Crystallographic Studies on *Ascaris suum* NAD-Malic Enzyme Bound to Reduced Cofactor and Identification of an Effector Site*

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The crystal structure of the mitochondrial NAD-malic enzyme from *Ascaris suum*, in a quaternary complex with NADH, tartronate, and magnesium has been determined to 2.0-Å resolution. The structure closely resembles the previously determined structure of the same enzyme in binary complex with NAD. However, a significant difference is observed within the coenzyme-binding pocket of the active site with the nicotinamide ring of NADH molecule rotating by 188° over the C-1-N-1 bond into the active site without causing significant movement of the other catalytic residues. The implications of this conformational change in the nicotinamide ring to the catalytic mechanism are discussed. The structure also reveals a binding pocket for the divalent metal ion in the active site and a binding site for tartronate located in a highly positively charged environment within the subunit interface that is distinct from the active site. The tartronate binding site, presumably an allosteric site for the activator fumarate, shows striking similarities and differences with the activator site of the human NAD-malic enzyme that has been reported recently. Thus, the structure provides additional insights into the catalytic as well as the allosteric mechanisms of the enzyme.

Malic enzyme (ME) is an oxidative decarboxylase that catalyzes the conversion of l-malate to pyruvate and carbon dioxide, using a divalent metal ion (Mg$^{2+}$ or Mn$^{2+}$) and NAD$^+$ or NADP$^+$ as cofactors (1–3). The enzyme is found in prokaryotes and eukaryotes and participates in diverse metabolic pathways such as photosynthesis, lipogenesis, and energy metabolism. Mitochondrial and cytosolic isoforms of the enzyme have been identified (1, 4). A sequence comparison of malic enzymes from different sources shows significant homology within the family but no homology to other proteins with the exception of the dinucleotide binding signature motif (5). Because of its functional importance, the enzyme has been isolated and characterized from several sources (3, 6). The mitochondrial NAD-malic enzyme (m-NAD-ME) from the parasitic nematode, *Ascaris suum*, plays a pivotal role in carbohydrate metabolism in parasitic worms (7). In the anaerobic metabolism of *A. suum*, malate, an intermediate in the worm’s glycolytic pathway, is transported into the mitochondria where it undergoes a dismutation and is converted to pyruvate and NADH via the malic enzyme reaction and to fumarate via the fumarase reaction. Fumarate is then converted to short, branched-chain fatty acids via succinate mediated by the NADH produced in the malic enzyme reaction. The succinate dehydrogenase reaction is also involved in a site 1 oxidative phosphorylation, the main source of mitochondrial ATP (8). Since malic enzyme generates reducing equivalents (NADH) for the conversion of fumarate to succinate, it is not surprising that fumarate regulates its own utilization by activating the malic enzyme reaction (9, 10).

The ascarid malic enzyme has been extensively studied in our laboratories from the standpoint of its kinetic, regulatory, chemical mechanisms and also physicochemical properties (3, 6). Recently, the crystal structure of the enzyme complexed with NAD has been determined to 2.3-Å resolution and compared with the structure of the human mitochondrial enzyme, also complexed with NAD (11–13). Whereas the two enzymes have similar tertiary and quaternary structures and exhibit similarities in domain structure, there are significant differences between the structures of the two enzymes. The ascarid enzyme has 30 additional residues at its amino terminus relative to the human enzyme, residues interacting with NAD differ between the two (11). The two enzymes also differ in the organization of the tetramer. The ascarid malic enzyme tetramer is more flattened compared with the human enzyme as a consequence of the difference in the positioning of the C domain in the two structures relative to the A and B domains and also a difference in the positioning of the two dimers within each tetramer. The most notable difference between the two enzymes is that the human enzyme has a second NAD-binding site (exo site), distinct from the active site, at the tetramer interface, which was originally thought to be an ATP-inhibitory site but later on was suggested to be of unknown function (13). The ascarid enzyme does not have this exo site for NAD (11) and is not inhibited by ATP at physiological concentrations.

In the previous structure of the ascarid malic enzyme (11),
the active site was located by the bound NAD and by compar-
ison with the closed and open forms of the human enzyme (12,
14). The NAD cofactor binds with the nicotinamide ring in the
anti conformation with the re face directed toward the solvent.
In the bound conformation, the nicotinamide ring closely ap-
proaches the pyrophosphate moiety, probably resulting in an
ionic interaction between N-1 of the nicotinamide ring and one
of the oxygens of the pyrophosphate backbone. In order to
determine the specific effects of reduction of the nicotinamide
ring on the coenzyme binding in the active site and the overall
catalytic mechanism, a structure of the Ascaris malic enzyme
has been solved with NADH bound at the active site. This is
the first report of the structure of a malic enzyme complexed
with the reduced coenzyme and reveals a huge movement of
the nicotinamide ring closer to the active site residues as a con-
sequence of reduction. Although tartronate was included in our
previous crystallization trials, we were not able to clearly iden-
tify bound tartronate in the electron density map of ME-NAD
crystals. However, the higher resolution data obtained in the
present study helped to locate tartronate bound to an allosteric
site that is shown to bind fumarate in the human enzyme.

MATERIALS AND METHODS

Crystalization and X-ray Data Collection—Malic enzyme was puri-
fied as described previously (11). Crystals of the ME-NAD complex
were obtained by the hanging drop vapor diffusion method from a
solution containing 100 mM Tris-SO4, pH 7.3, 100 mM sodium acetate,
15% polyethylene glycol 4000, 5 mM NADH, 10 mM tartrate, 20 mM
MgSO4, 10 mM 2-mercaptoethanol, and 0.02% sodium azide. The crys-
tals are typically 0.3 × 0.2 × 0.2 mm and are isomorphous with the
crystals of ME-NAD complex and belong to the space group P9321 with
an asymmetric unit of a = b = 139.95 Å, c = 149.13 Å, containing two
monomers. For cryoprotection, crystals were first soaked in a stabiliz-
ing solution containing 25% (v/v) polyethylene glycol 4000, 15 mM
NAD, and 10 mM 2-mercaptoethanol in 100 mM Tris-SO4, pH 7.5, for 2 h
and then soaked for 24 h in a cryoprotectant solution containing the above
components plus 20% ethylene glycol (v/v). Crystals were flash frozen
on a nitrogen loop by plunging them into liquid propane and stored
in liquid nitrogen prior to data collection. X-ray diffraction data to 2.0 Å
were collected using the Cornell synchrotron source (CHESS) FI1 line (λ =
0.947 Å) equipped with a CCD detector. Data were processed using the
DENZO/SCALEPACK programming package (15).

Model Building and Refinement—The starting model for refinement
was a dimer of the Ascaris ME-NAD complex solved to 2.3 Å in our
laboratory (Protein Data Bank code 1LLO) (11). NAD molecules were
omitted from the model for the first round of refinement. This initial
model was refined with the reflection data for the ME-NAD crystals by
simulated annealing with no symmetry constraints using the CNS
(version 1.1) programming package (16). Initial 2Fo − Fc and Fo − Fc maps
indicated no major differences between the two structures with the
exception of the nicotinamide portion of NAD molecule and in the
carboxyl-terminal region. The model was manually built using the
interactive graphics model building program O (17). Since differences
between the two crystallographically independent subunits (α and β)
were observed, as in the structure of the enzyme-NAD complex, inde-
dependent models were used for each monomer after the first two rounds
of refinement. A bulk solvent correction was applied to all refinements,
and the free-R factor method was used to monitor the refinement (16).
Sine electron density for the NADH cofactor and tartronate molecule
was clearly observed in the initial model, NADH and tartronate were
added during the first round of model building. The asymmetric unit for
the current model contains residues 2–603 (α) and residues 2–593 (β)
of the two crystallographically independent subunits, two NAD mole-
cules and two Mg2+ bound at the active sites, two tartronate molecules
bound in the dimer interface, and 410 water molecules. The subunits
superimposed with a root mean square (r.m.s.) deviation of 0.3 Å be-
tween 593 corresponding α carbons. Analysis of stereochemistry was
performed using the program PROCHECK (18) showed >90% of the residues in the most
favored region of the Ramachandran plot. Final refinement statistics
calculated using all data are given in Table I. Comparison between
subunits and domains and with the human ME structures was carried
out using O (17).

### Table I

|                  | ME-NADH | ME-NADα |
|------------------|---------|---------|
| Total observations | 683,156 | 308,683 |
| Unique observations | 104,791 | 65,007 |
| Resolution (Å)    | 50.0–2.0 | 25.0–2.3 |
| Completeness (%)   | 98.7    | 98.7    |
| Rmerge (nar)       | 5.8     | 4.7     |
| Rmerge (ref)       | 23.0 (50–2.0 Å) | 24.7 (25–2.3 Å) |
| Rsym             | 27.2 (50–2.0 Å) | 28.0 (25–2.3 Å) |
| Protein atoms     | 9519    | 9045    |
| Solvent atoms     | 410     | 58      |

* From Ref. 11.
* Rmerge = 100 × Σ |Fo −Fc|/Σ |Fo|.
* Rmerge = Σ |Fo −Fc|/Σ |Fo|.
* Rsym = R for a randomly selected 5% of the data excluded from the refinement.

RESULTS

Structure of the ME-NADH Complex—The tertiary and qua-
ternary structure of the Ascaris NAD-malic enzyme in complex
with the cofactor NADH is essentially identical to that of the
binary complex with NAD (11). Overall, the structure is a
tetramer, which is organized as a dimer of dimers (Fig. 1) (the
r.m.s. deviation between the α carbon backbones of the corre-
spanding monomers, dimers, and tetramers of the two com-
plexes are 0.25, 0.32, and 0.34 Å, respectively). Two types of
subunit interfaces have been defined: a dimer interface be-
tween two subunits composing two monomers and a tetramer
interface formed between two dimers (Fig. 1). The monomer is
organized into four domains (A, B, C, and D) as previously
described for Ascaris ME (11) and other malic enzymes (14).
The active site is a cleft, formed between the C and B domains,
and one NADH cofactor is bound to each of the four active sites
in the tetramer. The B and C domains in the ME-NADH com-
plex superimpose on the corresponding domains in the
ME-NAD complex with r.m.s. deviations of 0.18 and 0.30 Å,
respectively. The active site, like that of the ME-NAD complex
(11), is in an open conformation (i.e. the residues implicated in
substrate binding and catalysis (Arg181, Lys198, Asp238, Asp272,
and Asn441) are poorly positioned for their respective roles.

In the NAD-bound structure, electron density for three resi-
dues, Ala401, Ser402, and Met503 at the C terminus in the
D-domain (11) was either not present or weak. In the present
structure, the three residues could be unambiguously modeled
but do not have a significant impact on the overall structure.

The tetramer in the present structure has four NADH mole-
cules (one per monomer) bound in the active site and four
tartronate molecules (one per monomer) bound within the
dimer interface (Fig. 1).

The NADH-binding Site—The conformation of the NADH
cofactor and its interactions with the enzyme are similar to
those of the ME-NAD complex with the exception of a dramatic
difference in the conformation and interactions of the nicotin-
amide ring (Fig. 2, A and B). The nicotinamide ring in the
NADH complex rotates +198° about the N-glycosidic bond rel-
tive to its orientation in the ME-NAD complex, with the si face
exposed to the solvent-accessible region of the active site (Fig.
2A). Binding interactions observed in the ME-NAD complex
between the nicotinamide ring amide group and residues Gly577
and Asn479 are broken as a consequence of this rotation,
and new hydrogen bonds between the carbonyl side chain and
the enzyme are formed between active site residues Asp295
and Arg741 (Table II and Fig. 2B). A water molecule (wat139)
occupies the site vacated by the nicotinamide amide group and
forms hydrogen bonds with Gly577 and Asn479.

In addition to NADH, Mg2+ is also bound to the active site
(Fig. 2B). The metal ion is coordinated to the carboxylate oxy-
Fig. 1. Ribbon diagram of the structure of Ascaris mitochondrial ME in complex with NADH, Mg$^{2+}$, and tartronate. A, the tetramer viewed down one 2-fold axis (indicated by the black oval) with the tetramer and dimer interfaces indicated by the arrows. The four subunits are colored blue, yellow, green, and tan. The four NADH and four tartronate ligands are shown as red ball-and-stick models, and their binding sites are indicated for one dimer. B, the Ascaris ME dimer viewed down the 2-fold axis corresponding to the dimer interface showing the locations of the two tartronate binding sites more clearly. The NH$_2$ and COOH termini of each monomer are indicated. For one subunit, the two helices ($\alpha_A$ and $\alpha_A$) that contribute most of the residues involved in tartronate binding are colored purple and labeled. This figure was generated using MOLSCRIPT (31).

Fig. 2. NADH bound to the active site of Ascaris mME. A, electron density ($2F_o - F_c$, contoured at $1\sigma$) for NADH. The NAD molecule bound to the Ascaris mME-NAD complex is shown for comparison in this and the following diagram. B, stereo diagram of the NADH binding site. A water molecule (Wat139) and Mg$^{2+}$ bound within the active site are indicated as spheres. This figure was generated using MOLSCRIPT (31).
gens of Glu^{271}, Asp^{272}, and Asp^{295} with distances of about 2.3 Å. These residues are homologous to the residues that bind Mn\(^{2+}\) in the human enzyme (Asp\(^{255}\), Asp\(^{256}\), and Asp\(^{279}\)) with similar bonding distances (12).

The Tartronate Binding Site—Tartronate, a competitive inhibitor of the ascarid malic enzyme with respect to the substrate malate, was included in all A. suum ME crystallizations in order to characterize the malate-binding site. Although electron density is observed in the active site corresponding to the tartronate binding site as identified in the human ME (12), the density in the ascarid malic enzyme structure is weak and does not conclusively demonstrate that tartronate is bound to the active site (Fig. 3A). This may be due to partial occupancy of the malate-binding site. However, density for tartronate is observed at a different site, within the dimer interface near a noncrystallographic 2-fold axis (Fig. 3B). Here the tartronate molecule is bound to a highly positively charged pocket (Figs. 4, A and B) and is tightly anchored by strong hydrogen bond/ionic interactions with residues from two adjacent subunits. The majority of the residues contributing to the binding site are in a cleft formed between the \(\alpha_{A3}\) (residues 76–87) and \(\alpha_{A4}\) (residues 93–104) helices of a subunit (Figs. 3B and 5A). Oxygen atoms O-1 and O-2 from one tartronate carboxylate group are within hydrogen-bonding distance (Table III) to the N-\(\epsilon\) atom of the side chain amide group of Glu\(^{78}\) (Glu\(^{64}\) in human ME) and a salt bridge with the guanidinium group of Arg\(^{105}\) (Arg\(^{91}\) in human ME), respectively (Fig. 5, A and B).

Oxygen atom O-4 of the other carboxylate forms a salt bridge with the guanidinium group of Arg\(^{105}\) (Arg\(^{91}\) in human ME), whereas its O-5 atom forms a salt bridge with the NH\(_2\) of the guanidinium group of Arg\(^{81}\) (Arg\(^{67}\) in human ME). The hydroxyl oxygen atom (O-3) of tartronate is within hydrogen-bonding distance to the N-\(\epsilon\) of Arg\(^{81}\). The remainder of the tartronate binding site is formed by residues 140–142 from the adjacent subunit. These residues are part of a coil, which links the A and B domains of this subunit. The hydroxyl group of Tyr\(^{141}\) is within hydrogen bonding distance to a carboxylate oxygen of tartronate. Binding of tartronate to this site contributes to the subunit interaction across the dimer interface. It is interesting that the dimer interface in the A. suum malic enzyme contains several charged residues and provides a continuous, positively charged pocket (Figs. 4, A and B) for the binding of negatively charged molecules such as tartronate and malate. The tartronate site also contains a water molecule (wat174; Fig. 3B), which is bound to a tartronate carboxylate oxygen and the amide NH of Met\(^{84}\). Its location corresponds to a water molecule bound to the fumarate-binding site in the human ME-NAD.

Identification of bound tartronate in the A. suum ME-NADH complex also definitively indicated that tartronate is bound at the same site in the A. suum ME-NAD complex. In the ME-NAD complex, although density was observed at the tartronate binding site, the resolution of the data did not permit identification of this density as tartronate. Comparison of the ME-NADH and

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**TABLE II**

Comparison of the interactions between the cofactor and enzyme residues within the A. suum ME-NAD and ME-NADH binary complexes

A key to the atom names for the cofactor is shown below.

| Enzyme Moiety | atoms (cofactor/protein) | residues | distance (Å) |
|---------------|-------------------------|----------|-------------|
| nictinamide   | N07/C                   | G459     | 10.1        |
|               | N07/O                   | G477     | 10.4        |
|               | N07/O6                  | N479     | 5.4         |
|               | N07/O8                  | D295     | 2.8         |
|               | NO7/Ne1                 | R181     | 3.1         |
|               | NO7/Ne2                 | R181     | 3.3         |

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**Fig. 3.** Binding of tartronate to A. suum malic enzyme. A, \(F_o - F_c\) electron density map at 2.0-Å resolution for tartronate molecule in the active site. Tartronate (TTN) and active site residues shown as ball-and-stick models. B, ribbon diagram of the tartronate-binding site within the dimer interface of A. suum ME with tartronate and key residues indicated as ball-and-stick models and a water molecule bound to tartronate shown as a sphere. Electron density (2\(F_o - F_c\), contoured at 1\(\sigma\)) for tartronate is also shown. Generated using MOLSCRIPT (31), BOBSCRIPT (32), and RASTER-3D (33).
ME-NAD structures revealed that the unidentified density in ME-NAD was also tartronate and that the binding interactions are identical to those observed in ME-NADH.

**DISCUSSION**

**Overall Structure**—The present structure, the first of a malic enzyme with reduced dinucleotide bound to the active site, provides new insights into substrate binding modes and helps resolve some of the questions associated with the catalytic mechanism of the enzyme. In addition, the structure reveals the identity of the allosteric site, that binds the activator fumarate. The backbone conformation and the quaternary structure observed in the ME-NAD complex are very similar to those of the ME-NADH complex (the r.m.s. deviation between the main chain atoms of the two structures is 0.25 Å, monomer; 0.32 Å, dimer; and 0.34 Å, tetramer). There are no noticeable differences in the dimer and tetramer interactions. As stated above, the final three residues at the C terminus in the D-domain (Ala601, Ser602, and Met603) can be visualized but have no significant impact on the overall structure of the ME-NADH complex.

In earlier studies on the structure of the ME-NAD complex, it was speculated that the residues implicated in metal ion binding (Glu271, Glu272, and Asp295) were not properly positioned for binding the metal ion. In the present structure, the binding site for magnesium is clearly identified (Fig. 2B). Whereas the role of Asp295 in metal ion binding was further confirmed by earlier site-directed mutagenesis studies (19), a role for Glu271 and Asp272 was not postulated. These two residues are part of the malic enzyme sequence QFEDFA (positions 269–274), which agrees well with the consensus metal ion binding sequence of XXDDXX, where X is an uncharged or hydrophobic residue (20). All of the residues suggested as metal ion ligands are homologous to residues in the human enzyme (12). The fact that Mg$^{2+}$ was not observed bound at the corresponding site in the ME-NAD complex indicates that the metal ion affinity may be sensitive to the oxidation state of the cofactor or that the low resolution data for ME-NAD crystals does not reveal the metal ion-binding site. The binding of the metal ion is consistent with the required ordered addition of metal ion prior to malate.

**Table III**

Interaction distances between tartronate molecule and enzyme residues in the Ascaris ME-NADH complex

| residue | atom (tartronate**/protein) | distance (Å) |
|---------|-----------------------------|--------------|
| Arg105 (91) | O1/No                      | 2.92 (2.52)  |
|          | O1/Ne                      | 3.14 (2.62)  |
|          | O4/No                      | 3.12         |
| Arg81 (67) | O3/Ne                      | 2.92 (2.89)  |
|          | O5/Ne                      | 3.08         |
| Gln78 (64) | O2/Ne                      | 2.88 (3.04)  |
| Tyrl41   | O4/OH                      | 2.86         |

**A** A key to the atom names for the tartronate molecule is as follows:

![Tartronate model](image)

**Fig. 4.** A, electrostatic potential surface of a monomer of Ascaris mME as viewed in roughly the same orientation as in Fig. 3B. B, electrostatic potential surface of two monomers showing the dimer interface along the vertical axis. Two tartronates are indicated as red CPK models. This figure was generated using GRASP (28).
Malic Enzyme Structure

A

B

Fig. 5. Ribbon diagrams for the comparison between the tartronate and fumarate binding sites of Ascaris and human mME. A, tartronate binding site in Ascaris ME, B, fumarate binding site in human mME. The α and β subunits in both of the enzymes are colored blue and tan, respectively. Tartronate (TTN) in Ascaris ME and fumarate in human ME (FM) are shown as red and cyan CPK models. Key residues involved in binding ligands are shown as ball-and-stick models. The NH₃ and COOH termini of each monomer are indicated. This figure was generated using MOLSCRIPT (31) and RASTER-3D (33).

Although the active sites of the ME-NADH and ME-NAD complexes differ in their cofactor conformations and the presence of Mg²⁺ bound in ME-NADH complex, the active site main chain and side chain conformations are very similar to one another.

Conformational Change in the Coenzyme—A comparison of the coenzyme binding pockets in the present structure and the previously reported ME-NAD structure shows interesting changes in the conformation of the nicotinamide ring as a consequence of reduction (Fig. 2, A and B). In the ME-NAD complex, as NAD is bound, the re face of the nicotinamide ring is directed toward the solvent such that its carboxamido side chain is directed away from the catalytic pocket into the protein structure. In the present structure, however, the reduced cofactor has its nicotinamide ring moved into the active site through a rotation about the N-glycosyl bond by +198°, and its si face is now directed toward the solvent. The carboxamido side chain, which was originally hydrogen-bonded to a backbone carbonyl and the amide side chain of Asn¹⁷², now interacts with Arg¹⁸¹, which in turn interacts with the α-carboxylate of L-malate, and Asp²⁹⁵, the putative general base, which in turn interacts with the 2-hydroxyl of malate in the Michaelis complex (Table II). The proS proton of the reduced nicotinamide ring is now directed toward the active site. It is interesting to note that whereas the nicotinamide ring rotates by +198° into the active site, other catalytic residues are unaffected. As shown in Fig. 2A, the density around the nicotinamide ring in a difference map calculated from ME-NADH complex with and without NADH is very strong, and there are strong hydrogen-bonding contacts between the carboxamido group of the nicotinamide ring with Arg¹⁸¹ and Asp²⁹⁵, suggesting that the nicotinamide ring is indeed locked in this conformation and that the observed conformational change is not simply due to the conformational flexibility allowed by the Rossmann fold as in the case of lactate dehydrogenase (21, 22).

The mobility of the nicotinamide ring reported in this study is also observed in other enzymes, and it has been proposed to play a crucial role in the catalytic mechanism of at least two other enzymes, aldehyde dehydrogenase (23) and 6-phosphogluconate dehydrogenase (24).

Implications to Catalytic Mechanism—A three-step acid-base catalytic mechanism is proposed for ascarid malic enzyme based on pH and isotope partitioning studies (25–27). The assignment of the general acid, general base, and binding groups shown in Scheme 1 is based on the x-ray structure of the ME-NAD complex (11) and site-directed mutagenesis studies (19). Generally, L-malate is converted to oxaloacetate facilitated by Asp²⁹⁵, which acts as a general base to accept a proton from the 2-hydroxyl of L-malate (19). The hydride at C-2 of malate is transferred to the 4-position of the nicotinamide of NAD, with the positively charged pyridinium nitrogen providing the driving force for reduction. The guanidinium of Arg¹⁸¹ helps to orient malate for reaction by forming an ion pair/hydrogen bond with the α-carboxyl of malate, and Lys¹⁹⁹ acts in a similar role by interacting with the β-carboxyl. The metal ion (Mg²⁺) aids in the orientation of malate for catalysis and facilitates proton transfer to Asp²⁹⁵ by binding to the α-hydroxyl of malate. In the second step, oxaloacetate is decarboxylated to enolpyruvate with the metal ion acting as a Lewis acid and protonated Asp²⁹⁵ acting as a general acid to protonate the enolate oxygen. Finally, enolpyruvate is tautomized to pyruvate, with Asp²⁹⁵ acting as a general base and the e-amino group of Lys¹⁹⁹ acting as a general acid to protonate C-3 of enolpyruvate (29). Thus, the catalytic mechanism requires a protonated form of the general acid (Lys¹⁹⁹) and an unprotonated form of the general base (Asp²⁹⁵). The optimum protonation state for Asp²⁹⁵ (unprotonated) and Lys¹⁹⁹ (protonated) is observed in the V/K malate pH-rate profile (26), although the catalytic role of Lys¹⁹⁹ is not realized until CO₂ is released. The observation of the Lys¹⁹⁹ pK in the V/K profile indicates the importance of the protonated form of Lys¹⁹⁹ in binding malate. Whereas the existence of the protonated form of Lys¹⁹⁹ is important for optimum binding of the β-carboxylate of L-malate, it poses a problem in the decarboxylation step, where the ionic/hydrogen-bonding interaction between Lys¹⁹⁹ and the carboxylate would be anticycatalytic. Thus, during the subsequent hydride transfer step, the interaction between the carboxylate and Lys¹⁹⁹ must be eliminated.

The crystallographically observed conformational change in the nicotinamide ring may provide an explanation as to how the hydrogen bond between Lys¹⁹⁹ and the β-carboxyl of malate may be eliminated. A mechanism incorporating the structural data obtained in the present study is shown in Scheme 1. Malate is bound such that its α-carboxylate is hydrogen-bonded to the guanidinium of Arg¹⁸¹ and coordinated to Mg²⁺. The β-carboxylate of malate is proposed to hydrogen-bond to Lys¹⁹⁹,
and the β-hydroxyl of malate is hydrogen-bonded to Asp295 and coordinated to Mg$^{2+}$. The nicotinamide ring of NAD$^+$ is bound with its carboxamide side chain hydrogen-bonded to a backbone NH and carbonyl and to the amide side chain of Asn479. The distance between the pyridinium nitrogen of NAD$^+$ and the pyrophosphate moiety of the cofactor is about 5.7 Å. Hydride transfer from C-2 of malate to C-4 of the nicotinamide ring occurs and is partly driven by the positively charged pyridinium ring and general base catalysis by Asp295. The reduction of the nicotinamide ring results in a rotation by 198° about the N-glycosidic bond, placing the carboxamide side chain within hydrogen-bonding distance to Asp295 and Arg181. The rotation is thought to result, at least partly, from loss of the ionic interaction between the pyridinium nitrogen of NAD$^+$ and the pyrophosphate moiety of the cofactor. The movement of the cofactor is proposed to be reversible but with the equilibrium position favoring that shown in the structure (Fig. 2). The movement of the nicotinamide ring along with the sp$^3$ to sp$^2$ hybridization change at C-2 that occurs as oxalacetate is formed is proposed to shift the position of the bound oxalacetate such that 1) the β-carboxylate moves away from Lys199 and 2) the C-3–C-4 bond to the β-carboxylate is now orthogonal to the C-2–C-3 plane of oxalacetate, favoring decarboxylation. Decarboxylation then occurs facilitated by the Lewis acidity of the Mg$^{2+}$ and with Asp295 acting as a general acid. Finally, tautomerization occurs aided by general base-general acid catalysis via Asp295-Lys199 pair. The products pyruvate and NADH are then released. Final confirmation of the above hypothesis should come from the structure of a ternary complex of the enzyme with NADH and an analogue of malate or oxaloacetate.

In order to examine whether the active site of human enzyme could accommodate such a conformational change in the nicotinamide ring, NADH was modeled into the active site of the open structure of the human enzyme (14) such that the nicotinamide ring was rotated by 198° relative to its position in NAD molecule. The new conformation was indeed feasible without any steric hindrance, and the contact distances between the nicotinamide ring and the corresponding enzyme residues were similar to those observed in ascarid enzyme.

**The Tartronate Binding Site and Regulatory Mechanism**—
Tartronate, a dicarboxylic analogue of malate and fumarate, is tightly bound in an allosteric site via hydrogen-bonding interactions with the side chains of two arginines (Arg105 and Arg81) and a glutamine (Gln78). All of the residues shown are homologous to those in the fumarate allosteric site depicted in the recent structure of the human enzyme-ATP complex (Fig. 5, A and B) (13). The human mitochondrial ME is allosterically activated by fumarate. Although many of the residues that...
bind fumarate in human ME are homologous or functionally similar to those involved in tartronate binding in Ascaris ME, the binding modes of these ligands differ significantly between the two enzymes. A multiple sequence alignment for malic enzyme showed that the above residues. It is interesting to note that fumarate activation has not been reported in all of the species that have the conserved residues under consideration.

The Ascaris malic enzyme is also allosterically activated by fumarate with an activation constant of 40 μM (9, 10). Activation is expressed as a decrease in the off-rate for bound malate (10). Although the activation by fumarate under conditions of saturating NAD+ and Mg2+ is only 2-fold (9), with reactants maintained at estimated physiologic concentrations, the activation is 15-fold. Thus, the allosteric site where tartronate is bound is most likely an activator site for fumarate. This conclusion is further supported by the observation that the R105A mutant of the Ascaris malic enzyme, which has a V/E value identical to that of the wild type enzyme, is no longer activated by fumarate, consistent with tartronate being bound to the fumarate activator site.

The distance between the active site and the tartronate allosteric site is ~30 Å. However, there are structural relationships between the sites, which could allow for transmission of the allosteric signal. Within a subunit, Arg105 participates in binding tartronate. The backbone oxygen of the adjacent residue, Asp104, is hydrogen-bonded to the side chain of Lys143, which is part of a coil sequence (residues 140–142) that connects the A and B domains of a subunit (Fig. 5A). This coil is also directly involved in forming the second tartronate binding site within the dimer interface via Tyr141 (Fig. 5A). The amino-terminal end of this coil is linked to the coil connecting the A and B domains of a monomer (residues 140–143). Thus, the tartronate-binding site is linked to the coil connecting the A and B domains of a monomer (residues 140–143). It is interesting to note that the conformation of this coil differs significantly from that of the corresponding coil in human ME (residues 126–129) despite almost identical sequences (Figs. 5B and 6). In particular, there is no interaction corresponding to the Asp143-Lys144 pair, and the positions of the homologous residues Arg126 (Ascaris) and Arg128 (human) differ. In addition, in place of Tyr141 in the Ascaris enzyme, the human enzyme has a phenylalanine (Phe141).

Overall, the evidence presented in this study identifies an allosteric site and provides new insights into the mechanism of allosteric activation of the A. suum malic enzyme. However, the structure of the Ascaris malic enzyme with fumarate bound to this allosteric site will further confirm the identity of this site and enhance our understanding of the allosteric mechanism.

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