Mapping the Active Site Topography of the NAD-Malic Enzyme via Alanine-Scanning Site-Directed Mutagenesis†

William E. Karsten, Lilian Chooback, Dali Liu, Chi-Ching Hwang, Christopher Lynch, and Paul F. Cook*

Department of Chemistry and Biochemistry, University of Oklahoma, 620 Parrington Oval, Norman, Oklahoma 73019

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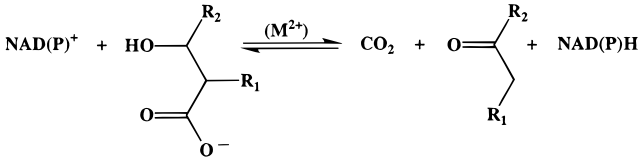
ABSTRACT: The NAD-malic enzyme cDNA has been subcloned into the pQE expression vector, expressed with a six-His tag, and purified. The His-tagged enzyme is purified by a combination of Ni−NTA and orange A agarose column chromatography with a yield of 45% and an estimated purity of >90%. The tag and linker have no effect on the kinetic parameters of the enzyme compared to the wild-type enzyme. Alanine-scanning site-directed mutagenesis has been carried out on all of the conserved neutral acid residues of the NAD-malic enzyme from *Ascaris suum*. Data obtained confirm the predicted role of D178 and D295 in metal ion binding, the likely role of D294, D361, and E440 in the NAD binding site, and the role of E58 and D272 in malate binding. Decreases in V/E_t by 10^4-fold and in $V/K_{malate}E_t$ by 10^7-fold, when D295 is changed to alanine, suggest that it is a likely candidate for the general base that accepts a proton from the malate hydroxyl in the oxidation step.

Pyridine nucleotide-linked β-hydroxyacid oxidative decarboxylases catalyze the general reaction shown in Scheme 1. Enzymes in this class include malic enzyme and isocitrate dehydrogenase (ICDH),[1] isopropylmalate dehydrogenase (IPMDH), 6-phosphogluconate dehydrogenase (6-PGDH), and tartrate dehydrogenase (*1*). All of the enzymes studied in detail to date have a random kinetic mechanism [malic enzyme (*2*, *3*), ICDH (*4*, *5*), 6-PGDH (*6*), IPMDH (*7*), and TDH (*1*)]. The kinetic mechanism of the pigeon liver malic enzyme was originally thought to be ordered with NADP adding prior to malate (*8*, *9*), but using the substrate dependence of deuterium isotope effects, we have shown that the mechanism is steady-state random (*10*). The oxidative decarboxylases noted above also have the same general acid−base mechanism [malic enzyme (*11*, *12*), ICDH (*13*, *14*), and 6-PGDH (*15*)]. Finally, the mechanism of oxidative decarboxylation appears to be stepwise in all cases with the native dinucleotide substrate. General base-catalyzed oxidation of the β-alcohol precedes decarboxylation to an enol or enediol, followed by general acid-catalyzed tautomerization to a ketone product [malic enzyme (*16*), ICDH (*14*), and 6-PGDH (*17*)].

Despite the similarities, there are significant differences among the oxidative decarboxylases noted above. Structurally, all but the malic enzyme, a homotetramer, are homodimeric (*18*). Although ICDH and IPMDH are only 30% identical, the two enzymes share in common the same protein fold (*19*−*21*). Tartrate dehydrogenase (TDH) is similar to IPMDH (*1*). In addition, the amino acid residues that line the active sites of both enzymes are very similar (*7*, *22*, *23*). Neither IPMDH nor ICDH has a relatively high degree of identity to 6-PGDH or malic enzyme, nor is malic enzyme homologous to 6-PGDH (*18*). Finally, malic enzyme, ICDH, and IPMDH require a divalent metal ion for catalysis [malic enzyme (*2*) and ICDH (*24*)], while 6-PGDH (*25*) does not.

Scheme 1: Proposed Mechanism for Malic Enzyme



R_1 = H (ME), OH (TDH, 6PGDH), $CH_2CO_2^-$ (ICDH), $CH(CH_3)_2$ (IPMDH)

R_2 = CO_2^- (ME, TDH, ICDH), $CH(OH)CH(OH)CH_2OPO_3^=$ (6PGDH)

The cDNA clone for NAD-malic enzyme from *Ascaris suum* has been sequenced (*26*) and expressed (*27*). To date, however, there has been no three-dimensional structure reported for a malic enzyme from any species, the only class of β-hydroxy acid oxidative decarboxylases for which a structure is not available. The E−NAD−Mg−tartronate quaternary complex of the NAD-malic enzyme from *A. suum* has been crystallized (*28*), but a structure has not been determined. The NADP-malic enzyme from duck liver has recently been crystallized (preliminary results). In anticipation of obtaining the three-dimensional structure of a malic enzyme, we report progress on mapping the active site topography of the NAD-malic enzyme from *A. suum* using alanine-scanning site-directed mutagenesis of ionizable neutral acids that are conserved for all malic enzymes that are available.

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[1] Abbreviations: ICDH, isocitrate dehydrogenase; IPMDH, isopropylmalate dehydrogenase; 6-PGDH, 6-phosphogluconate dehydrogenase; TDH, tartrate dehydrogenase; IPTG, isopropyl β-D-thiogalactopyranoside; NAD, nicotinamide adenine dinucleotide (the + sign has been omitted for convenience); NADH, reduced nicotinamide adenine dinucleotide; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PEPCK, phosphoenolpyruvate carboxykinase; PBS, phosphate-buffered saline.

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MATERIALS AND METHODS

Chemicals and Enzymes. Deoxynucleotide triphosphates were from Perkin-Elmer, while Taq Plus DNA polymerase and Pfu were from Stratagene. T_4 DNA ligase, T_4 polynucleotide kinase, restriction endonucleases, and IPTG were from GIBCO/BRL. The fmol^R DNA cycle sequencing kit, protein molecular mass markers, and *Escherichia coli* strain JM109 were from Promega. Protein concentrations were determined according to the Bradford method using the Bio-Rad protein assay kit with bovine serum albumin as a standard (*50*). The ampicillin, kanamycin, and NAD were from Sigma. Hepes buffer was from Research Organics. The DNA molecular weight ladder was purchased from New England Biolabs. DEAE-Spherilose was from Isco, and the orange A agarose was from Amicon. Mutagenesis and sequencing primers were purchased from either Biosynthesis or Promega. Site-directed mutagenesis was performed using the Altered Sites II Mutagenesis Kit purchased from Promega. The prokaryotic expression vector pKK223-3, GST purification modules which include the pGEX-4T-1 vector, and Glutathione Sepharose 4B were from Pharmacia Biotech, and the QIAexpress System which contained the pQE-30 vector, *E. coli* strain M15, and the Ni−NTA matrix was from QIAGEN. All other chemicals that were used were the highest quality available and were used without further purification.

New Expression System for the NAD-Malic Enzyme. The *A. suum* NAD-malic enzyme gene was subcloned into the pKK223-3 expression vector (*27*), and this system worked well for the wild-type enzyme. It was subsequently determined that one of the *E. coli* malic enzymes copurifies with the *Ascaris* enzyme. Two additional expression systems were used, and each is considered below.

(a) Subcloning of the Ascaris Malic Enzyme into Expression Vector pGEX-4T-1. The pKK223-3 vector containing the *A. suum* NAD-malic enzyme gene was digested with *Hin*dIII and the resulting 5′ overhang filled in with Klenow fragment to create a blunt end. The linearized DNA was then digested with *Eco*RI and the DNA fragment containing the gene gel purified. The pGEX-4T-1 vector was digested with *Eco*RI and *Sma*I (a blunt-end cutter), ligated to the malic enzyme gene-containing fragment, and transformed into *E. coli* strain JM109 (for storage and maintenance of the plasmid) or BL21 (for expression). The plasmid was designated pGEX.ME.

(b) Subcloning of Ascaris Malic Enzyme into Expression Vector pQE30. The malic enzyme cDNA in plasmid pAlt.ME was amplified using primer pairs 5′-GCATTGGATCCGAATGAAAAGT-3′ which created a *Bam*HI site (underlined) at the beginning of the coding sequence and 5′-TAGAATACTCAAGCTTAACCATCCAT-3′ which encompasses a *Hin*dIII site (underlined) at the end of the coding sequence. The resulting 1.8 kb cDNA fragment was subcloned into pQE30 at the corresponding sites, which adds six histidines and a short linker to the N-terminus. The resulting plasmid named pQE.ME was transformed into *E. coli* strain M15 using the Qiagen protocol. Frozen stocks of the strain harboring the plasmid were stored in LB/Amp/Kan medium containing 15% glycerol at −70 °C.

Growth Conditions. The BL21 strain containing pGEX.ME was grown in 500 mL of YT medium at 25 °C with vigorous shaking until the A_{600} reached 0.5. IPTG was added to a final concentration of 50 μM (higher concentrations of IPTG or higher temperatures led to an increased level of formation of inclusion bodies and lower levels of soluble protein), and the culture was allowed to grow for an additional 4 h. The cells were harvested by centrifugation at 6000*g* for 10 min, and suspended in 25 mL of PBS (phosphate-buffered saline) at pH 7. Growth conditions for the *E. coli* strain harboring pQE.ME were as previously described (*29*).

Protein Purification. The pGEX.ME bacterial cells were sonicated with a Misonix Inc. model XL ultrasonic liquid processor and centrifuged at 6000*g* for 10 min to pellet the cell debris. Most of the malic enzyme activity is present as inclusion bodies. The soluble activity was applied to a 5 mL bed volume column of glutathione Sepharose 4B. The column was washed with 10−12 bed volumes of PBS, and 4 mL of a solution of PBS containing 25 cleavage units of thrombin protease was drained into the column and incubated overnight. After incubation, malic enzyme was eluted from the column with about 8 mL of PBS. Under these conditions, the total number of units of enzyme in the crude extract was 22 and the number of units eluted from the column was 18 (80% yield), but this represents only the soluble fraction. SDS−PAGE reveals two bands in the column-purified enzyme solution, one at 65 kDa (malic enzyme) and a second slightly smaller band with an about equal intensity. The smaller band may be eliminated by applying the enzyme to an orange A agarose column according to previously published procedures (*30*). Preliminary kinetic results indicate the extra amino acids have no effect on the enzyme's kinetic parameters.

The pQE.ME bacterial cells were sonicated as described above and centrifuged to remove cell debris. The His-tagged malic enzyme was purified using the column chromatographic procedure supplied by Qiagen. The His-tagged malic enzyme was eluted with 0−0.3 M imidazole in wash buffer (pH 7.5). Solid ammonium sulfate was added to 80% saturation to precipitate the protein. The protein pellet was resuspended in orange A equilibration buffer and dialyzed overnight against the same buffer. The protein was then bound to the orange A column as previously described. Some of the mutant enzymes did not bind to the orange A column. Consequently, these enzymes were further purified on a DEAE-Spherilose column (17 cm × 1 cm) using a 10 to 400 mM potassium phosphate gradient (pH 7) to elute the enzyme using an Isco low-pressure liquid chromatography system.

Site-Directed Mutagenesis. The malic enzyme cDNA fragment was excised from plasmid pME.LC1 using *Eco*RI and *Hin*dIII restriction endonucleases. The 1.8 kb cDNA fragment was subcloned into the pAltered-1 vector from Promega via the *Eco*RI−*Hin*dIII sites located in the multiple cloning region. The resulting plasmid was designated pAlt.ME. This plasmid was used to produce the subsequent malic enzyme mutants.

Single-stranded pAltered vector containing the malic enzyme insert was generated using the R408 phage, and the single-stranded DNA was isolated according to the manufacturer's protocol. The site-directed mutant is made by annealing a mutagenic primer (Table 1) designed to create the desired base changes and an ampicillin repair oligonucleotide supplied by Promega which imparts ampicillin

Table 1: Sequence of Mutagenic Oligonucleotides[a]

D178A	TGTTGTAACG**GCT**GGTGAGCGAA
D187A	GGGATTGGGC**GCC**TTAGGCGCCT
D220A	TGTTCTATTG**GCC**GTCGGTACAA
D295A	TTCAATGAT**GCT**ATTCAAGGT
D295S	ATGTTCAATGAT**AGT**ATTCAAGGTACT
D295C	ATGTTCAATGAT**TGT**ATTCAAGGTACT
D294A	ACAATGTTCAAT**GCT**GATATTCAAGGT
D294C	TACAATGTTCAAT**TGT**GATATTCAAGGTAC
D272A	CAATTCGAA**GCT**TTTGCAAAT
E58A	GTTCAGCCTCTAC**GCG**CGACAATATCTCGG
E271A	ATACAATTC**GCA**GATTTTGCA
E440A	AGTAAAGCC**GCA**TGTACGGCC

[a] The sites of mutation are bold.

resistance to the resulting mutant DNA. The mutant strand is synthesized using T4 DNA polymerase in the presence of T4 DNA ligase. The heteroduplex DNA is transformed into the repair minus *E. coli* strain ES 1301 mutS, and the culture is grown in ampicillin-containing medium. Plasmid DNA is isolated from the mutS cells, and the DNA is used to transform *E. coli* JM109 cells which are plated on ampicillin-containing agar plates. Plasmid DNA is isolated from antibiotic resistant colonies, and the desired mutations are screened by DNA sequencing of the plasmid DNA. The mutation frequency is generally greater than 75% using this method. The plasmids identified as containing the mutation are then subcloned into the desired expression vector using the method described above for subcloning the wild-type enzyme. The insert is again sequenced to confirm that the mutation is still present, and that the cDNA is intact.

Initial Velocity Studies. All data were collected using a Beckman DU 640 spectrophotometer or a Hewlett-Packard 8453 diode array spectrophotometer to monitor the appearance of NADH at 340 nm. All assays were carried out at 25 ± 0.1 °C, and the temperature was maintained using a circulating water bath with the capacity to heat and cool the cell compartment. Typical assays contained 100 mM Hepes (pH 7.3) and 30 mM free Mg^{2+} (added as $MgSO_4$) and variable concentrations of free malate (0.25−10 mM) and free NAD (15−150 mM) or 2 mM free NAD^+ and variable levels of free Mg^{2+} (10−100 mM) and free malate (0.25−10 mM) (2). Initial velocity data were fitted with the appropriate rate equation and a BASIC version of the Fortran program developed by Cleland (*31*). Data conforming to a sequential initial velocity pattern were fitted using eq 1, while data conforming to a rapid equilibrium ordered initial velocity pattern were fitted using eq 2.

$$v = VAB/(K_{ia}K_b + K_aB + K_bA + AB) \quad (1)$$

$$v = VAB/(K_{ia}K_b + K_bA + AB) \quad (2)$$

where v and V represent initial and maximum velocities, respectively, A and B represent reactant concentrations, K_a and K_b are Michaelis constants for A and B, respectively, and K_{ia} is the inhibition constant for A.

Structural Characterization. The structural integrity of the mutant proteins was determined grossly using the far-UV CD spectrum recorded using an AVIV 62 DS circular dichroism spectrophotometer. In all cases, spectra were very similar to that obtained for the wild-type enzyme.

Table 2: Purification of Recombinant His-Tagged Mitochondrial NAD-Malic Enzyme[a]

	total protein (mg)	total units (mol/min)	specific activity[b] (units/mg)	fold purification	yield (%)
crude extract	859	1030	1.2	1	100
Ni−NTA	37	626	17	14	61
orange A agarose	16.3	463	28.4	23.7	45

[a] Purification was carried out with 32 g of wet cell paste. [b] Based on activity measured in an assay containing 100 mM Hepes (pH 7), 50 mM malate, 10 mM Mg^{2+}, and 0.5 mM NAD. Protein concentrations were determined using the method of Bradford (*50*) and bovine serum albumin as the protein standard.

RESULTS

Mutant Isolation and Structural Characterization. The mutant genes were subcloned into the pGEX or pQE expression vectors to take advantage of the specific affinity purification procedure that afforded separation of the mutant malic enzyme from the background *E. coli* malic enzyme activity. In this regard, both the pGEX and pQE expression systems worked well. However, in the pGEX system it was found that the thrombin cleavage of the GST fusion protein from malic enzyme was not reproducible and resulted in low enzyme yields. In addition, the overall expression levels were relatively low in the pGEX system since a large fraction of the enzyme was found in inclusion bodies. Since the activity of the mutant enzymes was low, a system that would improve expression and enzyme yields was sought, and this prompted subcloning the malic enzyme gene into the pQE vector. The level of malic enzyme expression is higher using the pQE expression vector than using pGEX, and the resulting His-tagged enzyme is reproducibly and easily purified in two steps (see below). The extra 14 amino acids on the N-terminus of malic enzyme consist of the sequence M-R-G-S-H-H-H-H-H-H-G-I-R-M with a molecular weight of 1481. Consequently, the His-tagged enzyme subunit molecular weight is 69 959 compared to the native enzyme molecular weight of 68 478.

The pQE.ME gave good expression, and is easy to purify from a crude extract obtained by sonication and centrifugation in two steps. The overall yield is 45% with a 24-fold-purification, which compares favorably with the 50% yield and 30-fold purification obtained by purification of enzyme expressed using the pKK223-3 vector (*27*). The final enzyme preparation is >90% pure. The kinetic parameters of the His-tagged enzyme are virtually identical to those obtained for the native enzyme (Table 3) (*12*).

To determine whether the mutations resulted in a loss of overall structural integrity, far-UV CD spectra were recorded for all mutant proteins. In all cases, the mutant proteins were expressed at a level equal to that of the wild-type enzyme. In all cases, the far-UV spectra were superimposable once corrected to the same protein concentration. Thus, changes in structure, if any, must be localized to the active site.

Kinetic Characteristics. The kinetic parameters of the wild-type and mutant malic enzymes are summarized in Table 3. Mutants can be divided generally by the kinetic parameters that are significantly altered as a result of the mutation. There are three instances where the turnover number (V/E_t) is decreased by ≥600-fold, i.e., D294A, D295A, and E440A. The most pronounced decrease, 10^4-fold, is obtained with

Table 3: Kinetic Constants of A Mutants of Conserved D and E Residues in NAD-Malic Enzyme

	K_{malate} (mM)	K_{NAD} (mM)	K_{Mg} (mM)	V/E_t (s^{-1})	$V/K_{malate}E_t$ (M^{-1} s^{-1})	$V/K_{NAD}E_t$ (M^{-1} s^{-1})
wild type[a]	0.53 ± 0.07	0.035 ± 0.007	4.5 ± 1.7	36	7×10^4	1×10^6
D178A	0.30 ± 0.15 (−)[b]	0.05 ± 0.01 (−)	200 ± 100 (∼50)	0.3 (120)	1.0×10^3 (70)	7×10^3 (140)
D272A	2.1 ± 0.7 (4)	0.023 ± 0.002 (−)	6 ± 4 (−)	0.17 (210)	80 (880)	8×10^3 (130)
D294A	2.0 ± 0.5 (4)	0.2 ± 0.1 (6)	2 ± 1 (−)	0.06 (600)	30 (2000)	250 (4000)
D294C	1.1 ± 0.2 (2)	0.030 ± 0.008[c] (−)	4 ± 1 (−)	2 (18)	2×10^3 (35)	7×10^5 (−)
D295A	500 ± 600[c] (≥1000)	ND	12 ± 15 (≥3)	0.004 (∼10^4)	0.007 (∼10^7)	ND
D295C	8 ± 2 (16)	0.028 ± 0.008 (−)	70 ± 20 (16)	0.2 (180)	28 (2500)	7600 (130)
D361A	1.4 ± 0.4 (3)	0.8 ± 0.2 (20)	4 ± 2 (−)	9 (4)	7×10^3 (10)	1×10^4 (100)
E58A	10 ± 2 (20)	0.12 ± 0.02 (3)	9 ± 2 (2)	0.5 (70)	50 (1400)	4×10^3 (250)
E440A	ND (−)	0.14 (4)	ND (−)	0.06 (600)	ND (−)	440 (2300)

[a] Data presented in ref *27* are in error. V/E_t should have been 30 s^{-1}. Data for the native enzyme (*12*) are as follows: V/E_t, 38 s^{-1}; $V/K_{NAD}E_t$, 4×10^6 M^{-1} s^{-1}; and $V/K_{malate}E_t$, 4×10^4 M^{-1} s^{-1}. [b] Values in parentheses are the approximated fold increases for K_m and fold decreases for V and V/K. [c] Although the K_m values are undefined, V/K values are well-defined.

the D295A mutant enzyme. There are changes in other parameters that are associated with the change in V/E_t in each case. Slight increases in K_{malate} and K_{NAD} are observed for D294A, while a marked increase in K_{malate}, accompanied by an increase in K_{Mg}, is obtained with D295A. In the case of E440A, the decrease in V/E_t is accompanied by an increase in K_{NAD}. Changes in V/E_t obtained with D294A and D295A are rescued by about the same amount (∼30-fold for D294 and ∼60-fold for D295) by changing the aspartates to C, but a substantial increase in K_{malate} is still obtained in the case of D295.

A second class of mutant is represented by significant changes in K_{Mg}, as seen with D178, while increases of 3−16-fold are observed with D295. Note that D295, as discussed above, also leads to significant changes in other parameters. Increases in K_{NAD} are observed with changes in D294, D361, and E58. The largest in this case is observed with D361, with modest changes observed for the others. Changes in D294 also give a low value of V/E_t. A final class of mutants includes D272 and E58, both of which give significant changes in the V/K for malate, generated by modest changes in V/E_t and K_{malate}.

DISCUSSION

Alanine-Scanning Site-Directed Mutagenesis of the A. suum NAD-Malic Enzyme. In this study, each of the conserved ionizable neutral acid residues was changed, one at a time, to alanine, in an attempt to assess their individual function and to begin to map the topography of the NAD-malic enzyme active site in anticipation of a three-dimensional structure determination. Neutral acid residues that are completely conserved for all malic enzyme sequences now available are aspartates D178, D187, D220, D272, D294 (conserved in 25 of 26 sequences), D295, and D361 and glutamates E58, E271, and E440. Each of these residues was converted to alanine, and their kinetic parameters were determined. The mutants D187A, D220A, and E271A appear to be expressed at very low levels, suggesting that the mutants are unstable. The aspartate at position 187 is in a highly conserved region identified by Kulkarni et al. (*26*) as being part of the ADP binding $\beta\alpha\beta$ fold thought to bind the ADP moiety of the dinucleotide. The aspartate at position 220 is part of a completely conserved sequence, DVG, in the 26 aligned malic enzyme sequences.

Little information presently exists about the identity of enzyme residues involved in catalysis and/or binding of reactants and the divalent metal ion activator. A thiol group was identified by modification with DTNB and shown to be at or near the malate binding site (*12*). Conversion of the thiol to a thiocyanoyl group leads to an active enzyme with a 30-fold higher K_{malate} (*38*). The sequence around the active site cysteine (C146, the N-terminal domain of the *Ascaris* NAD-malic enzyme) has been obtained (*39*). The cysteine has been mutated to a serine residue in the duck liver enzyme (*40*) and resulted in a $k_{cat}/(K_{NADP}K_{malate}K_{Mn})$ that was 3.7% of that of the native enzyme. The role of the cysteine is still unknown, but sequence alignments indicate that the cysteine is not conserved in all species. Phenylalanine-19 has been identified as an important residue in the binding of Mn^{2+} and malate, and in the subunit interface (*41*). Mutagenesis of F19 to small residues results in a monomeric form of the malic enzyme, while a change to larger residues gives enzymes that are altered by about 1 order of magnitude in malate and Mn^{2+} binding with a decrease of about 5-fold in V.

Metal Ion Binding. The divalent metal ion binding ligands in the pigeon liver NADP-malic enzyme have been identified in the elegant work of Chang and his colleagues (*42*−*44*). Using a chemical cleavage via Fenton chemistry, D141, D194, D258, and D465 were identified as ligands to the active site Mn^{2+} in the pigeon liver enzyme. The aspartate at position 465 is at a highly substituted position and may not be a ligand to the metal ion. In the case where site-directed mutagenesis was carried out to corroborate the chemical cleavage method, the D258E mutant gave a several 1000-fold increase in the dissociation constant for Mn^{2+} (*43*). The authors carried out sequence alignments with a number of other malic enzymes, including the *A. suum* NAD-malic enzyme, and showed that all of the aspartates are conserved in the latter, with the exception of D465. Three of the aspartate residues identified in the pigeon liver enzyme as metal ion ligands, D141, D194, and D258, are homologues of D178, D230, and D295 in the *Ascaris* enzyme. The C-terminal D465 could be either D498 or D500 in the *Ascaris* enzyme if this residue is in fact a metal ion ligand (see above).[2]

[2] The numbering of the *A. suum* NAD-malic enzyme in this report differs from the original numbering suggested by Kulkarni et al. (*26*). The leader sequence to the mitochondrial enzyme has been deleted in preparing the cDNA for expression (*27*), and thus, 12 must be subtracted from the numbering given in the previous reference. The numbering used in this article will be used for all mutagenesis and other studies. The His tag and linker are not counted in the numbering.

Hlavaty and Nowak (*45*) have identified a consensus metal binding sequence present in PEPCK, mitochondrial ICDH, and malic enzyme. The sequence is XXDDXX, where X is an uncharged or hydrophobic residue. Two such sequences exist in the malic enzyme. A sequence, QFEDFA, is seen in the malic enzyme sequence at positions 269−274. The ED in this sequence is completey conserved. However, mutation of D272 to alanine resulted in no change in K_{Mg} with the most significant effect being a decrease in V/E_t of about 200-fold. In addition, mutation of E271 gives an apparently unstable enzyme. Consequently, it appears that neither E271 nor D272 is a metal ion ligand, nor do they play a significant role in catalysis.

The sequence of amino acids at positions 292−297 is FNDDIQ and also conforms to the consensus metal binding sequence. A kinetic characterization of the D295A mutant indicates there is a change in the order of addition of malate and Mg^{2+} such that malate binds prior to the metal ion, likely due to the low binding affinity of the metal for the free enzyme. The large increase in K_{malate} (undefined) reflects the greatly decreased affinity of malate for the free enzyme. However, the relative decrease in affinity for malate must be less than that for Mg^{2+} since malate selectively binds to the enzyme prior to the metal ion. Karsten et al. (*46*), based on the inhibition constants for a number of malate analogues, showed that a major determinant for malate binding is the bound metal ion. The K_{Mg} for D295A is also undefined and likely quite high, but for the mutant, this value reflects Mg binding to E−malate rather than to free enzyme as in the wild-type malic enzyme. Significant increases in K_{malate} and K_{Mg} are also observed for the D295S mutant (data not shown). Some of the metal ion binding affinity is recovered in the D295C mutant where the K_{Mg} and K_{malate} are both increased by about 10-fold compared to those of the wild-type enzyme. The cysteine sulfur can likely replace the carboxylate group of aspartate as a metal ligand in D295C.

Little change is seen in K_{malate} or K_{NAD} for D178A, but a 50-fold increase in K_{Mg} is observed, consistent with a role in metal binding for D178. An approximately 100-fold decrease in V/E_t is also observed for D178A perhaps due to orientational changes for malate in the active site due to the alanine substitution. Thus, mutation to alanine of both of the amino acid residues suggested that, by identity to the pigeon liver NADP-malic enzyme, metal ion ligands give results that are consistent with their suspected role.

NAD-Binding Site. Kulkarni et al. (*26*), on the basis of sequence alignments, identified residues in NAD binding in the *Ascaris* malic enzyme in residues 327−361. D361 is located at the C-terminal end of the identified NAD binding region. The D361A mutant has relatively little effect on V/E_t, K_{malate}, and K_{Mg}, but a greater than 20-fold increase in K_{NAD}, supporting the role of D361 in NAD binding. NAD binding also appears to be affected by changing E440 to alanine, with a 3 order of magnitude decrease in V/K_{NAD}.

Finally, D294 is conserved in 25 of 26 aligned malic enzyme sequences with the one exception being a valine substitution in *Amaranthus hypochondriacus*. To assess the importance of this nearly conserved residue, D294 was converted to alanine and cysteine. The D294A mutant has about 0.2% (V/E_t) of the activity of the wild-type enzyme, and there is an even greater decrease in V/K_{NAD} (4000-fold) which is a result of the decrease in V and an increase in K_{NAD} of about 7-fold. Much of the activity is recovered in the D294C mutant where V/E_t is reduced to about 6% of the wild-type value. The K_{NAD} in D294C is actually 10-fold lower than that of the wild-type enzyme, while V/K_{NAD} is 69% that of the wild-type enzyme. Thus, D361 and E440 are likely involved in the NAD binding site. In addition, D294 could also be implicated in NAD binding, given the required proximity that must exist between the nicotinamide ring of NAD and C-2 of malate which form the reaction coordinate for hydride transfer.

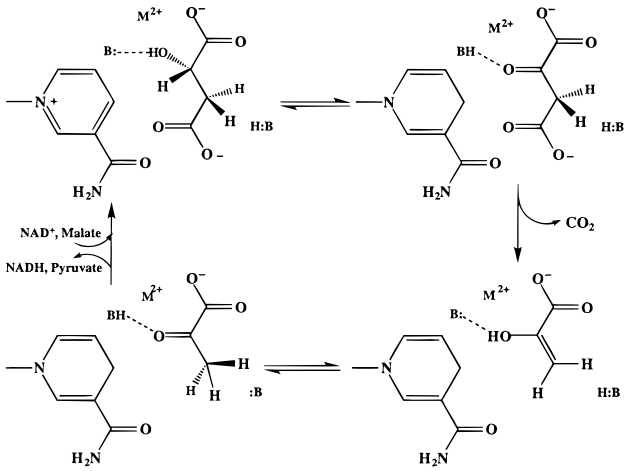

FIGURE 1

Malate Binding Site. Significant decreases in V/K_{malate}, about 1000-fold, are observed with changes at E58 and D272. Both residues are in the N-terminal domain and are likely involved in malate binding.

Candidates for the General Base. A proposed mechanism for the malic enzyme reaction is given in Figure 1. Mutation of the general base catalyst is expected to yield a significant decrease in the value of V/E_t and an increase in K_{malate}. How much of a decrease in V/E_t is expected in the case of the malic enzyme is problematic. Mutation of the general base in the Δ^5-3-ketosteroid isomerase gave a rate decrease of about 400000-fold (*47*). A recently obtained estimate of general base catalysis in the 6-phosphogluconate dehydrogenase reaction, a reaction homologue of the malic enzyme, is 10000-fold. Neither of these cases is a proper model for the malic enzyme reaction, however, which is thought to utilize Mg^{2+} as a Lewis acid, directly coordinated to the hydroxyl of malate. The Lewis acid could certainly be expected to stabilize any negative charge that develops in the transition state, so the advantage derived from general base catalysis may be significantly lower than either of the examples cited above. However, one might also expect a decrease in the affinity for malate and/or the metal ion in addition to changes in V/E_t. On the basis of these criteria, the best candidate for the general base is D295, which gives about 10^4- and 10^7-fold decreases in V/E_t and $V/K_{malate}E_t$, respectively, and an increase in K_{Mg}, whether the mutation is to A or C, although the former leads to much greater changes in kinetic parameters. Also, as stated, there is an analogy to the situation in the ICDH reaction, which is very similar to that catalyzed by malic enzyme. Dean and Dvorak (*7*) have suggested that in ICDH one of the metal ion ligands, D283, is positioned to act as a catalytic base abstracting a proton from the α-hydroxyl group during dehydrogenation.

Conclusions. A number of conserved aspartate and glutamate residues have been located in the N- and C-terminal domains of the NAD-malic enzyme. Alanine-scanning site-directed mutagenesis has been used to characterize the role of the conserved residues, and all those residues that have been characterized appear to function in catalysis and reactant binding. A reasonable candidate for the general base in the malic enzyme reaction is D295, which also may serve as a metal ion ligand. The mutants must now be further characterized to determine their exact role.

REFERENCES

1. Tipton, P. (1993) *Biochemistry 32*, 2822−2827.
2. Park, S.-H., Kiick, D. M., Harris, B. G., and Cook, P. F. (1984) *Biochemistry 23*, 5446−5454.
3. Mallick, S., Harris, B. G., and Cook, P. F. (1991) *J. Biol. Chem. 266*, 2732−2738.
4. Uhr, M. L., Thompson, V. W., and Cleland, W. W. (1974) *J. Biol. Chem. 249*, 2920−2927.
5. Northrop, D. B., and Cleland, W. W. (1974) *J. Biol. Chem. 249*, 2928−2931.
6. Berdis, A. J., and Cook, P. F. (1993) *Biochemistry 32*, 2036−2040.
7. Dean, A. M., and Dvorak, L. (1995) *Protein Sci. 4*, 2156−2167.
8. Hsu, R. Y., and Lardy, H. A. (1967) *J. Biol. Chem. 242*, 520−526.
9. Schimerlik, M. I., and Cleland, W. W. (1977) *Biochemistry 16*, 566−571.
10. Weiss, P. M., Gavva, S. R., Urbauer, J., Harris, B. G., Cleland, W. W., and Cook, P. F. (1991) *Biochemistry 30*, 5755−5763.
11. Schimerlik, M. I., Grimshaw, C. E., and Cleland, W. W. (1977) *Biochemistry 16*, 571−576.
12. Kiick, D. M., Harris, B. G., and Cook, P. F. (1986) *Biochemistry 25*, 227−235.
13. Cook, P. F., and Cleland, W. W. (1981) *Biochemistry 20*, 1797−1805.
14. Grissom, C. B., and Cleland, W. W. (1988) *Biochemistry 27*, 2927−2934.
15. Berdis, A. J., and Cook, P. F. (1993) *Biochemistry 32*, 2041−2046.
16. Hermes, J. D., Roeske, C. A., O'Leary, M. H., and Cleland, W. W. (1982) *Biochemistry 21*, 5106−5114.
17. Rendina, A. R., Hermes, J. D., and Cleland, W. W. (1984) *Biochemistry 23*, 6257−6762.
18. Hurley, J. H., Dean, A. M., Koshland, D. L., Jr., and Stroud, R. M. (1991) *Biochemistry 30*, 8671−8678.
19. Hurley, J. H., Thorsness, P., Ranalingham, D. E., Helmers, N., Koshland, D. E., Jr., and Stroud, R. M. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 8635−8639.
20. Imada, K., Sato, M., Tanaka, N., Katsube, Y., Matsuura, Y., and Oshima, T. (1991) *J. Mol. Biol. 222*, 725−738.
21. Thorsness, P. E., and Koshland, D. E., Jr. (1987) *J. Biol. Chem. 262*, 10422−10425.
22. Lee, M. E., Dyer, D. H., Klein, O. D., Bolduc, J. M., Stoddard, B. L., and Koshland, D. E., Jr. (1995) *Biochemistry 34*, 378−384.
23. Kadono, S., Sakurai, M., Moriyama, H., Sato, M., Hayashi, Y., Oshima, T., and Tanaka, N. (1995) *J. Biochem. 118*, 745−752.
24. Villafranca, J. J., and Colman, R. F. (1974) *Biochemistry 13*, 1152−1160.
25. Pontremoli, S., de Flora, A., Grazi, E., Mangiarotti, G., Bonsignore, A., and Horecker, B. L. (1961) *J. Biol. Chem. 236*, 2975−2980.
26. Kulkarni, G., Cook, P. F., and Harris, B. G. (1993) *Arch. Biochem. Biophys. 300*, 231−237.
27. Chooback, L., Karsten, W. E., Nalabolu, S. R., Kulkarni, G., Harris, B. G., and Cook, P. F. (1997) *Protein Expression Purif. 10*, 51−54.
28. Clancy, L. L., Rao, G. S. J., Finzel, B. C., Muchmore, S. W., Holland, D. R., Watenpaugh, K. D., Krishnamurthy, H. M., Sweet, R. M., Cook, P. F., Harris, B. G., and Einspahr, H. M. (1992) *J. Mol. Biol. 226*, 565−569.
29. Chooback, L., Price, N. E., Karsten, W. E., Nelson, J., Sundstrom, P. R., and Cook, P. F. (1998) *Protein Expression Purif. 13*, 251−258.
30. Karsten, W. E., and Cook, P. F. (1994) *Biochemistry 33*, 2096−2103.
31. Cleland, W. W. (1979) *Methods Enzymol. 63*, 103−138.
32. Bagchi, S., Wise, L. S., Brown, M. L., Bregman, D., Sul, H. S., and Rubin, C. S. (1987) *J. Biol. Chem. 262*, 1558−1565.
33. Kobayashi, K., Doi, S., Negoro, S., Urabe, I., and Okada, H. (1989) *J. Biol. Chem. 264*, 3200−3205.
34. Stoddard, B. L., Dean, A., and Koshland, D. E., Jr. (1993) *Biochemistry 32*, 9310−9316.
35. Stoddard, B. L., and Koshland, D. E., Jr. (1993) *Biochemistry 32*, 9317−9322.
36. Corpet, F., Gouzy, J., and Kahn, D. (1999) *Nucleic Acids Res. 27*, 263−267.
37. Fischer, D., and Eisenberg, D. (1996) *Protein Sci. 5*, 947−955.
38. Gavva, S. R., Weiss, P. M., Harris, B. G., and Cook, P. F. (1991) *Biochemistry 30*, 5764−5769.
39. Satterlee, J., and Hsu, R. Y. (1991) *Biochim. Biophys. Acta 1079*, 247−252.
40. Hsu, R. Y., Glynias, M. J., Satterlee, J., Feeney, R., Clarke, A. R., Emery, D. C., Roe, B. A., Wilson, R. K., and Goodridge, A. G. (1992) *Biochem. J. 284*, 869−876.
41. Chou, W.-Y., Liu, M.-Y., Huang, S.-M., and Chang, G.-G. (1996) *Biochemistry 35*, 9873−9879.
42. Wei, C.-H., Chou, W.-Y., Huang, S.-M., Lin, C. C., and Chang, G.-G. (1994) *Biochemistry 33*, 1931−1936.
43. Wei, C.-H., Chou, W.-Y., and Chang, G.-G. (1995) *Biochemistry 34*, 7949−7954.
44. Chou, W.-Y., Tsai, W.-P., Lin, C.-C., and Chang, G.-G. (1995) *J. Biol. Chem. 270*, 25935−25941.
45. Hlavaty, J. J., and Nowak, T. (1997) *Biochemistry 36*, 3389−3403.
46. Karsten, W. E., Gavva, S. R., Park, S.-H., and Cook, P. F. (1995) *Biochemistry 34*, 3253−3260.
47. Kuliopulos, A., Talalay, P., and Mildvan, A. S. (1990) *Biochemistry 29*, 10271−10280.
48. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) *Nucleic Acid Res. 22*, 4673−4680.
49. Jia, Y. (1998) Ph.D. Dissertation, University of Maryland, College Park, MD.
50. Bradford, M. (1976) *Anal. Biochem. 72*, 248−254.

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