The Three-dimensional Structure of Porcine Heart Mitochondrial Malate Dehydrogenase at 3.0-Å Resolution*

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The oxidation of L-malate to oxalacetate in nearly all eukaryotic organisms is catalyzed by at least two distinct NAD-dependent dehydrogenases termed cytoplasmic and mitochondrial malate dehydrogenase. Although both enzymes are nuclear gene products, the mitochondrial isoenzyme is synthesized with a 24-residue amino-terminal transit peptide which is proteolytically cleaved and removed upon translocation of the enzyme to the mitochondrial matrix (Mihara et al., 1982; Grant et al., 1983). Mitochondrial malate dehydrogenase functions in the tricarboxylic acid cycle. Both isoenzymes are components of a malate-aspartate shuttle by which reducing equivalents, in the form of malate, are shuttled across the inner mitochondrial membrane.

The three-dimensional structures of the NAD-dependent dehydrogenases lactate dehydrogenase (Adams et al., 1970), cytoplasmic malate dehydrogenase (Hill et al., 1972), glycerol-3-phosphate dehydrogenase (Buehner et al., 1974), and alcohol dehydrogenase (Eklund et al., 1976) have been determined by x-ray diffraction methods. These studies have revealed similarities in the polypeptide chain fold of an NAD-binding domain (Hill et al., 1972; Rossmann et al., 1975). A catalytic domain, however, is similar only for the cytoplasmic malate and lactate dehydrogenases which catalyze the oxidation of 2-hydroxyacid substrates.

The subunits of tetrameric lactate dehydrogenase are related by three mutually perpendicular and intersecting 2-fold rotation axes (Adams et al., 1970). One of these, termed the Q axis, is common to both lactate and cytoplasmic malate dehydrogenase (Hill et al., 1972).

An amino acid sequence alignment of various lactate dehydrogenases, porcine heart mitochondrial malate dehydrogenase, and an "x-ray" sequence of porcine heart cytoplasmic malate dehydrogenase indicated that although the malate and lactate dehydrogenases are only about 20-25% homologous, a number of residues are absolutely conserved (Birktoft et al., 1982a). Among these are a histidine and aspartic acid residue thought to comprise a proton relay system from the 2-hydroxyl oxygen of the reduced substrate to the histidine imidazole ring and an arginine residue which may serve as a counterion to the 1-carboxylate group of the substrate (Blow et al., 1969; Parker and Holbrook, 1977; Bernstein and Everse, 1978; Birktoft et al., 1982b). The conservation of these residues is consistent with the more recently determined amino acid sequences of porcine heart cytoplasm malate dehydrogenase* (Birktoft et al., 1982a) and rat liver mitochondrial malate dehydrogenase.

Partial peptide and nucleotide sequences for malate dehydrogenase from Escherichia coli corresponding to the first 40 residues have also been reported (Fernley et al., 1981; Sutherland and McAlister-Henn, 1986). These results reveal a greater degree of sequence homology between the malate dehydrogenases from E. coli and porcine heart mitochondria than between the porcine heart mitochondrial and cytoplasmic isoenzymes.

In a previous report, we described the similarity of an α-carbon model of cytoplasmic malate dehydrogenase to an electron density map of porcine heart mitochondrial malate dehydrogenase (Roderick and Banaszak, 1983). The interpretation of electron density maps at higher resolution presented in this report has resulted in an α-carbon model of mitochondrial malate dehydrogenase and a more precise representation of the coenzyme binding site. These results confirm the structural similarity of mitochondrial malate dehydrogenase to its cytoplasmic isoenzyme and to lactate dehydrogenase.

MATERIALS AND METHODS

Preparation of Crystals—The purification of porcine heart mitochondrial malate dehydrogenase has been described elsewhere (Weininger and Banaszak, 1978). The enzyme used in this study is the mature form which does not contain the amino-terminal transit peptide responsible for translocation of the enzyme to the mitochondrial matrix. Crystals were grown from polyethylene glycol 6000

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1 R. A. Bradshaw, personal communication.

2 P. Grant, S. L. Roderick, L. J. Banaszak, and A. W. Strauss, manuscript in preparation.
solutions buffered at pH 5.8 with citrate and belong to the monoclinic space group P2₁ with a = 72.8, b = 146.9, c = 67.6 Å, and β = 108.4°.

Fourier density maps were computed as in the preliminary study (Roderick and Banaszak, 1983) with the exception of those crystals soaked in the heavy atom compound ethylmercurithiosalicylic acid. The concentration of this reagent was lowered from 5 to 1 mM in order to reduce the radiation sensitivity of the enzyme crystals. Unfortunately, these conditions also produced lower heavy atom binding site occupancy. Because of this, an additional heavy atom derivative was prepared by soaking native crystals in 1 mM cis-Pt(NH₃)₂Cl₂ for 5 days at 4 °C.

Data Measurement and Processing—Diffraction data was measured with an Enraf-Nonius CAD4 diffractometer using a ω-2θ stepscan with exposure times of about 35 s. Five crystals each of the apoenzyme, holoenzyme, and each heavy atom derivative were used to measure shells of x-ray diffraction to 3.0-Å resolution. Native diffraction data to 2.4-Å resolution was measured with six additional crystals.

The data was processed with a modified version of an x-ray diffraction data processing program package which employs Gaussian curve fitting to x-ray reflection profiles (Hanson et al., 1979). Modifications included an estimated background value derived from background measurements for neighboring reflections. A scale constant and temperature factors were used to adjust the shells of native diffraction data to a subset of high intensity data in order to produce a single native data set. The discrepancy between native and derivative data sets was reduced by applying a local scale factor based on the Miller indices of reflections, their resolution, or radiation exposure time.

Phasing of Electron Density Maps—The heavy atom sites of the low resolution study were used to compute the phases of a difference Fourier map of the newly incorporated cis-Pt(NH₃)₂Cl₂ derivative. Eleven additional heavy atom sites were located in this map. One constellation of four sites was present at chemically equivalent locations in each of the four subunits. The three remaining chemically distinct sites were present in only two or three subunits. Least squares refinement of all heavy atom coordinates and occupancies at 3.0-Å resolution resulted in a mean figure-of-merit of 0.52 (Bowie and Crick, 1975).

The electron density map computed with these phases was subjected to a density modification procedure as previously described (Bricogne, 1976; Roderick and Banaszak, 1983). The improved phases produced in this way were transferred directly to either the measured native amplitudes to produce a protein electron density map or to the difference between the measured data from holoenzyme and apoenzyme crystals to produce a difference Fourier map of the coenzyme.

These electron density maps were then averaged over the four symmetry-related positions of the tetramer and the unique electron density corresponding to either a single averaged subunit or coenzyme density map computed at 3.0-Å resolution. The progress of the model building was frequently checked by reference to the side chain type identified from the chemical sequence and the alignment of the chemical sequence within the pattern of secondary structure predicted by sequence homology studies of the malate and lactate dehydrogenases. The model is also consistent with the location of three ethylmercurithiosalicylic acid-binding sites near the sulphydryl groups of cysteine residues 69, 188, and 261. A fourth site is very close to the electron density corresponding to the sulphydryl groups of cysteine 104 and 108.

RESULTS AND DISCUSSION

The 314 residues corresponding to a single subunit of mitochondrial malate dehydrogenase were placed in an electron density map computed at 3.0-Å resolution. The progress of the model building was frequently checked by reference to the side chain type identified from the chemical sequence and the alignment of the chemical sequence within the pattern of secondary structure predicted by sequence homology studies of the malate and lactate dehydrogenases (Birktoft et al., 1982a). The model is also consistent with the location of three ethylmercurithiosalicylic acid-binding sites near the sulphydryl groups of cysteine residues 69, 188, and 261. A fourth site is very close to the electron density corresponding to the sulphydryl groups of cysteine 104 and 108.

The α-carbon coordinates of the crystalline tetramer may be generated from the subunit model by rotation of one subunit about the three intersecting molecular 2-fold axes to generate a tetramer with 222-point symmetry. However, because porcine heart mitochondrial malate dehydrogenase is a dimeric enzyme, only one of the three 2-fold axes relating subunits of the crystalline tetramer exists in solution and presumably in vivo.

The α-carbon model of the crystalline tetramer and the three 2-fold rotation axes relating the four subunits are presented in Fig. 1. The orientation of the figure emphasizes the sparse contacts between the “upper” and “lower” dimers. The contacts between these dimers are formed only by a loop region present in each of the four subunits.

The 2-fold Q axis oriented vertically in Fig. 1 corresponds to the 2-fold axis which we believe exists in vivo. We believe the two 2-fold axes P’ and R’, which generate dimers with sparse intersubunit contacts, are formed only in the crystalline state. Although the orientation of these 2-fold axes is similar to the P and R axes of lactate dehydrogenase, they intersect the Q axis at a different point. The result is a “head-to-head” packing arrangement of mitochondrial malate dehydrogenase dimers in these crystals and a “tail-to-tail” packing of the Q axis dimers of tetrameric lactate dehydrogenase (Rossmann et al., 1975; Roderick and Banaszak, 1983).

Fig. 2 is a stereodiagram of a single dimer which has been oriented with the vertical 2-fold axis of Fig. 1 nearly perpendicular to the plane of the page. The position and orientation of the coenzyme NAD in the extended conformation identified from the difference Fourier map is also included.

The overall conformation of the enzyme is very similar to cytoplasmic malate dehydrogenase and lactate dehydrogenase (Hill et al., 1972; Adams et al., 1970). The coenzyme binds to the carboxyl-terminal edge of a six-stranded parallel β-sheet domain within an amino-terminal coenzyme-binding domain. The extended conformation of NAD as it is bound to mitochondrial malate dehydrogenase is very similar to that of cytoplasmic malate dehydrogenase and lactate dehydrogenase (Birktoft et al., 1982b).

The interface between the subunits is formed by three helices and their counterparts related by the 2-fold Q axis. These helices have been denoted αB, αC, and α3G in one nomenclature (Adams et al., 1970; Hill et al., 1972) and correspond to the residue ranges 11–22, 38–47, and 220–245.
Structure of Mitochondrial Malate Dehydrogenase

FIG. 1. Stereodiagram of the α-carbon model of crystalline mitochondrial malate dehydrogenase. The diagram depicts the crystalline tetramer of porcine heart mitochondrial malate dehydrogenase and the three mutually perpendicular 2-fold rotation axes. The orientation of the diagram emphasizes the sparse contacts between the "upper" and "lower" dimers which are formed by the association of loop regions corresponding to residues 82–92 present in each of the four subunits. These loop regions are displayed with bold lines.

FIG. 2. Stereodiagram of the α-carbon model of dimeric mitochondrial malate dehydrogenase viewed parallel to the 2-fold Q axis. The orientation of the 2-fold axis Q depicted in Fig. 1 is nearly perpendicular to the plane of the page in this figure. The residue numbering for the subunit depicted with bold lines is given as well as the location of the coenzyme molecules bound to both subunits.

in the mitochondrial malate dehydrogenase sequence. This interface is very similar to those described for the three-dimensional structures of cytoplasmic malate and lactate dehydrogenase (Rossmann et al., 1975).

The loop of polypeptide chain corresponding to residues 82–89 is equivalent to the so-called "NAD-binding loop" thought to undergo a conformational change in lactate dehydrogenase upon binding the coenzyme (Holbrook et al., 1975). This is the same loop which is involved in the loose contacts between dimers of mitochondrial malate dehydrogenase in the crystalline state as illustrated in Fig. 1. A conformational change of this coenzyme-binding loop may explain the disordering and occasional cracking of mitochondrial malate dehydrogenase crystals into which NADH has been diffused. However, NAD does not cause any such disruption of the crystalline lattice. This is consistent with an NAD-binding conformation of the enzyme in these crystals and also the proposal that citrate, present in the crystallization solution, binds preferentially to this conformation (Mullinax et al., 1982).

Various lines of chemical and structural evidence support the view that the active site of the malate and lactate dehydrogenases contains a histidine and aspartic acid residue which forms a relay system responsible for proton transfer from the reduced substrate 2-hydroxyl group to the histidine imidazole ring and then to solvent (e.g. Parker and Holbrook, 1977; Bernstein and Everse, 1978; Birktoft and Banaszak, 1983). These residues have been identified as His-176 and Asp-149 in the porcine heart mitochondrial malate dehydrogenase sequence. In addition, an arginine residue is thought to serve as a counterion to the 1-carboxylate group of the substrate in the active site of cytoplasmic malate dehydrogenase. The homologous residue in the mitochondrial isoenzyme sequence has been identified as Arg-152.

These three residues have been located in the structure of mitochondrial malate dehydrogenase and adopt a very similar orientation to that reported for the cytoplasmic malate and lactate dehydrogenases (Rossmann et al., 1975). Reference to Fig. 2 indicates that His-176 is placed at a turn between two anti-parallel β-strands called βG and βH. Asp-149 and Arg-152 are located on one side of the α-helix α2F corresponding to residues 148–160. The guanidinium group of Arg-152 prob-
ably serves as a counterion for a buffer anion bound at the active site substrate location for which significant electron density is observed in the electron density map. The side chain density corresponding to Asp-149 also extends toward the amino-terminal end of a2F and the active site.

In conclusion, the overall conformation of porcine heart mitochondrial malate dehydrogenase, the interpretation of its quaternary structure, the location of the NAD-binding site, and the position of several catalytically important residues are similar to cytoplasmic malate and lactate dehydrogenase. The more detailed comparison of mitochondrial and cytoplasmic malate dehydrogenase will be possible in the immediate future when the analysis of side chain positions of mitochondrial malate dehydrogenase has been completed. The correlation of this structural evidence with various lines of chemical evidence will provide for an improved understanding of the catalytic mechanism and evolution of NAD-dependent dehydrogenases.

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