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CRYSTALLIZATION NOTE

Preliminary Crystallographic Studies of Human Mitochondrial NAD(P)$^+$-Dependent Malic Enzyme

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Human mitochondrial NAD(P)$^+$-dependent malic enzyme was overexpressed in *Escherichia coli* and purified by anion-exchange, ATP affinity, and gel filtration chromatography. The protein was crystallized with the hanging-drop vapor diffusion method. Many different crystal forms were observed, five of which were characterized in some detail. A 2.5-Å multiple-wavelength anomalous diffraction data set and a 2.1-Å native data set were collected using synchrotron radiation on crystals containing selenomethionyl residues. These crystals belong to space group *B*2, with *a* = 204.4 Å, *b* = 107.0 Å, *c* = 59.2 Å, and γ = 101.9°. Self-rotation functions demonstrated that the tetramer of this enzyme obeys 222 symmetry. © 1999 Academic Press

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Malic enzyme (ME) catalyzes the oxidative decarboxylation of malate to pyruvate, together with the reduction of the cofactor NAD^+ or $NADP^+$ (Hsu, 1982; Loeber *et al.*, 1991; Moreadith and Lehninger, 1984b). A divalent cation (Mg^{2+} or Mn^{2+}) is also required for the activity. The enzyme has been found in most living organisms and has highly conserved amino acid sequences, suggesting that ME may have important biological functions. The enzymes are generally homo-tetrameric, with monomers having about 550 amino acid residues (60 kDa).

The human mitochondrial NAD(P)$^+$-dependent ME (m-NAD-ME) is found mostly in rapidly dividing cells and particularly tumors (Loeber *et al.*, 1991). The enzyme is believed to play an important role in the metabolism of glutamine, which is a major energy source for many tumor cells (Baggetto, 1992; McKeehan, 1982; Moreadith and Lehninger, 1984a). In contrast to the other malic enzymes, m-NAD-ME is a cooperative enzyme, and its activity is allosterically controlled with fumarate as an activator and ATP as an inhibitor (Loeber *et al.*, 1991; Moreadith and Lehninger, 1984b; Sauer, 1973). The enzyme prefers NAD^+ as the cofactor at physiological pH, but can also use $NADP^+$ as the cofactor, especially at lower pH.

With the exception of two copies of the ADP-binding βαβ signature motifs (Wierenga *et al.*, 1986) (GXGXXG/A; 168-GLGDLG-173 and 311-GAGEAA-316 in m-NAD-ME), malic enzymes show no recognizable amino acid sequence homology to other oxidative decarboxylases, dehydrogenases, and other proteins in general. Crystallization of the malic enzyme from rat liver (Baker *et al.*, 1987) and from the parasitic nematode *Ascaris suum* (Clancy *et al.*, 1992) has been reported. However, no structural information is currently available on this important class of enzymes.

Human m-NAD-ME was overexpressed in *Escherichia coli* BL21 cells using an expression vector (pRH281) under the control of the inducible trp promoter system (Loeber *et al.*, 1991). The mitochondrial leader sequence (residues 2–18) was not included in the expression construct as it may interfere with the activity of the enzyme (Loeber *et al.*, 1991). The identity of the construct was verified by DNA sequencing. The bacterial cells were grown at 37°C until an OD_{600} of 0.4 was reached. m-NAD-ME expression was induced by the addition of indoleacrylic acid (Sigma) to 50 mg/liter. The cells were harvested 3 h after induction and stored at −80°C.

The purification of m-NAD-ME was carried out at 4°C and followed a protocol similar to that reported previously (Loeber *et al.*, 1991). The cell pellet was resuspended in lysis buffer, containing 30 mM Tris

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(pH 7.4), 0.2 mM DTT, 0.2% NP-40, 3 mM $MgCl_2$, 0.2 mM EDTA, and 0.5 mM PMSF. Lysozyme was added to a final concentration of 0.5 mg/ml, and the suspension was sonicated on ice 45 min later. After centrifugation, the supernatant was diluted 1:1 with buffer A (30 mM Tris (pH 7.4), 3 mM $MgCl_2$, and 0.2 mM EDTA) and loaded onto a TMAE anion-exchange column (Merck) preequilibrated in buffer A. The enzyme was eluted with a 0–300 mM KCl gradient in 6 column volumes. The fractions were assayed for m-NAD-ME activity using malate as the substrate and monitoring the increase in OD_{340} due to NADH (Loeber *et al.*, 1991), and those fractions containing the activity were pooled and brought to 1 mM $MnCl_2$ and 2 mM fumarate. The solution was loaded onto an ATP-agarose affinity column (Sigma) preequilibrated in buffer B (buffer A supplemented with 1 mM $MnCl_2$ and 2 mM fumarate). The enzyme was eluted with buffer B containing 4 mM NAD^+. Fractions containing m-NAD-ME activity were pooled and concentrated using a small (0.5–1 ml) TMAE column. The last step of purification was gel filtration chromatography with a Sephacryl S-300 column (Amersham Pharmacia). The running buffer contained 30 mM Tris (pH 7.4), 250 mM KCl, 3 mM $MgCl_2$, and 0.2 mM EDTA. The protein eluted as a tetramer from the gel filtration column, with a small tail at the dimer position. The protein sample was then concentrated to about 20 mg/ml (Centricon) and stored at −80°C. The purity of the protein sample was greater than 98% as judged by Coomassie-stained SDS–polyacrylamide gels. The purified enzyme had very high specific activity, ranging between 45 and 70 units/mg.

The selenomethionyl protein was produced in order to determine the structure with the multiple-wavelength anomalous diffraction (MAD) technique (Hendrickson, 1991). m-NAD-ME contains 14 Met residues in each monomer, not counting the initiator methionine residue. It was expressed using a methionine auxotroph grown in a defined medium (Hendrickson *et al.*, 1990) supplemented with Kao and Michayluk vitamins and purified using essentially the same protocol as for the wildtype enzyme, except for the inclusion of 5 mM DTT in all the buffers. The successful incorporation of the selenomethionyl residues was confirmed by mass spectrometry analysis. The specific activity of selenomethionyl m-NAD-ME, assayed under the same conditions as the wildtype enzyme, was comparable to that of the wildtype enzyme, suggesting that the introduction of selenomethionyl residues did not have a major impact on the structure of the enzyme.

Crystals of m-NAD-ME were obtained at 4°C by the hanging-drop vapor diffusion method. The initial condition was identified by sparse matrix screening (Jancarik and Kim, 1991) with a commercial kit (Hampton Research). Optimization of this condition led to crystal form A (Table I). The protein solution contained 8 mg/ml m-NAD-ME, 250 mM KCl, 5% glycerol, 10 mM DTT, 6 mM $MgSO_4$, 6 mM fumarate, 1 mM NAD^+, and a fourfold molar excess of tartronic acid, a substrate-analog inhibitor. The reservoir solution contained 14% PEG 8000 and 35 mM KH_2PO_4 (pH 4.5). The crystals generally had the shape of rectangular plates, the largest of which measured $0.3 \times 0.4 \times 0.1$ mm^3. These crystals belong to space group $P2_1$ (Table I), with a tetramer of m-NAD-ME in the asymmetric unit. A weak X-ray diffraction data set to 3-Å resolution was collected on a cryo-protected crystal, using an R-axis imaging plate system mounted on a rotating anode X-ray generator. Self-rotation functions, calculated with the program GLRF (Tong and Rossmann, 1990), clearly showed the presence of three mutually perpendicular noncrystallographic twofold axes (Fig. 1). This demonstrates that tetramers of m-NAD-ME obey 222 point group symmetry.

Based on the average protein partial specific volume of 0.74 cm^3/g, the volume of the m-NAD-ME tetramer was estimated to be 300 000 $Å^3$. This corresponds to the volume of a sphere with a radius of about 42 Å. Such a sphere could not fit into this crystal unit cell due to the short *a* axis. A packing

TABLE I

Crystal Forms of Human Mitochondrial $NAD(P)^+$-Dependent Malic Enzyme

Crystal form	Space group	Unit cell parameters	Asymmetric unit	V_m ($Å^3$/Da)	Resolution (Å)	R_{merge} (%)	Completeness (%)
A	$P2_1$	a = 69.6 Å, b = 106.9 Å, c = 190.6 Å, γ = 107.7°	Tetramer	2.8	3.0	7.0	83
B	$P3_121$	$a = b$ = 200 Å, c = 168 Å	—	—	4.0	8.5	91
C	$B2$	a = 204.8 Å, b = 143.8 Å, c = 57.7 Å, γ = 129.7°	Dimer	2.7	2.9	6.8	90
D	$B2$	a = 204.4 Å, b = 107.0 Å, c = 59.2 Å, γ = 101.9°	Dimer	2.6	2.1	4.3	87
E	$P1$	a = 111.3 Å, b = 119.0 Å, c = 125.9 Å, α = 116.5°, β = 94.8°, γ = 102.8°	Two tetramers	2.9	2.9	5.3	85

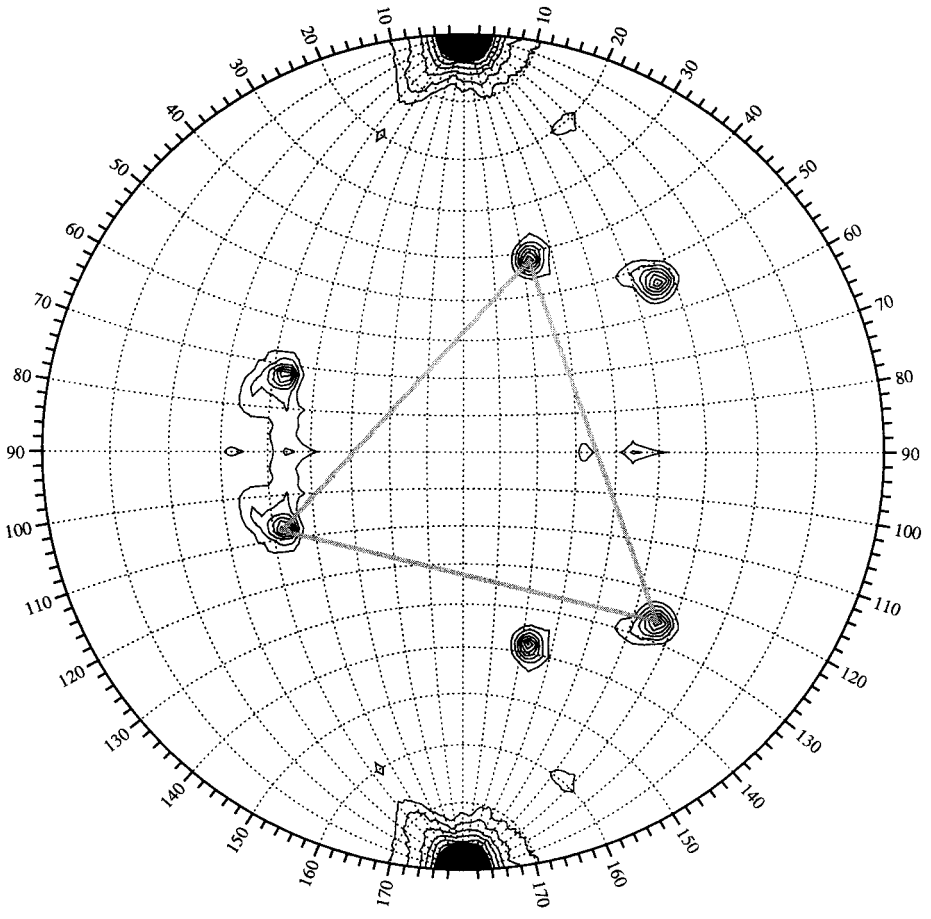

FIG. 1. Stereographic projection of the $\kappa = 180°$ section of the self-rotation function for crystal form A. Reflection data between 10- and 3.5-Å resolution were used in the calculation with the program GLRF (Tong and Rossmann, 1990). The large-term cut-off was 1.5, and the radius of integration was 35 Å. The peaks at $\psi = 0°$ and 180° (scaled to an arbitrary height of 1000) correspond to the crystallographic twofold axis. The starting contour level is at 390, and the increment is 25. The three peaks corresponding to the twofold axes of one tetramer are connected by thick gray lines. The other three peaks correspond to the twofold axes from the crystallographically related tetramer.

analysis was carried out using ellipsoidal shapes, aligning the three axes of the ellipsoid with the directions of the NCS twofold axes from the self-rotation function (Fig. 1). The lengths of the axes were varied while maintaining a volume of about 300 000 Å^3, and the packing of the ellipsoids in the unit cell was examined manually with the program Frodo (Jones, 1978). This analysis showed that an ellipsoid with dimensions of about $105 \times 105 \times 55$ Å^3 could fit into the unit cell (centered at around (1/4, 1/4, 0)). Therefore, the tetramer is likely organized in a planar rather than a tetrahedral fashion, consistent with electron microscopy observations for the pigeon liver ME (Nevaldine *et al.*, 1974). The packing analysis also suggested that the radius of gyration of the m-NAD-ME tetramer should be about 55 Å. Dynamic light scattering experiments showed that m-NAD-ME was tetrameric and mono-disperse in solution and that the radius of gyration was about 55 Å (data not shown).

Unfortunately, these $P2_1$ crystals were rather difficult to manipulate and many attempts at producing heavy atom derivatives were unsuccessful. By changing the pH of the crystallization condition and lowering the salt concentration in the protein buffer, several new crystal forms were obtained. Four of these crystal forms were characterized in some detail (Table I). Both the selenomethionyl protein and the wildtype enzyme were used for crystallization, and they generally produced similar crystals under similar crystallization conditions. For all these crystal forms, the protein solution contained 8 mg/ml m-NAD-ME, 125 mM KCl, 3 mM NAD^+ or ATP, and 10 mM DTT. For crystal forms B and C (Table I), the reservoir solution contained 100 mM Hepes (pH 7.0), 8% PEG 8000, 5% glycerol, 6 mM $MgSO_4$, and 10 mM tartronic acid. These crystals often appeared in the same drop, with crystal form B as hexagonal bipyramids and crystal form C as thin plates. The hexagonal bipyramids can grow to a substantial size, mea-

suring $1.0 \times 1.0 \times 0.6$ mm^3. The X-ray diffraction of these crystals was, however, very poor, extending to only about 4-Å resolution even with synchrotron radiation. X-ray diffraction to about 3-Å resolution was observed for crystal form C on a rotating anode generator. However, most of these crystals had smeared diffraction patterns, and individual reflections along the long axis of the unit cell oftentimes could not be resolved. A clean diffraction pattern could be obtained only after examining many crystals. From a screen that was carried out to search for a replacement of glycerol by other alcohols (ethylene glycol, isopropanol, *t*-butanol, MPD), it was discovered that inclusion of 5% MPD instead of glycerol in the reservoir solution produced crystals that gave sharp diffraction spots to about 3-Å resolution on a rotating anode generator. These crystals have the same morphology as crystal form C and belong to the same space group. However, they have different unit cell parameters (crystal form D, Table I). Crystal form E was produced when lutetium ions (Lu^{3+}) were introduced into the protein solution at 0.5 mM concentration, in an attempt to take advantage of the large anomalous signal of this atom for structure determination. This ion has roughly the same radius as Mn^{2+}, and kinetic studies showed that Lu^{3+} is a potent inhibitor of m-NAD-ME activity (data not shown). This suggests that the ion might compete for binding at the divalent cation site. For crystal forms C, D, and E, microseeding was important for obtaining the crystals reproducibly.

X-ray diffraction data sets have been collected on crystals belonging to forms B through E at Brookhaven National Laboratory (BNL) and/or the Cornell High Energy Synchrotron Source (CHESS) (Table I). Crystal forms C and D contain a dimer of m-NAD-ME in the asymmetric unit. Self-rotation functions showed two strong peaks for NCS twofold axes perpendicular to the crystallographic twofold axis (not shown). This suggested that one of the twofold axes of the tetramer is coincident with the crystallographic twofold axis, which was confirmed by a cross-rotation function between crystal forms A and C. The lengths of the crystallographic twofold axes in crystal forms C and D (about 58 Å) are also in agreement with the packing analysis described above. Crystals grown in the presence of Lu^{3+} were found to be in the *P*1 space group, with two tetramers in the unit cell, limiting their usefulness for structure determination.

A data set to 2.1-Å resolution was collected on a crystal in form D at the F-2 beamline of CHESS (Table I). A MAD data set was collected on a R-axis IV imaging plate system at the X4A beamline at BNL on a crystal containing selenomethionyl residues. The crystal diffracted to 2.0-Å resolution at the beginning of the data collection, but decayed to about 2.7-Å resolution after 60 h of data collection, even though it was maintained at cryo-temperature. After data processing with HKL (Otwinowski, 1993), a good quality MAD data set to 2.5-Å resolution was produced. The MAD structure determination is well under way, as we have already located the 28 selenium sites in the asymmetric unit.

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