clusters. The data unambiguously demonstrate that this protein contains four iron atoms arranged in two spectroscopically distinguishable [2Fe-2S] clusters. A Mössbauer study of the protein reacted with benzaldehyde shows partial reduction of the [2Fe-2S] clusters, indicating the involvement of the clusters in the process of substrate oxidation.

### MATERIALS AND METHODS

#### Protein purification and sample preparation

<sup>57</sup>Fe enrichment of the bacterial mass was accomplished as reported in [14]. The *D. gigas* aldehyde oxido-reductase was isolated essentially as described in [11], using an additional HPLC purification step on a Ultrapack TSK DEAE 35W column (LKB). The protein was purified aerobically and the isolated enzyme was in the oxidized state. All subsequent protein manipulations were conducted under anaerobic con-

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Abbreviation. EXAFS, extended X-ray-absorption fine structure.

ditions (purified argon atmosphere). Protein samples were prepared in different redox states using variable equilibration times in the presence of sodium dithionite and after reaction with benzaldehyde (in the presence of 2,6-dichlorophenol in-dophenol). Experimental conditions are indicated in the figure legends. EPR and Mössbauer samples were prepared in parallel in order to assess the level of reduction of the iron-sulfur centers and the type of molybdenum(V) species generated.

### Metal content determination

The iron and molybdenum contents were determined by plasma emission (Jarrell-Ash Atom Comp 750 Spectrometer) and protein was determined by the Lowry method [15]. Different batches of purified protein were used. After recording the visible/ultraviolet spectra, the protein solution was diluted to the appropriate concentration and this same protein solution was used for metal analysis and protein determination in order to directly correlate these parameters and estimate the molar absorption coefficient values.

The molecular mass of the native enzyme was determined both by gel filtration on a HPLC column and by electrophoresis in the presence of SDS [16, 17]. Both high-molecular-mass and low-molecular-mass standard kits (Pharmacia) were used.

### Mössbauer and EPR spectroscopy

Both the weak-field and the strong-field Mössbauer spectrometers are of the constant-accelerating type with a standard transmission arrangement. The weak-field spectrometer was equipped with a Ranger VT-700 velocity transducer and a top-loading Janis 8DT SuperVaritemp cryostat. The strong-field spectrometer was equipped with a top-loading Janis 12 CNDT/SC SuperVaritemp cryostat completed with an American Magnetics 8 T superconducting magnet and a home-built Doppler velocity transducer. The velocity scale was calibrated using room-temperature Mössbauer spectra of a metallic iron foil. The zero velocity was referred to the centroid of these spectra. EPR spectra were recorded on a Bruker ER-200-D-SRC spectrometer equipped with an Oxford Instruments ESR-9 flow cryostat.

## RESULTS

### Molecular mass, metal content and absorption coefficient

The minimum molecular mass of the protein was determined to be 120 kDa by electrophoresis under denaturing conditions and by HPLC. No indication of subunits was obtained. The metal content was determined for twelve protein samples isolated from different batches of cells grown in medium enriched with  $^{57}\text{Fe}$  as well as in medium without the enrichment. The iron and molybdenum analyses were performed with and without extensive dialysis prior to the determination. Only these two metals were detected by plasma emission and the results were not affected by the dialysis process. The average values determined/120 kDa protein are  $3.5 \pm 0.1$  mol (Fe),  $0.53 \pm 0.07$  mol (Mo) and  $7.2 \pm 1.3$  for iron/molybdenum ratio. (The reported metal stoichiometries are based on a protein determination estimated by the Lowry method, a common method for protein determination. However, we would like to point out that, depending on the protein, this method may introduce a systematic error larger than the quoted uncertainties.) The iron content varies with the 462-nm

absorption and tends to maximize at around 4 mol iron/protein molecule. The molybdenum content is relatively constant and centered around 0.5 mol molybdenum/protein molecule, suggesting that some protein molecules may be devoid of molybdenum. Based on the Fe content analysis, a re-evaluation of the absorption coefficient at 462 nm yields a value of  $24.6 \text{ cm}^{-1} \cdot \text{mM}^{-1}$ , assuming 4 Fe/protein molecule. This value agrees with those reported for proteins containing only a [2Fe-2S] cluster [approximately  $5.0 \text{ cm}^{-1} \cdot \text{mM}^{-1} \cdot (\text{mol iron})^{-1}$ ] [18], suggesting that molybdenum does not contribute significantly to the spectrum in this region. The recent discovery of a molybdenum enzyme containing no iron-sulfur cluster (dimethyl sulfoxide reductase) confirms this suggestion [19].

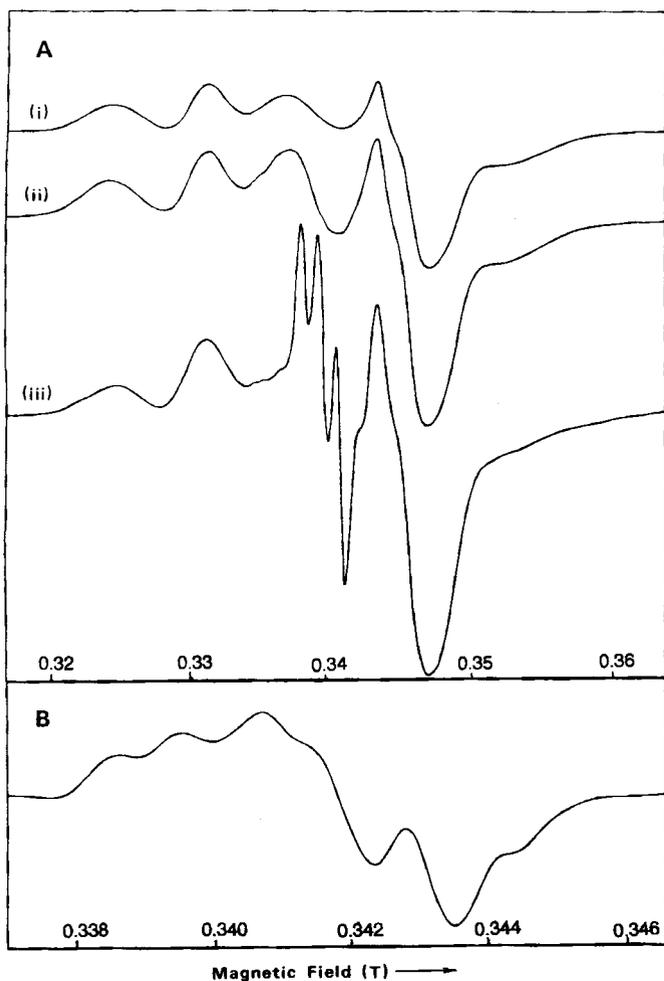
### EPR data

The EPR data reported in this section essentially defines the redox states of the [Fe-S] centers and the molybdenum site after reduction with dithionite or reaction with benzaldehyde. The results are complementary to and should be correlated with the Mössbauer data presented in the following sections. Details on the EPR-active species referred to here can be found in [1] and [11]. In agreement with previous work [11], the native samples exhibit a weak EPR signal (not shown) attributable to molybdenum(V) (termed the 'resting signal'). Reduction with dithionite for 15 s to 1 min yields samples with variable amounts of reduced [2Fe-2S] signals and the molybdenum(V) rapid EPR signal (Fig. 1A). The molybdenum(V) rapid signal is a characteristic signal for molybdenum hydroxylases observed during enzymatic turnover and represents active species [1]. The same molybdenum(V) rapid EPR species were also generated after reacting with benzaldehyde (Fig. 1B). Longer reduction with dithionite generates molybdenum(V) EPR signals of the 'slow type' due to inactive species (Fig. 1A, iii). Samples prepared for the Mössbauer spectroscopic study include the following redox states of the enzyme; oxidized, partially reduced [with molybdenum(V) rapid signal], reduced (with molybdenum(V) slow signal) states and enzyme reacted with benzaldehyde [with molybdenum(V) rapid signal].

### Mössbauer data

#### Oxidized protein

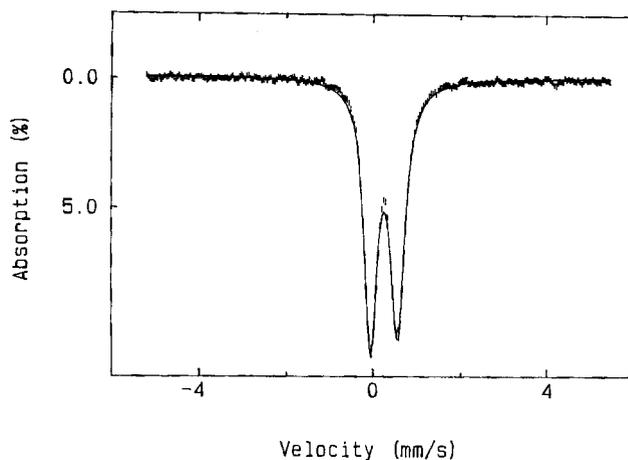
Fig. 2 shows the Mössbauer spectrum of the isolated enzyme recorded at 4.2 K with a magnetic field of 50 mT applied parallel to the  $\gamma$ -beam. A single quadrupole doublet is observed. The Mössbauer parameters (quadrupole splitting  $\Delta E_Q = 0.62 \pm 0.02$  mm/s and isomer shift  $\delta = 0.27 \pm 0.01$  mm/s), obtained by least-squares fitting of the data, indicated that all the iron sites are high-spin ferric and are identical to those of the oxidized [2Fe-2S] clusters in various iron-sulfur proteins [20–22]. The observed isomer shift is typical for ferric ion with tetrahedral-sulfur coordination. The linewidths of 0.36 mm/s and 0.40 mm/s for the low-energy and high-energy lines of the quadrupole doublet, respectively, are broader than the instrumental linewidth, but are very similar to those observed for the [2Fe-2S] clusters in ferredoxins [20, 21], suggesting that the iron sites are slightly inequivalent. Strong-field spectra further indicate that the iron sites are diamagnetic, which is another common property of oxidized [2Fe-2S] clusters where the two ferric ions are spin-coupled to form a diamagnetic state.



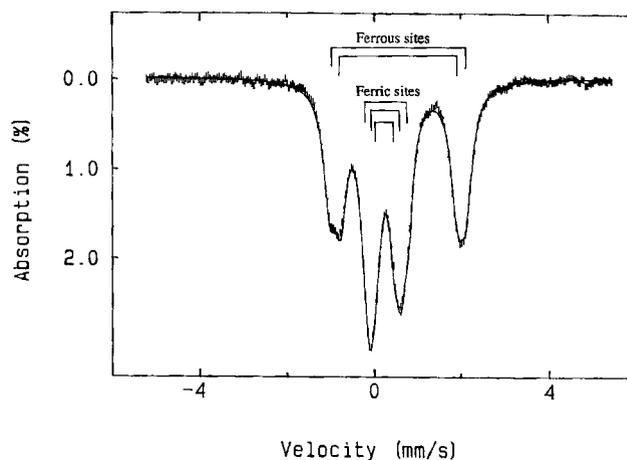
**Fig. 1.** X-band EPR spectra of reduced states of  $^{57}\text{Fe}$ -enriched *D. gigas* aldehyde oxido-reductase. (A) (i) 15 s, (ii) 1 min and (iii) 15 min dithionite-reduced enzyme. Temperature, 20 K; modulation amplitude, 1 mT; microwave frequency, 9.42 GHz; microwave power, 2 mW. (B) Benzaldehyde-reacted enzyme in the presence of 2,6-dichlorophenol indophenol. Detail of the  $g = 2$  region. Similar spectra were observed for the 15 s and 1 min dithionite-reduced enzyme. Temperature, 100 K; modulation amplitude, 1 mT; microwave frequency, 9.40 GHz; microwave power, 10 mW.

#### Dithionite-reduced protein

Fig. 3 shows a Mössbauer spectrum of a dithionite-reduced aldehyde oxido-reductase recorded at 180 K. The sample was reduced by dithionite for 30 min in an argon atmosphere before it was frozen with liquid nitrogen for Mössbauer measurements. Two apparent quadrupole doublets are observed. The outer doublet exhibits parameters ( $\Delta E_Q \approx 2.9$  mm/s and  $\delta \approx 0.57$  mm/s) that are typical of high-spin ferrous ions and the inner doublet ( $\Delta E_Q \approx 0.7$  mm/s and  $\delta \approx 0.25$  mm/s) is characteristic of high-spin ferric ions. The observed isomer shifts are characteristic for tetrahedral-sulfur-coordinated iron atoms in their respective oxidation states. A reduced  $[\text{2Fe-2S}]$  cluster is composed of a high-spin ferrous ion ( $S = 2$ ) and a high-spin ferric ion ( $S = 5/2$ ) that are spin-coupled to form a paramagnetic  $S = 1/2$  state. Consequently, the high-temperature Mössbauer spectrum of a reduced  $[\text{2Fe-2S}]$  cluster exhibits two well-resolved quadrupole doublets of



**Fig. 2.** Mössbauer spectrum of the isolated  $^{57}\text{Fe}$ -enriched *D. gigas* aldehyde oxido-reductase. The data were recorded at 4.2 K with a magnetic field of 50 mT applied parallel to the  $\gamma$  beam. The solid line is a least-squares fit to the data. A single quadrupole doublet is assumed in the fit.



**Fig. 3.** Mössbauer spectrum of a dithionite-reduced *D. gigas* aldehyde oxido-reductase recorded at 180 K. The data were least-squares fitted with five quadrupole doublets and the solid line is the result of such a fit. Positions of the five doublets are indicated by the brackets.

approximately equal intensity; one doublet corresponding to the ferrous ion and the other to the ferric ion [19–21]. The spectrum shown in Fig. 3 is therefore consistent with the presence of reduced  $[\text{2Fe-2S}]$  clusters in the reduced proteins. The percent absorption of the ferrous-type doublet in the reduced aldehyde oxido-reductase is estimated to be  $42 \pm 4\%$ , indicating that approximately 84% of the clusters are reduced while 16% remains oxidized.

A detailed examination of the spectrum shown in Fig. 3 revealed that the shape of the absorption lines of the ferrous doublet are not Lorentzian and a shoulder is observed for the low-energy line at  $-1.3$  mm/s. This observation suggests the existence of different types of  $[\text{2Fe-2S}]$  clusters in this aldehyde oxido-reductase, with distinguishable ferrous sites (and possibly different ferric sites). With this understanding, the data were fitted with five quadrupole doublets; two ferrous-type and three ferric-type doublets (two ferric-type doublets for the reduced clusters and one for the oxidized clusters). The

**Table 1.** Mössbauer parameters for the dithionite-reduced *D. gigas* aldehyde oxido-reductase obtained at 180 K.

Oxido-reductase	Parameter	
	$\Delta E_Q$	$\delta$
Reduced [2Fe-2S]	mm/s	
Ferrous sites	$3.14 \pm 0.02$	$0.55 \pm 0.01$
	$2.69 \pm 0.02$	$0.57 \pm 0.01$
Ferric sites	$0.97 \pm 0.02$	$0.28 \pm 0.01$
	$0.42 \pm 0.02$	$0.24 \pm 0.01$
Oxidized [2Fe-2S]	$0.70 \pm 0.02$	$0.25 \pm 0.01$

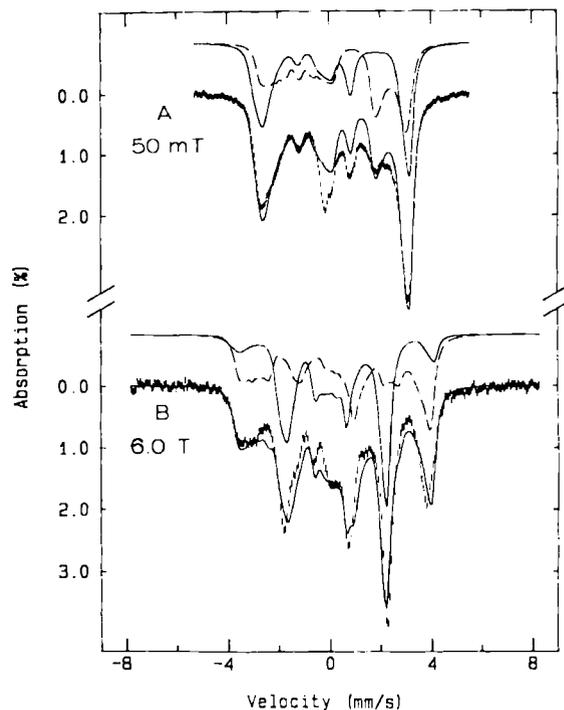
intensities of the two ferrous-type doublets were found to be almost equal and the solid line plotted in Fig. 3 is the result of a least-squares fit with the constraint that the intensities of the two ferrous doublets and the two corresponding ferric doublets are the same. The parameters obtained are listed in Table 1. Since the ferric-type doublets are unresolved, the parameters obtained for them can not be taken literally. Nevertheless, it is interesting to note that the parameters for the oxidized cluster are very similar to that of the isolated protein. The  $\Delta E_Q$  values for the ferrous sites are typical for high-spin ferrous ions, but are larger than those generally observed for reduced [2Fe-2S] clusters [20, 21]. Similar to other reduced [2Fe-2S] clusters, the  $\Delta E_Q$  values of the two ferrous-type doublets are temperature dependent [ $\Delta E_Q(1) = 3.12 \pm 0.03$  mm/s and  $\Delta E_Q(2) = 2.69 \pm 0.03$  mm/s at 180K;  $\Delta E_Q(1) = 3.42 \pm 0.03$  mm/s and  $\Delta E_Q(2) = 2.93 \pm 0.03$  mm/s at 85 K) while those of the ferric-type doublets are not.

At low temperatures (below 30 K), the electronic relaxation of the reduced [2Fe-2S] clusters is slow in comparison with the nuclear precession, and the clusters exhibit paramagnetic Mössbauer spectra. To characterize the reduced [2Fe-2S] clusters in the aldehyde oxido-reductase in further detail, we recorded the low-temperature spectra of the dithionite-reduced protein. Spectra representing the reduced [2Fe-2S] clusters were then prepared by removing the contribution of the oxidized cluster from the raw data. The spectra of the oxidized cluster were simulated using the parameters obtained for the isolated enzyme and assuming diamagnetism. Fig. 4 shows the prepared 4.2-K spectra for the reduced [2Fe-2S] clusters in parallel-applied fields of 50 mT (Fig. 4A) and 6T (Fig. 4B). Although two reduced [2Fe-2S] clusters are spectroscopically differentiable at high temperatures, the resolution of the low-temperature spectra is not good enough to distinguish them. Nevertheless, these low-temperature spectra show characteristics that are common for [2Fe-2S] clusters and can be understood by a spin-coupling model proposed for the [2Fe-2S] clusters [23]. They can be decomposed into two components of equal intensity, one for the ferric site and one for the ferrous site. Each component can be simulated by an  $S = 1/2$  spin Hamiltonian (Eqn 1).

$$\hat{H} = \beta \vec{S} \cdot \vec{g} \cdot \vec{H} + \vec{S} \cdot \vec{A} \cdot \vec{I} + \frac{cQV_{zz}}{4} \left[ I_z^2 - I(I+1)/3 + \frac{\eta}{3} (I_x^2 - I_y^2) \right] - g_n \beta_n \vec{H} \cdot \vec{I} \quad (1)$$

where  $\eta = (V_{xx} - V_{yy})/V_{zz}$ .

The two components share a common electronic  $\vec{g}$  tensor, but have different  $\vec{A}$  and  $\vec{V}$  tensors values [20–22]. Table 2 lists the hyperfine parameters which yielded theoretical spectra



**Fig. 4.** Mössbauer spectra of the reduced [2Fe-2S] clusters of the *D. gigas* aldehyde oxido-reductase. The data were prepared by removing the contribution of the oxidized clusters from the spectra of the dithionite-reduced sample (see text). The experiments were performed at 4.2 K with the indicated magnetic fields applied parallel to the  $\gamma$  beam. The solid lines plotted over the experimental data are theoretical simulations using the parameters listed in Table 2. Theoretical simulations for the subcomponents, the ferric site (—) and the ferrous (— · —), are also shown on top of each spectrum.

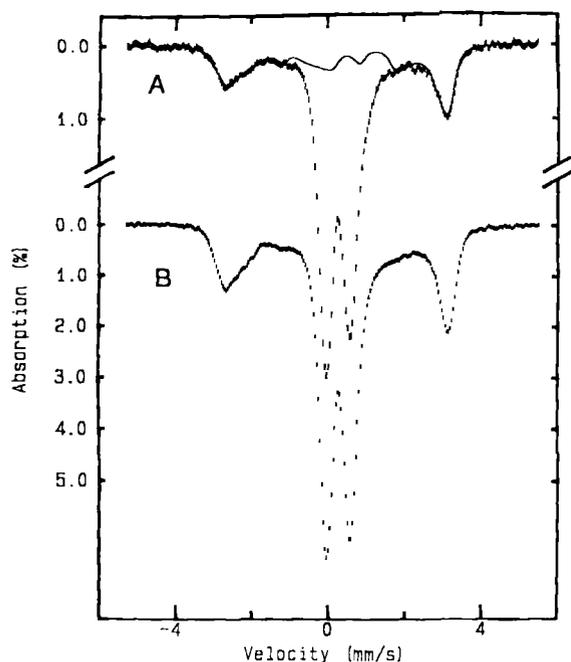
that best resemble the experiments. The theoretical simulations are plotted in Fig. 4 and the subcomponents are also shown. The theoretical spectra agree reasonably well with the experiments, except at the central region where the contribution from the oxidized cluster has been removed. The parameters obtained are very typical for a reduced [2Fe-2S] cluster. The  $\vec{A}$  tensor for the ferrous site is positive and anisotropic, while that of the ferric site is negative and relatively isotropic. The opposite signs of the  $\vec{A}$  tensors reflect the anti-ferromagnetic coupling nature of the [2Fe-2S] cluster and have been observed for all the reduced [2Fe-2S] clusters studied so far.

#### Benzaldehyde-reacted and partially-reduced states

Fig. 5 shows the Mössbauer spectrum of the benzaldehyde-reacted sample recorded at 4.2 K in a parallel-applied field of 50 mT (A). The spectrum is a superposition of two spectral components corresponding to the oxidized and reduced states of the [2Fe-2S] clusters, indicating partial reduction of the iron-sulfur clusters in this substrate-reacted sample. For comparison, the spectrum of a sample reduced by dithionite for 1 min, recorded under the same conditions, is also shown in Fig. 5 (B). It is obvious that both spectra are very similar. The only difference is in the degree of reduction. Approximately 35% of the clusters are reduced in the benzaldehyde-reacted sample, while 40% of the clusters are reduced in the dithionite-reduced sample. To demonstrate the degree of reduction of the iron-sulfur cluster in the benzaldehyde-

**Table 2. Hyperfine parameters for the reduced [2Fe-2S] clusters in the *D. gigas* aldehyde oxido-reductase.** The values in parentheses give the uncertainties in the last significant digits, which were estimated by visually comparing the data with theoretical simulations. The  $\Delta E_Q$  values were evaluated by extrapolations of the high-temperature data down to 4.2 K.

	Parameters					
	$A_{xx}/g_n\beta_n$	$A_{yy}/g_n\beta_n$	$A_{zz}/g_n\beta_n$	$\Delta E_Q$	$\delta$	$\eta$
T	mm/s					
Ferric site	-38(3)	-32(3)	-30(2)	1.0(5)	0.0(5)	0.30(3)
Ferrous site	8(2)	8(2)	22(2)	-3.6(5)	-1.5(5)	0.62(3)



**Fig. 5. Mössbauer spectra of the *D. gigas* aldehyde reductase after reacting with (A) benzaldehyde and (B) partially reduced by dithionite for 1 min.** The data were recorded at 4.2 K with a parallel-applied field of 50 mT. The solid line in A is a theoretical spectrum of the reduced [2Fe-2S] cluster and is plotted with an absorption area normalized to 35% of the total iron absorption of the experimental spectrum.

reacted sample, the theoretical spectrum of the reduced [2Fe-2S] cluster described in the previous section is plotted as a solid line over the experimental data in Fig. 5A. The absorption area of the theoretical spectrum is normalized to 35% that of the experimental data, and good agreement between experiment and theory is observed.

As mentioned in the previous section, the two iron-sulfur clusters are distinguishable in the reduced state by their high-temperature Mössbauer spectra. In order to investigate whether preferential reduction of the clusters exists, high-temperature measurements were performed for both samples (data not show) and no preferential reduction was observed. This observation is consistent with our previous EPR kinetic measurements [11] revealing that the iron-sulfur clusters are concomitantly reduced during enzymatic turnover. For the class of molybdenum hydroxylases isolated from the eukaryotic organism, EPR measurements have also been used to demonstrate that, upon reaction with substrate, the electrons flow

from the molybdenum center through the iron-sulfur clusters toward the flavin group [1].

## DISCUSSION

We have demonstrated that, in the *D. gigas* aldehyde oxido-reductase, all the iron atoms are organized in the form of [2Fe-2S] clusters. The general properties of these clusters are quite similar to those observed for [2Fe-2S]-containing ferredoxins [20, 21]. For the oxidized clusters, the iron sites are high-spin ferric in character and the observed isomer shift suggests tetrahedral-sulfur coordination. The two ferric ions are anti-ferromagnetically coupled to form a diamagnetic cluster. In their reduced forms, each cluster is composed of a ferric and a ferrous ion, and the Mössbauer spectra can be explained by a spin-coupling model proposed for [2Fe-2S] cluster [23] where a high-spin ferrous ion ( $S = 2$ ) is anti-ferromagnetically coupled to a high-spin ferric ion ( $S = 5/2$ ) to form an  $S = 1/2$  paramagnetic cluster.

At high-temperatures, two ferrous sites with different  $\Delta E_Q$  values are observed in the dithionite-reduced sample, indicating the presence of two types of [2Fe-2S] clusters. The observed absorption intensity further suggest that they exist in equal quantities. Earlier EPR studies [10, 11] and the current EPR data (see Fig. 1) have also detected two  $S = 1/2$  EPR signals corresponding to two types of Fe-S clusters. In correlation with the iron determination which yields approximately 4 Fe atoms/molecule, it is concluded that this *D. gigas* aldehyde oxido-reductase contains two spectroscopically distinguishable [2Fe-2S] clusters. In an earlier redox-titration study [11] monitored by EPR, three types of Fe-S clusters with different redox potentials were suggested; type IA ( $-260$  mV), type IB ( $-440$  mV) and type II ( $-285$  mV). In view of the current data, this previous notion must be re-examined. It is intriguing to note that only two types of EPR signals are observed for the Fe-S centers in the *D. gigas* aldehyde oxido-reductase and that type IA and type IB clusters exhibited identical EPR spectra (Type I).

Besides the larger  $\Delta E_Q$  values observed for the ferrous sites in the reduced [2Fe-2S] clusters, the hyperfine parameters obtained for the Fe-S clusters in the *D. gigas* aldehyde oxido-reductase are very similar to those of the [2Fe-2S] clusters in ferredoxin [20, 21]. Since other molybdenum hydroxylase also contain [2Fe-2S] clusters, it would be interesting to employ Mössbauer spectroscopy to further characterize these hydroxylase. The only Mössbauer study of molybdenum hydroxylases prior to this work was performed on a non-enriched sample of milk xanthine oxidase [24] and an unusually large  $\Delta E_Q$  (3.2 mm/s at 175 K) is also observed for the ferrous site of one of the [2Fe-2S] clusters.

A classification of molybdenum-containing enzymes, based on amino acid sequence data and spectroscopic properties, has recently been made [25]. A high degree of similarity, both in the amino acid sequence and prosthetic-group composition, is found in the molybdenum-containing enzymes isolated from eukaryotic organisms, including xanthine oxidase, aldehyde dehydrogenase and aldehyde oxido-reductase. A large diversity, however, is observed for the molybdenum hydroxylases found in prokaryotic systems. In particular, significant differences are found both in the content and spectroscopic properties of the iron-sulfur clusters [26–27]. The current investigation, which demonstrates the presence of two types of [2Fe-2S] clusters in the *D. gigas* aldehyde oxido-reductase, and the earlier EPR studies [11] which shows the different forms of the molybdenum centers, have established the close relationship between the *D. gigas* enzyme and the molybdenum hydroxylases from the eukaryotic systems. Important differences, however, do exist in that the *D. gigas* protein is isolated as a single unit of 120 kDa and lacks a FAD group. Molybdenum-containing proteins similar to the *D. gigas* enzyme have been found in *Desulfovibrio desulfuricans* of strains ATCC 27774, Berre eau and Berre sol [B. A. S. Barata, J. J. G. Moura, I. Moura and J. LeGall, unpublished results]. However, unique molybdenum [iron-sulfur] proteins have also been isolated from *Desulfovibrio africanus* [28] and *Desulfovibrio salexigens* [29]. The visible spectrum is distinct from that of the *D. gigas* enzyme and information regarding to the type of iron-sulfur cluster and redox properties of the molybdenum site is not yet available.

This work is supported by grants from *Instituto Nacional de InvestigaçãO Científica* (JJGM and IM), *Junta Nacional de InvestigaçãO Científica e Tecnológica* (JJGM), Po-Lis Food NATO Science for Stability (JJGM), the National Institutes of Health grants GM 41482 (JLG and JJGM) and GM 32187 (BHH), and the National Science Foundation grant DMB 9001530 (BHH). This research is also supported in part by the University Research Committee of Emory University.

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