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Electron Microscopic Analysis and Biochemical Characterization of a Novel Methanol Dehydrogenase from the Thermotolerant *Bacillus* sp. C1*

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Methanol dehydrogenase from the thermotolerant *Bacillus* sp. C1 was studied by electron microscopy and image processing. Two main projections can be distinguished: one exhibits 5-fold symmetry and has a diameter of 15 nm, the other is rectangular with sides of 15 and 9 nm. Subsequent image processing showed that the 5-fold view possesses mirror symmetry. The rectangular views can be divided into two separate classes, one of which has 2-fold rotational symmetry. It is concluded that methanol dehydrogenase is a dcameric molecule, and a tentative model is presented. The estimated molecular weight is 430,000, based on a subunit molecular weight of 43,000. The enzyme contains one zinc and one to two magnesium ions per subunit. N-terminal amino acid sequence analysis revealed substantial similarity with alcohol dehydrogenases from *Saccharomyces cerevisiae*, *Zymomonas mobilis*, *Clostridium acetobutylicum*, and *Escherichia coli*, which contain iron or zinc but no magnesium. In view of the aberrant structural and kinetic properties, it is proposed to distinguish the enzyme from common alcohol dehydrogenases (EC 1.1.1.1) by using the name NAD-dependent methanol dehydrogenase.

All thermotolerant methanol-utilizing *Bacillus* spp. studied to date oxidize methanol by means of a novel NAD-dependent methanol dehydrogenase (MDH)¹ (1, 2). The enzyme oxidizes primary C₁–C₄ alcohols and 1,3-propanediol and also catalyzes the NADH-dependent reduction of the corresponding aldehydes. Oxidation of alcohol substrates is strongly stimulated by a $M_r = 50,000$ activator protein. Purification of the activator protein and its effects on the kinetics of alcohol oxidation are described in the accompanying paper (3).

NAD-dependent alcohol dehydrogenases (ADH, EC 1.1.1.1) have been characterized from many sources (4). In general,

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¹ The abbreviations used are: MDH, NAD-dependent methanol dehydrogenase; ADH, alcohol dehydrogenase.

the enzymes possess a low affinity, if any, for methanol and are not involved in methanol metabolism (4, 5). Alcohol dehydrogenases have been the subject of many investigations, among others for determinations of phylogenetic relationships (6, 7). The currently described alcohol dehydrogenases can be divided in three classes, depending on their amino acid sequences, subunit chain lengths, quaternary protein structures, and metal contents (6). Representatives of the first class are horse liver ADH and bakers' yeast ADH. These enzymes are composed of long chain subunits ($M_r = 40,000$ approximately) and contain zinc at the active site. Alcohol dehydrogenases of a second class, exemplified by *Drosophila* ADH, are composed of short chain subunits ($M_r = 25,000$ approximately) and lack a metal ion at the active site. These enzymes reveal no clear sequence similarity with enzymes of the first class (8). A third class of ADH is represented by *Zymomonas mobilis* ADH2, *Saccharomyces cerevisiae* ADH4, butanol dehydrogenase from *Clostridium acetobutylicum*, and propanediol dehydrogenase from *Escherichia coli*, which may either contain iron (ADH2) or zinc (ADH4; 9–13). Each of these enzymes shares 35 to 54% of amino acid sequence identity with any of the other enzymes within this class, but does not exhibit significant similarity with primary structures of other alcohol dehydrogenases (11, 12).

Alcohol dehydrogenases possess either dimeric or tetrameric quaternary protein structures (6). In a previous paper (2) we reported the purification and initial characterization of MDH from *Bacillus* sp. C1. This enzyme was found to be a homopolymer of $M_r = 43,000$ subunits, but possessed a native M_r of approximately 280,000 as indicated by gel filtration studies (2, 3). This strongly suggested that MDH possesses an unusual subunit structure.

Here we present the quaternary structure of MDH as analyzed by electron microscopy and image processing. Electron microscopy of protein molecules can reveal details of their morphology, and by alignment and averaging of similar molecular projections the signal to noise ratio can be improved considerably. To distinguish between slightly different projections, the recently developed techniques of correspondence analysis (14) followed by multivariate statistical classification (15, 16) are very useful.

In addition, the N-terminal amino acid sequence of MDH as well as its metal composition are compared with those of other alcohol dehydrogenases.

EXPERIMENTAL PROCEDURES

Purification—MDH was purified from *Bacillus* sp. C1 cells grown in a methanol limited chemostat culture at $D = 0.1 \text{ h}^{-1}$ according to the procedures described previously (2).

Protein Determination—Protein was determined by the method of Bradford (17) using the Bio-Rad protein assay kit with bovine serum albumin as standard.

Electron Microscopy—Specimens for electron microscopy were made by applying freshly thawed MDH, in a concentration of 100 $\mu\text{g}/\text{ml}$, on grids covered with a carbon-coated formvar film which had been treated by a glow discharge in pentylamine immediately before use. The specimens were blotted with filter paper and negatively stained with 2% (w/v) sodium silicotungstate (pH 7.3).

Electron microscopy was performed on a Philips CM12 operating at 80 kV. A low dose system was used for focussing on an area adjacent to the area to be imaged, in order to avoid unnecessary irradiation. Images were recorded on Agfa Scientia 23D56 film at an electron optical magnification of 60,000.

Image Processing—Electron micrographs checked by optical diffraction for proper focussing and lack of drift and astigmatism were digitized on a Joyce-Loebl Scandig 3 rotating drum densitometer using a step size of 25 μm , corresponding to 0.4 nm at the specimen level.

Image analysis was carried out on a Convex C1-XP mini-supercomputer using the Imagic software system (18). Interactively selected images were band-pass-filtered to suppress the very high and very low spatial frequencies (which do not contribute significantly to the image but influence the alignment), surrounded by a circular mask and normalized to an average of zero and a standard variance. The normalized images were aligned rotationally and translationally relative to a reference image. As initial reference, a well preserved particle showing a characteristic view was chosen; after several alignments, 20 images having the highest correspondence with this reference were averaged and used as a second reference. The images were aligned again, and finally 100 images or more showing the highest correspondence were averaged. Correspondence analysis (14) and multivariate statistical classification (15, 16) were used to distinguish different classes within the data set. Finally, all images belonging to the same class were averaged.

Computer Model Building—Model building was done on a Commodore Amiga 2000 using the Sculpt-Animate-3D program (Byte by Byte Corp., Austin, TX).

Enzyme Assays and Inhibitor Studies—MDH activity, formaldehyde reductase activity (MDH activity in the reverse direction), and stimulating activity of MDH-activator protein were measured using the spectrophotometric assays described in the accompanying paper (3). The effects of potential inhibitors were tested against formaldehyde reductase activity of MDH. Before use, the purified enzyme preparation was desalted against 50 mM potassium phosphate buffer (pH 7.5) by passage through a Sephadex G-25 gel filtration column (Bio-Rad PD10). The inhibitors, at final concentrations of 1 mM or 5 mM, were preincubated with the enzyme for 5 min before addition of NADH. Reactions were started with formaldehyde.

Molecular Weight Determination—The molecular weight of MDH was determined by the ultracentrifugal sedimentation equilibrium method (19) using a MSE Centriscan 75 ultracentrifuge, equipped with an ultraviolet/visible monochromator. Equilibrium was established at $3,300 \times g$ (20 °C). The sample cell, containing MDH dissolved in buffer A (0.4 mg of protein/ml), and reference cell were scanned at 280 nm after 80, 176, and 248 h.

Sedimentation velocities of purified MDH were measured at 20 °C and at 40 °C at a rotor speed of 45,000 rpm ($148,000 \times g$) using the ultracentrifuge and other conditions as described above. The sample and reference cells were scanned at 280 nm at time intervals of 6 min.

Metal Analyses—The metal composition of purified MDH was determined by atomic absorption spectrophotometry using a Perkin Elmer Zeeman/3030 atomic absorption spectrophotometer. The enzyme was dialyzed extensively against 10 mM Tris-HCl buffer (pH 7.0) (buffer B) containing 1 mM EDTA and subsequently against buffer B only. The final protein concentration was 190 $\mu\text{g}/\text{ml}$. The following elements were analyzed in duplicate: zinc, magnesium, iron, and copper.

Amino Acid Analysis—Purified methanol dehydrogenase (0.4 mg) was dialyzed thoroughly against buffer B and hydrolyzed in 6 N HCl containing 0.1% thioglycolic acid for 20 h at 108 °C *in vacuo*. Half-cystine was determined as cysteic acid after performic acid oxidation and hydrolysis. Proline was analyzed with an LKB Alpha Plus amino acid analyzer using ninhydrin detection. All other amino acids were analyzed in duplicate with a Biotronik amino acid analyzer LC5001, using fluorescence detection of the *o*-phthalaldehyde derivatives.

N-terminal Sequence Analysis—The N-terminal amino acid se-

quence of purified MDH was analyzed by automated Edman degradation (20). Analysis of about 2 nmol of purified MDH was carried out on a 477 A pulse-liquid Sequenator (Applied Biosystems). The phenylthiohydantoin amino acid derivatives were separated and identified by an on-line phenylthiohydantoin analyzer model 120 A with a PTH-C₁₈ column (Applied Biosystems) as described by the manufacturer.

Materials—All chemicals were reagent grade. Protein dye reagent and bovine serum albumin were obtained from Bio-Rad.

RESULTS

Electron Microscopy—Fig. 1 shows an electron micrograph of MDH. Two views can easily be distinguished: a 5-fold symmetrical structure with a diameter of 15 nm and a rectangular view with a length of 15 nm and a width of 9 nm. Because the diameter of the disc is equal to the length of the rectangle, it is likely that they represent two different views of a cylindrical molecule with a diameter of 15 nm and a height of 9 nm. We shall refer to the pentagonal profile as the top view and to the rectangular view as the side view, respectively. The 5-fold symmetry of the top view indicates that the number of subunits per molecule is a multiple of 5, probably either 5 or 10. Many side views appear to be divided in four by two lines of stain, one parallel to the 15 nm side and the other perpendicular to it. This suggests the presence of two layers of subunits.

When the protein was kept at room temperature or at 4 °C, it dissociated into smaller fragments, probably monomers or dimers. No dissociation occurred when the isolated protein was stored frozen. For this reason, the frozen protein stock was not thawed and diluted until immediately before use.

Image Processing—Because top views and side views were easily distinguishable, they were processed separately.

For analysis of the top views, 250 images were selected from several micrographs. Because correspondence analysis did not show any significant differences between the images, the whole set was averaged. The average top view is shown in Fig. 1. The five protein masses constituting the molecule each contain a mirror line. The mirror symmetry of the building blocks indicates that they consist of two equal parts in up and down orientations, placed next to or above each other.

Among the side views, the heterogeneity was much larger than among the top views, and, for this reason, a much larger number of images were selected, namely 1376. For classification, 15% of the images were rejected and the remaining 85% divided into 8 classes. The class averages are shown in Fig. 2. Apart from some "bad" classes, consisting of molecules which were badly aligned or damaged or in a less frequently occurring

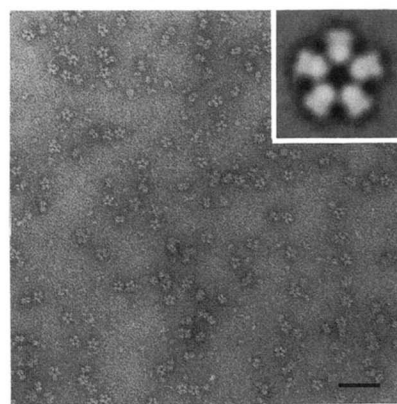


FIG. 1. Electron micrograph of MDH negatively stained with 2% sodium silicotungstate. Pentagonal and rectangular profiles can be distinguished. The bar represents 50 nm. Inset, average structure of 250 aligned pentagonal views.

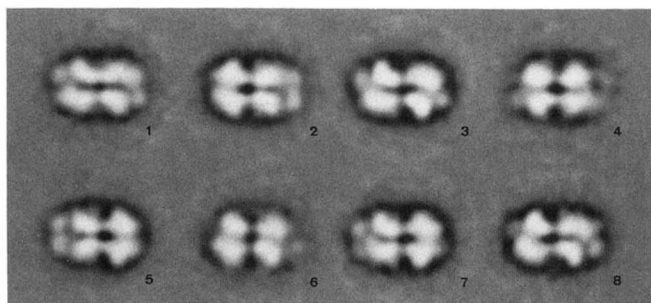


FIG. 2. Class averages of the side view of MDH. After alignment of 1376 side view images and correspondence analysis, the images were classified by multivariate statistical classification. 15% of the images were rejected, and the remaining 85% were divided into 8 classes. All images belonging to the same class were averaged. The classes contain 117, 195, 159, 98, 194, 123, 109, and 106 images, respectively.

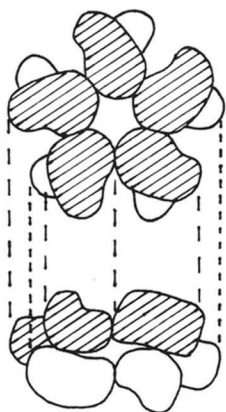


FIG. 3. Interpretation of the top view and side view of MDH. The top layer of the molecule is shown shaded. Broken lines indicate the boundaries of the subunits in both views.

orientation, two distinct projections can be distinguished, both of which show six protein masses. The first type is represented by the classes 3 and 8 in Fig. 2, and the second by the classes 2, 5 and 7. The second type occurs in two orientations differing in a 180° rotation, due to the fact that a symmetrical image of the first type was used as the reference, which makes either orientation equally likely.

The first type has 2-fold rotational symmetry. This means that the top and bottom halves of the molecule are equal, which would not be possible if the number of (asymmetrical) subunits is odd. Therefore, it can be concluded that the MDH molecule is a decamer in which the 10 subunits are organized in two rings of 5, stacked face to face.

We found three projections of the MDH molecule: the top view and two distinct side views. In the side view position, subunits are projected from several different angles, which further increases the number of independent views of a subunit. This made it possible to draw more conclusions about the three-dimensional structure of the MDH molecule. The 2-fold rotational symmetry of the first side view (classes 3 and 8 in Fig. 2) shows that the molecule is standing on two dimers in this projection. From the dimensions of the different views of the subunits in the side view, deductions can be made on the contributions of both monomers to the dimer projection seen in the top view, as shown in Fig. 3. Clearly, the two subunits forming a dimer point away from each other on the outer rim of the molecule. It can be seen that the molecule is resting on just two subunits in the side view.

The second side view, shown in the classes 2, 5, and 7 of Fig. 2, must also represent a stable orientation of the molecule

on the support film. An obvious stable orientation would be one in which the molecule is standing on both subunits of one dimer, instead of two subunits belonging to adjacent dimers. This would, however, give rise to a projection with 2-fold rotational symmetry. Another possibility is that the molecule rests on three rather than just two subunits. For this, a slight tilt of the molecule is needed, which would increase the width of the projection, and this is indeed observed.

Presumably, the protein is not in all cases completely embedded in stain, depending on the height of the molecule in the orientation in question. In the side view, no stain-excluding material is present in the center of the molecule, where the projection of the top dimer would be expected (compare Fig. 3). It thus appears likely that the top dimer in the side view orientation sticks out too far to be imaged by negative staining. The top view projection, however, shows mirror symmetry, indicating that in this case the molecule is completely embedded, and the resulting image is a complete projection from both the top and bottom half. The height of the molecule is 9 nm, considerably less than the diameter, which is 15 nm, and this may explain why the molecule is completely embedded in one orientation but not in the other.

A model of the MDH molecule, constructed by computer graphics, is shown in Fig. 4 in orientations corresponding to the three projections described. The construction of the model was based on the top view and the first type of side view, but it turned out to be in agreement with the second side view as well, which is a further indication that the basic ideas are correct.

Amino Acid Composition—The amino acid composition of MDH is presented in Table I. The predominant residues are Gly, Ala, Glx, Asx, and Val.

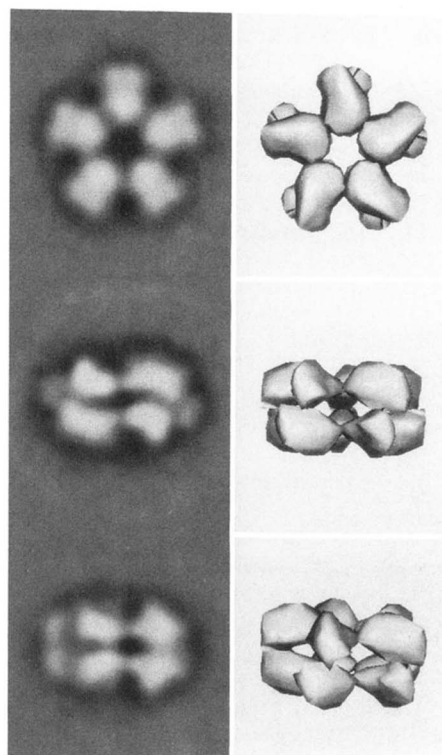


FIG. 4. The three views of the MDH molecule found by image processing and a tentative model of the MDH molecule, shown in corresponding orientations. The model is shown in a surface representation. Therefore, all subunits are visible if they are not obscured by others, independent of their distance from the front, whereas on the electron micrographs the subunits sticking out of the staining layer are not imaged.

molecular weight can be calculated from the amino acid composition and is at least 37,000, depending on the number of tryptophan residues, which agrees reasonably well with the value of 43,000 determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (2). The molecular weight of native MDH therefore is approximately 430,000. An exact determination of this value by biochemical methods was complicated by the tendency of the protein to dissociate upon storage. The molecular weight was best approximated by sedimentation equilibrium studies and corresponds reasonably well to reported molecular weights of enzymes with sedimentation coefficients similar to MDH. The markedly lower molecular weight of 280,000 as determined by gel filtration studies (2, 3) may be caused by interactions between protein and gel material. The appearance of a symmetric gel filtration peak of approximately $M_r = 50,000$ (see accompanying paper (3)), consisting of dissociated subunits, suggests that dissociation of MDH most likely does not proceed via stable intermediate pentameric or dimeric forms. Fragments larger than monomers or dimers were never observed in the electron microscopical studies.

A decameric alcohol dehydrogenase was first reported by Hou *et al.* (23). This enzyme, an NAD-linked 1,2-propanediol dehydrogenase present in propane-grown *Pseudomonas fluorescens* cells, consisted of 10 identical subunits of $M_r = 77,600$. The authors did not rule out the possibility that the observed decameric structure might have resulted from oligomerization during enzyme purification. Gel filtration studies with purified *Bacillus* sp. C1 MDH and freshly prepared extracts revealed identical native molecular weights, indicating that the MDH subunit structure remained unaffected during purification and that decameric alcohol dehydrogenases indeed do exist in nature (3). Although 1,2-propanediol dehydrogenase and MDH may possess similar quaternary protein structures, several differences suggest that the two proteins belong to two different classes of alcohol dehydrogenases. The *P. fluorescens* enzyme possesses a considerably larger subunit molecular weight, the protein does not contain metal ions, and the enzyme does not oxidize primary and secondary alcohols.

The metal composition of MDH seems unusual, as there are no other reports of zinc-containing alcohol dehydrogenases which also possess magnesium ions. In analogy with most zinc-containing alcohol dehydrogenases, MDH activity was inhibited by 1,10-phenanthroline, which is probably due to chelation of zinc from the active site (4). The presence of one zinc ion per MDH subunit suggests that each subunit is catalytically active. The thiol reagent iodoacetate is known to inactivate the bakers' yeast and horse liver alcohol dehydrogenases by alkylation of cysteine residues that serve as a ligand of the active site zinc (24). MDH was not significantly inhibited by thiol reagents, which indicates that cysteine residues (Table I) are not accessible for the inhibitors or may not be essential for either activity or maintenance of a proper enzyme structure. The precise function of magnesium in MDH remains to be elucidated. Extensive dialysis of MDH against 1 mM EDTA-containing buffer did not result in loss of magnesium, which suggests that the metal is tightly bound to the protein. Magnesium may be involved in the stabilization of the native protein structure (25). Alternatively, magnesium may play a role in the interaction between MDH and activator protein. Stimulation of MDH activity strictly required the presence of magnesium ions (3).

Many bacterial NAD(P)-dependent alcohol dehydrogenases have been reported to require or contain zinc, but not many of these enzymes have been sequenced. Complete amino acid sequences have been obtained for only *Alcaligenes eutrophus*

ADH (26), ADH type 1 from *Z. mobilis* (27), and the secondary ADH from *T. Brockii* (28). In addition, N-terminal sequences have been obtained for *B. stearothermophilus* ADH (29), and the benzyl alcohol dehydrogenases of *Acinetobacter calcoaceticus* and *Pseudomonas putida* (30). All six enzymes, including two alcohol dehydrogenases from thermophilic organisms, have been found to show significant homologies with the class of long chain, zinc-containing ADH enzymes exemplified by horse liver ADH and yeast ADH. Although in this class of enzymes fewer than 10% of all amino acid residues are strictly conserved, several amino acid residues were identified to be important for the structure and function of the enzyme (4).

It is clear that the zinc-containing MDH of *Bacillus* sp. C1 does not belong to this class of proteins. A strictly conserved cysteine 46, considered to be an essential active site zinc ligand, and a conserved asparagine 49, which binds to the zinc-liganding histidine 67 (6), are not present in the N-terminal sequence of MDH from *Bacillus* sp. C1. Furthermore, no sequence similarity was found with zinc-lacking short chain alcohol dehydrogenases from *Drosophila*, which form a separate class of alcohol dehydrogenases (31). In contrast, considerable similarity was found with the sequences of a new class of alcohol dehydrogenases, composed of *Z. mobilis* ADH2, *S. cerevisiae* ADH4, propanediol dehydrogenase from *E. coli*, and butanol dehydrogenase from *C. acetobutylicum* (11, 12). This class of enzymes thus comprises an iron-containing tetrameric alcohol dehydrogenase (ADH2; Ref. 3), a zinc-containing dimeric alcohol dehydrogenase (ADH4; Ref. 13), and a zinc- plus magnesium-containing decameric alcohol dehydrogenase (*Bacillus* sp. C1 MDH). Data on metal composition and subunit structure therefore do not provide a firm basis for the classification of alcohol dehydrogenases.

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