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# Subunit composition, crystallization and preliminary crystallographic studies of the *Desulfovibrio gigas* aldehyde oxidoreductase containing molybdenum and [2Fe-2S] centers

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The *Desulfovibrio gigas* aldehyde oxidoreductase contains molybdenum bound to a pterin cofactor and [2Fe-2S] centers. The enzyme was characterized by SDS/PAGE, gel-filtration and analytical ultracentrifugation experiments. It was crystallized at 4 °C, pH 7.2, using isopropanol and $MgCl_2$ as precipitants. The crystals diffract beyond 0.3-nm (3.0-Å) resolution and belong to space group $P6_122$ or its enantiomorph, with cell dimensions $a = b = 14.45$ nm and $c = 16.32$ nm. There is one subunit/asymmetric unit which gives a packing density of $2.5 \times 10^{-3}$ nm³/Da (2.5 Å³/Da), consistent with the experimental crystal density, $\rho = 1.14$ g/cm³. One dimer (approximately $2 \times 100$ kDa) is located on a crystallographic twofold axis.

The detailed knowledge of the structure and the catalytic mechanism of molybdenum-containing enzymes has considerably increased during the last decade [1–6] and, in particular, the biochemistry of aldehyde oxidoreductases has been extensively studied [4, 5].

Molybdenum is a relevant transition metal in biological systems. Two groups of molybdo-proteins have been described. In the first group Mo is associated with iron in a complex cluster type structure (FeMocofactor) in the nitrogenase enzyme. Recently, a structural model of the FeMoco was proposed based on crystallographic analysis, consisting of [4Fe,3S] and [1Mo,3Fe,3S] clusters bridged by three non-protein ligands [7, 8]. In the second group Mo is contained in an organic structural component (pterin), designated as Mocofactor (molybdopterin), generally associated with iron-sulfur centers and/or a flavin or heme center, as in the case of molybdenum oxotransferases [6].

From sulfate-reducing bacteria (strict anaerobes) enzymes related to the second group have been isolated and characterized [9–17]. They represent unique situations for the presence of such a group of enzymes in the prokaryotic world, since most often these proteins are studied in eukaryotes (e.g. plants, insects and higher animals).

The molybdenum iron-sulfur protein (MOP) isolated from *D. gigas* has analogies with the molybdenum hydroxylases but does not contain a flavin moiety [9, 10, 12]. The visible absorption spectrum of the protein is rather similar to those observed for the deflavo-forms of xanthine and aldehyde oxidases. CD and preliminary EPR studies were complemented with Mössbauer and EPR spectroscopic studies (X and Q bands) [12–14], revealing the presence of two types of [2Fe-2S] cores, named Fe/S I and Fe/S II centers. An important advance on these studies was the possibility to isolate the enzyme from $^{57}$Fe-enriched media with obvious interest for an iron-sulfur-center site labelling, enhanced sensitivity of the Mössbauer studies (an advantage with respect to mammalian systems) and the possibility of a direct measurement of substrate binding [14].

Previously described molybdenum (V) resting-type and slow-type EPR signals and the extended-X-ray-absorption fine-structure (EXAFS) spectrum of the molybdenum center indicated close similarities to desulfo-xanthine oxidase (inactive) [10–12]. In addition, it was later demonstrated for MOP, a new Mo(V) EPR signal (rapid type 2) centered at EPR average g-value ($g_{av}$) of 1.9742, similar to those observed for xanthine and aldehyde oxidases [12]. These rapid EPR signals have been shown, in these proteins, to be physiologically significant, as they develop within the enzyme-turnover time scale. A molybdenum cofactor, liberated from the protein, was active in the nit-1 *Neurospora crassa* nitrate-reductase assay [12]. However, the complete identification of the pterin moiety has not been achieved.

In contrast with the recent developments in the field of the molybdenum-containing nitrogenases [7, 8], the lack of fine structural information on the molybdenum-containing hydroxilating enzymes, especially with regard to the relative spatial arrangement of the metal sites and the nature of Moco, hampered the detailed understanding of the mecha-

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*Abbreviations.* MOP, molybdenum iron-sulfur protein; EXAFS, extended X-ray absorption fine structure; $g_{av}$, EPR average g-value.

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*Note.* *R* factor is defined as $\Sigma(\langle I_H \rangle - I_H)/\Sigma I_H$, where $\langle I_H \rangle$ is the averaged intensity and $I_H$ is an individual measurement ($R_{merge}$) or a Friedel mate.

nisms operating in these enzymes. We report now, as a first step towards the structure determination of *D. gigas* aldehyde oxidoreductase, the crystallization and preliminary X-ray diffraction studies of this enzyme. The subunit composition of the protein is also revised on the basis of the known data.

## MATERIAL AND METHODS

### Organism and enzyme purification

*D. gigas* (NCIB 9332) cells were grown as indicated in [18], using a lactate-sulfate medium. The *D. gigas* aldehyde oxidoreductase was isolated as previously described [10], using an additional HPLC purification step on an Ultrapack TSK DEAE 35 W column (LKB) [15].

### Amino-acid composition and $NH_2$-terminal sequence determination

Protein samples were hydrolyzed in the gas phase according to procedures described in [19]. Amino-acid analyses were performed on a Beckman 6300 amino-acid analyser according to the instructions of the manufacturer. The results for the amino-acid composition were in agreement with previously reported values [9], with the exception of Arg, for which the data were non-reproducible.

Sequence determination was performed on an Applied Biosystem 477A protein sequencer coupled to an Applied Biosystem 120A analyser.

### Molecular mass and subunit composition

Molecular mass of the protein was re-evaluated by two independent methods. SDS/PAGE was performed [20] using the Sigma high-molecular-mass kit as standard for calibration. The sample was incubated with 10% SDS and 2-mercaptoethanol, with or without boiling step (Fig. 1A). Gel filtration on a Superose 12 HR 10/30 Pharmacia column was also performed, using standards as indicated in Fig. 1B, with 0.3 M NaCl, 0.1 M Tris/HCl, pH 7.0, as elution buffer. Protein concentration was 7 mg/ml. The detection wavelength used was 275 nm.

Sedimentation equilibrium experiments were performed in a Beckman analytical ultracentrifuge Model E (Beckman Instruments, Inc.) equipped with a photoelectric scanning system, at 277.2 K. The detection wavelength used was 280 nm. An AnH rotor with a filled Epon 6-channel Yphantis cell [21] and saphire windows were employed.

The experiments were performed with enzyme solutions in 10 mM Tris/HCl pH 7.6 with $A_{280}$ of 0.8 (corresponding to a concentration of about 0.3 mg/ml) and centrifuged at 6800 rpm. The enzyme solutions were then pooled (460 µl) and adjusted to pH 1–2 with 20 µl HCOOH. This sample was used for a second experiment. Equilibrium was reached at 7200 rpm. For the calculation of the molecular mass, a partial specific volume of 0.73 $cm^3/g$ was assumed.

### Crystallization and crystal stabilization

Crystallization was performed by the vapour-diffusion method using sitting drops and a reservoir containing 0.1 M Hepes, pH 7.5, 0.2 M $MgCl_2$ and 30% isopropanol. Droplets were prepared by mixing 4 µl of a 13 mg/ml protein solution in 10 mM Tris, pH 7.6, with 2 µl reservoir solution. Single crystals were obtained within 3–6 weeks at 4°C and had the shape of hexagonal bipyramids (Fig. 2). The crystals are red and grow to a size of 0.2–0.6 mm in their maximal dimension. These crystals were very fragile and sensitive to increase in temperature as well as to exposure to the X-rays. When harvesting them with a solution similar to the precipitating solution (0.1 M Hepes, 0.4 M $MgCl_2$, 40% isopropanol), the crystals diffracted to less than 0.5-nm (5-Å) resolution and were destroyed after 2 h of exposure. Stabilization and improvement of the quality of these native crystals was successfully achieved by treatment with a harvesting buffer containing 20% poly(ethylene glycol) 4000 in the original reservoir solution. After 1–2 weeks in the new buffer, the crystals could be much more easily handled and withstood some temperature increase. These treated crystals diffracted to beyond 0.3-nm (3.0-Å) resolution on a Rigaku rotating anode operated at 5.4 kW as X-ray source, and a complete intensity-data set could be obtained.

### Diffraction experiments

X-ray-diffraction intensity data were measured with a MAR-Research X-ray imaging plate system (Hendrix/Lantfer) installed on a Rigaku rotating anode generator operated at 5.4 kW. Graphite monochromatized $CuK_\alpha$ radiation was used. The crystal-to-plate distance was 145 mm and frames of 1.2° rotation were taken in 20 min for each. The crystal was cooled to about 0°C by a modified Colder cooling device (Kager) operated with pressurized air. The raw data were evaluated with MOSFLM package, Version 4.0 [25].

## RESULTS AND DISCUSSION

### Molecular mass of *D. gigas* aldehyde oxidoreductase

Polypeptide composition of the enzyme was analysed by SDS/PAGE (Fig. 1A). When the sample was boiled (with SDS and 2-mercaptoethanol) before electrophoresis, a single band was observed around 100 kDa.

The molecular mass determined by calibrated gel exclusion is around 200 kDa (Fig. 1B) for the native protein at an initial concentration of 7 mg/ml in 0.3 M NaCl, 0.1 M Tris/HCl, pH 7.0, indicating, together with the gel-electrophoresis results, that the protein is isolated as a dimer. Previous results had shown a molecular mass of 120 kDa [10, 12]. Those measurements were conducted in 1 M NaCl and 0.02 M potassium phosphate, pH 7.6, suggesting that the protein is present in those conditions as a monomer.

A lower molecular mass (≈65 kDa) was sometimes observed by SDS/PAGE when omitting the heating procedure. A similarly abnormal electrophoretic mobility of a protein was also reported for $Me_2SO$ reductase [22]. The origin of this abnormality is unclear.

Sedimentation equilibrium experiments were performed with samples of enzyme at a concentration of 0.3 mg/ml under two different conditions: (a) in 10 mM Tris/HCl, pH 7.6, and (b) in Tris/HCOOH, pH 1–2. A molecular mass of about 73 kDa was obtained from the former. For preparation (a) no straight line was obtained in a plot according to Yphantis [21], indicating heterogeneity. The plot may be explained by a bimodal sedimentation distribution with species of molecular mass 96 kDa and 180 kDa.

These data suggest that the protein is a dimer with molecular mass 200 kDa composed of two identical subunits under the conditions of the gel-filtration experiment and that it

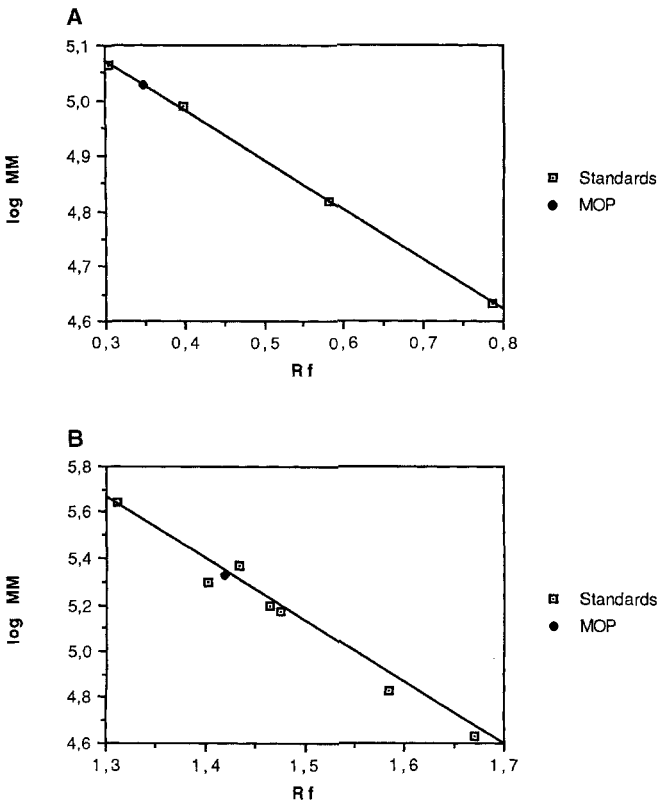


**Fig. 1. Molecular mass determinations.** (A) Electrophoretic mobility of *D. gigas* aldehyde oxidoreductase in SDS/PAGE (10%). The experiments were performed as described in Materials and Methods using the following Sigma protein standards: β-galactosidase (116 kDa); phosphorylase b (97.4 kDa; bovine serum albumin (66 kDa); ovalbumin (45 kDa). Molecular mass determined for MOP was 107000 ± 3000 Da. (B) Gel-filtration calibration curve performed on a Superose 12 HR 10/30 Pharmacia column, using as standards: ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa) and ovalbumin (43 kDa) from Pharmacia, and β-amilase (200 kDa) and alcohol dehydrogenase (150 kDa) from Sigma. The molecular mass determined for *D. gigas* aldehyde oxidoreductase is 212000 ± 8000 Da.

tends to dissociate in the sedimentation-equilibrium experiment, at low protein concentration (a), and is fully dissociated in acid (b).

## $NH_2$-terminal amino-acid sequence

The amino acid sequence of the first 33 residues of the $NH_2$-terminal portion of *D. gigas* aldehyde oxidoreductase was identified as (results from three different determinations):

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 1 | | | | 5 | | | | | 10 |
| Met | Ile | Gln | Lys | Val | Ile | Thr | Val | Asn | Gly |
| 11 | | | | 15 | | | | | 20 |
| Ile | Glu | Gln | Asn | Leu | Phe | Val | Asp | Ala | Glu |
| 21 | | | | 25 | | | | | 30 |
| Ala | Leu | Leu | Ser | (Asp) | Val | Leu | (Arg) | Gln | Asp |
| | | | | | | | | | Gln |
| 31 | | | | | | | | | |
| () | () | Leu | Thr | | | | | | |

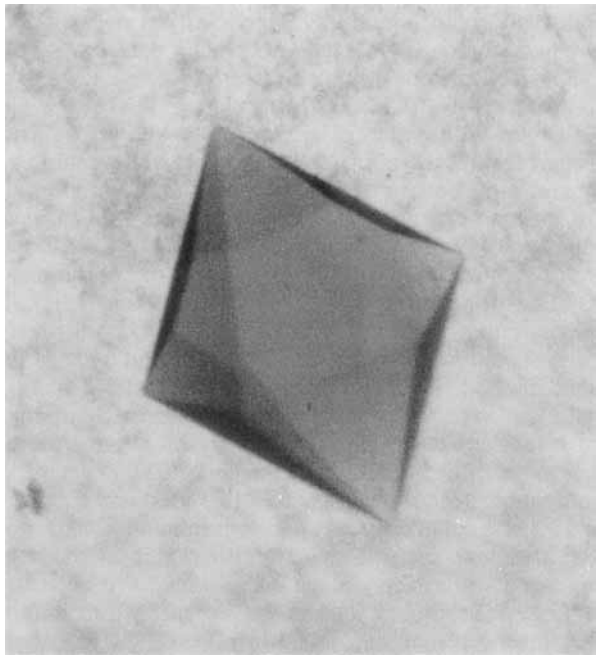

**Fig. 2. Crystal of *D. gigas* aldehyde oxidoreductase grown as described in the experimental section.** The maximum dimensions here are 0.6 × 0.3 × 0.3 mm.

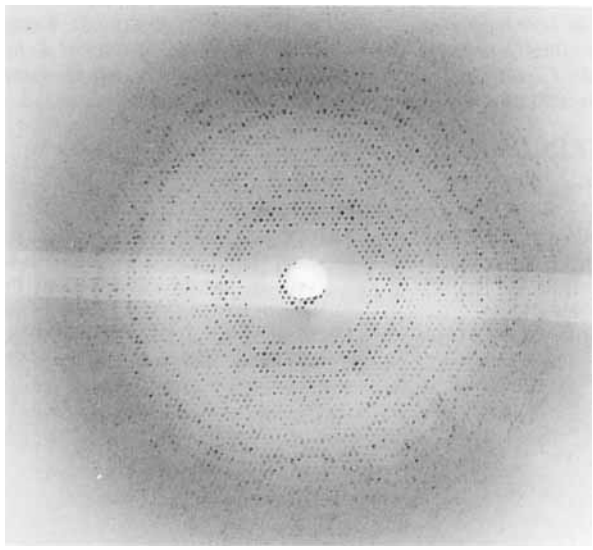

**Fig. 3. Screenless precession photograph of the hkN projection of a crystal from *D. gigas* aldehyde oxidoreductase.** Taken at a crystal-to-film distance of 100 mm, at 4 °C.

## Preliminary crystallographic studies

According to the symmetry of the reciprocal lattice (Fig. 3) and systematic extinctions along 00 l, these crystals belong to the hexagonal space group $P6_122$ or to its enantiomorph $P6_522$. The unit cell constants of the native crystals are $a = b = 14.45$ nm and $c = 16.32$ nm, $\alpha = \beta = 90°$, $\gamma = 120°$.

Diffraction data, collected with an X-ray-imaging plate system, were processed using the MOSFLM software system [25], and programs ROTAVATA/AGRAVATA from the CCP4 package. Reflections were merged and loaded with PROTEIN [23] . From 66708 measured reflections, 19688 were unique, with an $R_{merge}$ for individual measurements of 0.094,

and between independently averaged Friedel pairs of 0.031. Two separately measured native crystals scaled with $R$ of 0.07. The completeness of the data was 90% (2−0.3 nm; 20−3 Å) and 94% (0.32−0.31 nm; 3.2−3.1 Å).

Self-rotation function calculations showed no significant peak apart from those corresponding to crystallographic symmetry and independent of the resolution ranges chosen. The size of the unit cell and the symmetry correspond to a packing density of $2.5\times10^{-3}$ nm³/Da (2.5 Å³/Da) for 12 molecules of approximately 100 kDa (or for 6 dimers of ≈200 kDa sitting on a crystallographic twofold axis). This corresponds to a solvent content of 50%. To confirm these data, the crystal packing density was measured by the Ficoll-gradient method [24]. The experimental crystal density $\rho_{exp}$ of 1.14 g/cm³ is in rough agreement with the calculated value $\rho_{calc}$ of 1.18 g/cm³ corresponding to a crystal volume/unit molecular mass of $2.5\times10^{-3}$ nm³/Da (2.5 Å³/Da). These data are compatible with the presence of two identical subunits of about 100 kDa related by a crystallographic diad axis.

The structure analysis by crystallographic methods is in progress and some heavy-atom derivatives have been found and analysed. Preliminary phase information from multiple-isomorphous replacement data has allowed us to calculate an electron-density map, which confirms the packing of the protein as a dimer of about $2\times100$ kDa with internal crystallographic symmetry.

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