Lysine 199 Is the General Acid in the NAD-Malic Enzyme Reaction†

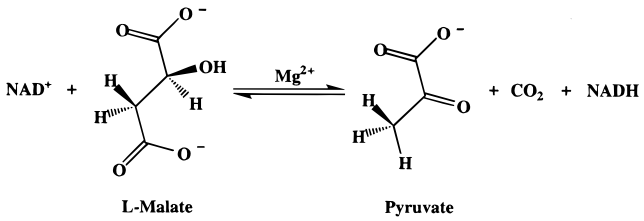
Dali Liu, William E. Karsten, and Paul F. Cook*

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

Received April 7, 2000; Revised Manuscript Received July 17, 2000

ABSTRACT: Site-directed mutagenesis was used to change K199 in the *Ascaris suum* NAD-malic enzyme to A and R and Y126 to F. The K199A mutant enzyme gives a 10^5-fold decrease in V and a 10^6-fold decrease in V/K_{malate} compared to the WT enzyme. In addition, the ratio for partitioning of the oxalacetate intermediate toward pyruvate and malate changes from a value of 0.4 for the WT enzyme to 1.6 for K199A, and repeating the experiment with A-side NADD gives isotope effects of 3 and 1 for the WT and K199A mutant enzymes, respectively. The K199R mutant enzyme gives only a factor of 10 decrease in V, and the pK for the general acid in this mutant enzyme has increased from 9 for the WT enzyme to >10 for the K199R mutant enzyme. Tritium exchange from solvent into pyruvate is catalyzed by the WT enzyme, but not by the K199A mutant enzyme. The Y126F mutant enzyme gives a 10^3-fold decrease in V. The oxalacetate partition ratio and isotope effect on oxalacetate reduction for the Y126F mutant enzyme are identical, within error, to those measured for the WT enzyme. Thus, Y126 is important to the overall reaction, but its role at present is unclear. Data are consistent with K199 functioning as the general acid that protonates C3 of enolpyruvate to generate the pyruvate product in the malic enzyme reaction.

Malic enzyme (EC 1.1.1.39) catalyzes the reversible oxidative decarboxylation of L-malate to pyruvate and CO_2 with the concomitant generation of NAD(P)H[1] (*1*). In the parasite *Ascaris suum*, the NAD-malic enzyme plays a central role in the production of reducing equivalents for the production of short branched-chain fatty acids, an end-product of metabolism (*47*). The adult worm has an anaerobic metabolism necessitated by its physiologic niche in the intestine, and the end-product of glycolysis is L-malate, with PEP first converted to oxalacetate by PEP carboxykinase and then reduced to malate by malate dehydrogenase. The malate produced is then transported into the mitochondrion where it serves as a substrate for the malic enzyme.



Isotope effect data have shown that the oxidative decarboxylation of L-malate to enolpyruvate proceeds via a stepwise mechanism with hydride transfer preceding decarboxylation (*2*−*5*). A general base/general acid mechanism has been suggested (Scheme 1) based on the pH dependence of kinetic parameters, for the oxidative decarboxylation of L-malate (*6*) and the decarboxylation of oxalacetate (*7*). The general base has a pK of 5, while the general acid has a pK of 9 (*6*, *7*). The general base accepts a proton from the 2-hydroxyl of L-malate concomitant with hydride transfer and then shuttles the proton between itself and the C2 oxygen throughout the reaction, ultimately accepting it as pyruvate is formed. The general acid presumably plays a role in only the last of three steps, viz., the tautomerization of the enol of pyruvate to the keto product.

Recent data have suggested that D295[2] likely functions as the general base in the *Ascaris suum* NAD-malic enzyme reaction (*8*). The carboxylate of the D295 side chain is in the general vicinity of where L-malate is expected to bind in the three-dimensional structure of the homologous enzyme from human mitochondria (*9*). Based on the pK of 9, and the presence of lysine in other β-hydroxyacid oxidative decarboxylases, we reasoned that a lysine likely served as the general acid in the malic enzyme reaction. Sequence alignment of all of the known sequences of malic enzyme from a wide variety of species indicates a single lysine (K199) is conserved in all species (Table 1). The homologous lysine is found in the active site of the human mitochondrial NAD-malic enzyme (*9*). In addition, a conserved tyrosine residue is present in the isocitrate and isopropylmalate dehydrogenases (*10*) and may be catalytically important in the isocitrate dehydrogenase reaction (*11*).

In this manuscript, site-directed mutagenesis is used to change lysine 199 to alanine and arginine, and to change

† This work was supported by a grant from the Oklahoma Center for the Advancement of Science to P.F.C. and funds for P.F.C. from an endowment to the University of Oklahoma to fund the Grayce B. Kerr Centennial Professorship in Biochemistry.

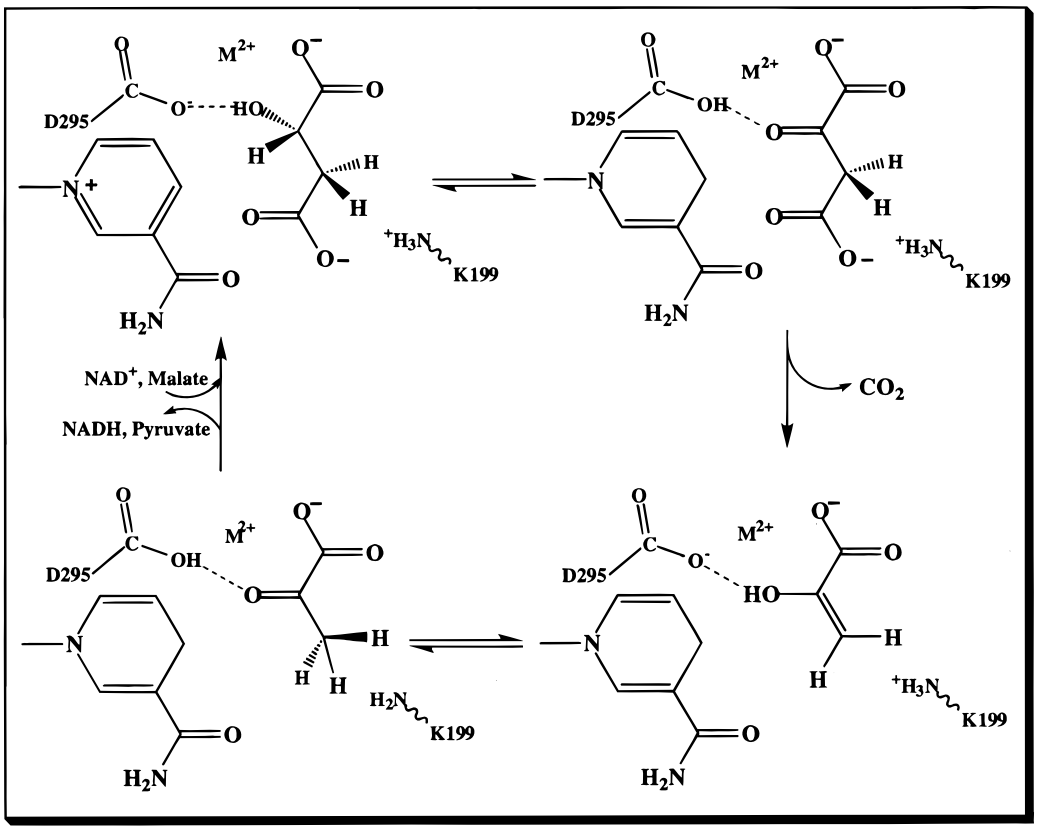
* To whom correspondence should be addressed. Fax: (405) 325-7182. E-mail: pcook@chemdept.chem.ou.edu.

[1] Abbreviations: 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; EDTA, ethylenediaminetetraacetate.

[2] Numbering of residues in the *Ascaris suum* NAD-malic enzyme is that used in ref *8*.

10.1021/bi000790p CCC: $19.00 © 2000 American Chemical Society
Published on Web 09/01/2000

Scheme 1: Proposed Acid−Base Chemical Mechanism for Malic Enzyme[a]



[a] No geometry is implied by the malate conformation shown.

Table 1: Sequence Alignment around the Active Site Lysine and Tyrosine

source	sequence around K199	sequence around Y127	ref
Populus trichocarpa	CQGIGIPVG**K**LSLYTAL	EELLPVV**Y**TPTVGEA	*19*
grape	CQGMGIPVG**K**LSLYTAL	EELLPVV**Y**TPTVGEA	*20*
Flaveria pringlei	CQGMGIPVG**K**LSLYTAL	EELLPIV**Y**TPTVGEA	*21*
Flaveria trinervia	CQGMGIPVG**K**LALYTAL	EELLPIV**Y**TPTVGEA	*22*
tomato	CQGMGIPVG**K**LSLYTAL	EELLPIV**Y**TPTVGEA	*23*
kidney bean	CQGMGIPVG**K**LSLYTAL	AELLPVV**Y**TPTVGEA	*24*
Ice plant	CQGMGIPVG**K**LSLYTAL	EELLPLV**Y**TPTVGEG	*25*
maize	CQGMGIPVG**K**LSLYTAL	VELLPFV**Y**TPTVGEG	*26*
rice	CYGMGIPVG**K**LALYTAC	EELLPVV**Y**TPTVGEA	*27*
duck	CYGMGIPVG**K**LALYTAC	ERFMPIV**Y**TPTVGLA	*28*
pigeon	CNGMGIPVG**K**LALYTAC	ERFMPIV**Y**TPTVGLA	*29*
pig	CNGMGIPVG**K**LALYTAC	EKFMPIV**Y**TPTVGLA	*30*
mouse	CYGMGIPVG**K**LALYTAC	EKFMPIV**Y**TPTVGLA	*31*
rat	CYGMGIPVG**K**LALYTAC	EKFMPIV**Y**TPTVGLA	*32*
human cytosolic	CYGMGIPVG**K**LALYTAC	EKFMPIV**Y**TPTVGLA	*33*
human mitochondrial (NADP)	CYGMGIPVG**K**LALYTAC	EKFMPIV**Y**TPTVGLA	*34*
human mitochondrial (NAD)	VYGMGIPVG**K**LCLYTAC	ESLMPIV**Y**TPTVGLA	*35*
Amaranthus hypochondriacus	VHGIGVAIG**K**LDLYVAA	EEYAPIVSTPTVGLV	*36*
potato 62 kDa isoform	IQGIGIAIG**K**LDLYVAA	EEYAPIV**Y**TPTVGLV	*37*
potato 59 kDa isoform	VQGIGIPIG**K**LDMYVAA	KDFAPII**Y**TPTVGLV	*37*
Mycobacterium tuberculosis	VGGIQIAVG**K**LALYTAG	PELMPVV**Y**TPTVGEA	*38*
fission yeast	VGGVLISVA**K**GHLMTLC	IEMIPII**Y**TPTEGDA	*39*
bakers yeast	IGGVRIAIS**K**LALMTLC	KELVPII**Y**TPTEGDA	*40*
Escherichia coli	GGMGIPIG**K**LSLYTAC	DEMMPVI**Y**TPTVGAA	*41*
Bacillus subtilis	GMPVMEG**K**AALFDQL	LAELGRV**Y**TPGVADV	*42*
Bacillus stearothermophilus	AMPVMEG**K**AMLEKEF	RDDLSRV**Y**TPGVARV	*43*
Haemophilus influenzae	LAGKPVMEG**K**GVLFKK	QRDLALA**Y**SPGVAEP	*44*
Ascaris suum	YGIGIPVG**K**LALYVAL	KELMPIV**Y**TPTVGLA	*45*

tyrosine 126 to phenylalanine. Data support the assignment of the lysine side chain at position 199 in the *Ascaris suum* malic enzyme as a general acid. Implication of the assignment is discussed. During the time these studies were completed, the three-dimensional structure of the human mitochondrial malic enzyme with NAD^+, oxalate, and Mn^{2+} in the active site was published (*12*) and corroborates the assignments made in this manuscript.

MATERIALS AND METHODS

Chemicals and Enzymes. Deoxynucleotide triphosphates were from Perkin-Elmer, while IPTG and restriction endonucleases were from GIBCO BRL. The fmol^R DNA cycle sequencing kit, T_4 DNA ligase, T_4 kinase, Taq Plus DNA polymerase, protein molecular mass markers, and *Escherichia coli* strain JM109 were from Promega. Protein concentrations were determined according to Bradford using the Bio-Rad protein assay kit with bovine serum albumin as a standard (*13*). The ampicillin, kanamycin, Dowex AG 1X8-2000, lactate dehydrogenase, Na_4EDTA, and NADPH were from Sigma. Hepes buffer was from Research Organics. The DNA molecular weight ladder was purchased from New England Biolabs. The Orange-A agarose was from Amicon. Mutagenesis and sequencing primers were purchased either from GIBCO BRL or Promega. Site-directed mutagenesis was performed using the Altered sites mutagenesis kit from Promega. The QIA express System, which contained the pQE-30 vector, *E. coli* strain M15 and the Ni−NTA matrix was from QIAGEN. Malic enzymes were purified according to Karsten et al. (*8*). All other chemicals used were the highest quality available and were used without further purification.

Site-directed mutagenesis, subcloning into the pQE30 vector, expresssion of mutant enzymes, and purification were carried out as in ref *8*. Primers utilized for the K199A, K199R, and Y126F mutants are as follows: 5′-GTATCCCGGGC**GCG**CTAGCCCTTTATG-3′, 5′-GTATCCCGGGCA**GG**CTAGCCCTTTATG-3′, and 5′-AATGCCAATTGTC**TTT**ACGCCCACTGTTGG-3′. The site of mutation is in bold.

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 (ϵ_{340} = 6220 M^{-1} cm^{-1}). 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 μM) or 2 mM free NAD^+ and variable free Mg^{2+} (10−100 mM) and free malate (0.25−10 mM) (*14*). Initial velocity data were fitted with the appropriate rate equation and a BASIC version of the Fortran programs developed by Cleland (*15*). Saturation curves obtained as a function of pH for the K199R mutant enzyme were fitted using eq 1. Data conforming to a sequential initial velocity pattern were fitted using eq 2

$$v = VA/(K_a + A) \quad (1)$$

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

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

The ratio of partitioning of the oxalacetate intermediate toward pyruvate or malate was measured according to Karsten and Cook (*5*), after Grissom and Cleland (*46*).

Tritium Exchange. Exchange of tritium from ^3H_2O into pyruvate was measured according to Bratcher and Hsu (*48*). Exchange reactions were carried out at pH 7 and 25 °C. A typical reaction mixture contained the following in a final volume of 1 mL: 100 mM Hepes, pH 7; 2.6 × 10^9 cpm ^3H_2O; 100 mM pyruvate; 10 mM $MnSO_4$; 5 mM $NaHCO_3$; 1 mM NADH, and 600 μg (∼8 μM) *Ascaris suum* NAD malic enzyme (either the wild-type or the K199A mutant enzyme). Reactions were allowed to proceed for 2 h at room temperature, and were quenched by the addition of 0.1 mL of 1 M EDTA to a 0.2 mL aliquot of the reaction mixture. Parallel reactions monitored spectrophotometrically were arrested upon addition of the EDTA.

The terminated reaction mixture was then applied to a 0.9 × 2 cm Dowex AG 1X8-2000 column. The column was washed extensively to remove unreacted ^3H_2O. Pyruvate, lactate and malate were eluted with 0.05 N HCl, and 6 mL fractions were collected. Radioactivity in the 6 mL fractions was measured using a Packard Tri-Carb 2100 TR liquid scintillation counter. The amount of pyruvate in the sample was estimated by enzymatic endpoint using lactate dehydrogenase.

Structural Characterization. The structural integrity of the mutant proteins was determined grossly using the far-UV CD spectrum measured using an AVIV 62 DS circular dichroism spectrophotometer.

RESULTS

Mutant Isolation and Structural Characterization. The mutant genes were subcloned into pQE expression vectors in order 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 (*8*). The pQE.ME gave good expression, and the resulting enzyme 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.

To determine whether the mutations affected resulted in a loss of overall structural integrity, far-UV CD spectra were measured for all mutant proteins. In all cases, the mutant proteins were expressed at a level equal to that of the WT enzyme. In all cases, the far-UV spectra were superimposable once corrected to the same protein concentration (data not shown). Thus, changes in structure, if any, must be localized to the active site.

Initial Velocity Studies. The kinetic parameters of the wild-type and mutant malic enzymes are summarized in Table 2. Lysine 199 was changed to either A, which is expected to eliminate its function, or to R, which is expected to modulate its function. Neither change affects the binding of NAD as indicated by K_{NAD},[3] but the binding affinity for malate is decreased by a factor of 6−7.5. The K199A mutant shows drastically reduced activity with V/E_t, and both V/K values decreased by a factor of 10^5−10^6. Much of the loss in activity is regained by preparing the K199R mutant, which exhibits a modest decrease of 10-fold in V and V/K_{NAD}, and a slightly greater 75-fold decrease in V/K_{malate}. In addition, although the pH dependence of V/K_{malate} for the WT enzyme exhibits a pK of 9 on the basic side of its pH-rate profile, the V and V/K_{malate} pH-rate profiles for the K199R mutant enzyme are independent of pH from 7 to 10 (data not shown).

[3] K_{NAD} is the dissociation constant for the E:NAD:Mg:malate complex (*6*).

Table 2: Kinetic Constants of Mutants at K199 and Y126 in NAD-Malic Enzyme[a]

	K_{mal} (mM)	K_{NAD} (mM)	V/E_t (s^{-1})	$V/K_{mal}E_t$ (M^{-1} s^{-1})	$V/K_{NAD}E_t$ (M^{-1} s^{-1})
WT[b]	0.53 ± 0.07	0.035 ± 0.007	36	7×10^4	1×10^6
K199A	3 ± 1	0.034 ± 0.003	$(2.7 \pm 0.4) \times 10^{-4}$	0.08 ± 0.02	8 ± 0.5
	(6)	(−)	(1.3×10^5)	(8×10^5)	(1.2×10^5)
K199R	3.8 ± 0.4	0.032 ± 0.003	3.5 ± 0.1	930 ± 65	$(1 \pm 0.1) \times 10^5$
	(7.5)	(−)	(10)	(75)	(10)
Y126F	5.1 ± 0.9	0.088 ± 0.017	0.023 ± 0.001	4.6 ± 0.6	$(2.6 \pm 0.4) \times 10^2$
	(10)	(2.5)	(1.5×10^3)	(1.5×10^4)	(4×10^3)

[a] Values in parentheses are approximated fold-increase for K_m and fold-decrease for V and V/K. [b] Data were collected at pH 7, 100 mM Hepes, and 25 °C. Data for the WT enzyme are from ref *8*.

Table 3: Pyruvate/Malate Partition Ratios for Oxalacetate Partitioning for the NAD-Malic Enzyme from Ascaris suum[a]

enzyme	r_H	r_D	r_H/r_D
wild-type	0.4 ± 0.1	1.2 ± 0.1	0.33 ± 0.08
K199A	1.60 ± 0.25	1.6 ± 0.4	1.0 ± 0.3
Y126F	0.30 ± 0.15	1.0 ± 0.2	0.30 ± 0.17

[a] The partition ratios reported are the average of triplicate determinations.

The Y126F mutant enzyme exhibits modest changes in the affinity for NAD (2.5-fold) and malate (10-fold). The decreases in V and V/K_{NAD} are about 10^3-fold, while that in V/K_{malate} is about 10^4-fold, and all are 2 orders of magnitude less than the changes observed with the K199A mutant enzyme.

Oxalacetate Partitioning Studies. Partitioning of the oxalacetate intermediate in the E:NADH:Mg:oxalacetate complex toward either the E:NADH:Mg:enolpyruvate or E:NAD:Mg:malate complexes was measured for the WT, K199A, and Y126F mutant enzymes, Table 3. The partition ratio, measured as the ratio of the rates of formation of pyruvate and malate, is 0.4 ± 0.1 for the WT enzyme, in agreement with the ratio measured by Karsten and Cook (*5*) previously. The partition ratios (r_H) for the K199A and Y126F mutant enzymes are 1.60 ± 0.25, and 0.30 ± 0.15, respectively. Thus, although mutation of Y126 does not substantially alter the rates of hydride transfer and decarboxylation relative to one another, mutation of K199 preferentially decreases the rate of oxalacetate reduction with respect to its decarboxylation.

Measurement of the partition ratio for the WT enzyme with A-side NADD gives an r_D of 1.2 ± 0.1 and an isotope effect (r_H/r_D) of 0.30 ± 0.08, in agreement with the data of Karsten and Cook (*5*). The isotope effect on r_H/r_D reflects an effect of 3.3 ± 0.9 on the reduction of oxalacetate to malate (*5*). Partition ratios and isotope effect data for the K199A and Y126F mutant enzymes are given in Table 3. The isotope effects on the reduction of oxalacetate are 1.0 ± 0.3 and 3.0 ± 1.6 for the K199A and Y126F mutant enzymes, respectively.

Tritium Exchange. The enzyme-catalyzed exchange of tritium from solvent into pyruvate was measured for the WT and K199A mutant enzymes. A minus enzyme control gave 8.7×10^4 cpm in 2 h in the pyruvate, lactate, and malate mixture (corrected to the 1 mL reaction volume). In the same time period, the WT enzyme was responsible for incorporating 3.95×10^5 cpm into the organic acid mixture, while the K199A mutant enzyme incorporated 8.3×10^4 cpm. The amount of remaining pyruvate estimated in the three reaction mixtures was 93, 96, and 94 mM, respectively. Denaturing the WT or K199A mutant enzymes made no difference in the exchange rate, with values of 8.6×10^4 and 8.7×10^4 cpm, respectively, for the 2 h incubation. A repeat of the above experiment at a slightly longer time gave 4.6×10^5, 1.2×10^5, and 1.1×10^5 cpm for the WT, K199A mutant enzyme, and the minus enzyme control, respectively; and 1.3×10^5 and 1.2×10^5 cpm for the denatured WT and K199A mutant enzyme controls. Replacing malic enzyme with an equimolar amount of BSA gave counts identical to the minus enzyme control value.

DISCUSSION

Lysine 199 is the General Acid. Replacement of the completely conserved lysine 199 with alanine decreases the rate of the overall reaction by a factor of 10^5. Much of the activity lost (10^4-fold) by changing the lysine to alanine is regained by replacing the lysine with arginine. This behavior is predicted for lysine as a general acid, since the arginine can also serve in the same capacity as a general acid. It is further predicted that the pK for arginine as a general acid would be increased from 9 to 11, given the difference in the pK values for the ϵ-amine of lysine (10.5) and the δ-guanidino group of arginine (12.5). The pH dependence of V for the K199R mutant is identical to that obtained for the WT enzyme (*6*), and reflects binding of malate to the correctly protonated form of the enzyme, that is with the general base unprotonated and the general acid protonated, but with a pH dependent conformational change of the E:NAD complex that gives a pK of 5 (*6*, *16*, *17*). The V/K_{malate} profile for the WT enzyme exhibits a pK of 9 for the general acid, while the V/K_{malate} pH-rate profile for the K199R mutant enzyme is pH independent from 7 to 10. Data are consistent with the expected perturbation of the general acid pK to about 11 (data cannot be collected above pH 10 because of a decreased stability of the enzyme). Tritium exchange from solvent into pyruvate is catalyzed by the malic enzyme general acid (*48*). Although significant exchange is observed in the case of the WT enzyme (~4.2−4.5-times the control), no exchange is observed for the K199A mutant enzyme. The incorporation of tritium in the minus enzyme (or denatured enzyme) controls results from the Mn-catalyzed enolization of pyruvate. Data are thus fully consistent with the general acid role of K199.

The partition ratio for the oxalacetate intermediate increases in favor of pyruvate by a factor of 4 for the K199A mutant enzyme compared to the WT enzyme, that is decarboxylation is favored with respect to reverse hydride transfer. It is very likely that K199 donates a hydrogen bond

to the 4-carboxyl of malate and thus contributes to its binding affinity. The proposed interaction with malate would place a positive charge in the vicinity of the β-carboxyl, and this would certainly hinder decarboxylation. The latter hydrogen-bonding scheme is observed in the 6-phosphogluconate dehydrogenase reaction with the general acid (E190) hydrogen-bonded to the 1-carboxyl of 6PG in the E:NADP:6PG complex (*18*). The hydrogen bond has been eliminated in the E:NADPH:3-keto-6PG complex (*18*). The same may be true in the case of the malic enzyme reaction, and this is corroborated by the recent structure of the E:NAD:Mn:oxalate complex of the human mitochondrial enzyme,[4] which shows K183 (homologous to K199 of the *Ascaris* enzyme) within hydrogen-bonding distance (3.3 Å) to an oxygen of oxalate that would be equivalent to C3 of malate (*12*). The lack of a deuterium isotope effect on the reduction of oxalacetate suggests that the hydride transfer step no longer limits the overall rate. Rather, a step after hydride transfer, probably an isomerization of the E:NAD:Mg:malate complex to release reactants, likely limits. The presence of such an isomerization is documented by the difference in structure of the E:NAD (*9*) and E:NAD:Mn:oxalate (*12*) complexes, respectively. That the effect of mutating K to A is more pronounced on the isomerization than on the catalytic step(s) is suggestive of a dual role of the lysine. The residue may be in the "hinge" region and thus intimately involved in the isomerization. There are a number of examples from the literature for catalytic residues that are found in the hinge region of loops that close to generate the catalytic conformation. Enzymes in this category include loops in triosephosphate isomerase [TIM (*49*)], the α-subunit of tryptophan synthase (*50*), enolase (*51*), ribulose bisphosphate carboxylase (*52*), and *Yersenia* protein tyrosine phosphatase (*53*−*55*). The best-documented example is that of TIM (*49*), which has a catalytic loop (residues 166−176) comprised of a 3 amino acid N-terminal hinge, a 5 amino acid hydrophobic lid, and a 3 amino acid C-terminal hinge (*56*). The catalytic general base, E165, is adjacent to the N-terminal hinge. Sampson and Knowles (*49*) originally suggested that the loop closes upon formation of the Michaelis complex and closure is linked to orientation of active site residues and substrate functional groups, with the concomitant deprotonation of C-1 of dihydroxyacetone phosphate by E165.

A similar arrangement may be true in the case of the *Ascaris* malic enzyme. Indeed, K183 (the human NAD-malic enzyme equivalent of K199) is found toward the C-terminal end of helix αB2 (*9*). The B-domain is comprised of two subdomains that include residues 131−277 and 467−538. The catalytic lysine is in the vicinity of the subdomain interface of the B-domain and at the juncture of the B- and C-domains. Closure of the open form to generate the catalytic conformation will be centered around the juncture of B- and C-domains. The small effect on K_{malate} may indicate a nearly equal affinity for opened and closed (*catalytic*) forms of the enzyme.

[4] Although the human and *Ascaris* NAD-malic enzyme structures are not identical, the active site residues are conserved and the active site structure is very similar in both cases (unpublished results). The distance between lysine and the oxalate oxygen in the human can thus be taken as evidence consistent with, but not proof for, the proposed role of K199 in the *Ascaris* enzyme.

One would expect a complete regain in activity when comparing the K199A and K199R mutant enzymes, if R perfectly complemented the loss of the lysine side chain. The 10-fold decrease in V and 7.5-fold increase in K_{malate}, however, indicates that R is not a perfect mimic, and may interfere with optimum binding as a result of its bulkier side chain.

Tyrosine 126. Tyrosine 126 is conserved in all malic enzymes sequenced to date with one exception, Table 1. The malic enzyme from *Amaranthus hypochondriacus* has a serine in place of the tyrosine. The decrease in activity observed when Y126 is replaced with F is significant, but not as substantial as that observed for the K199A mutant enzyme. The tyrosine is not completely conserved, but is obviously important to the overall reaction. However, its role in the overall reaction is not clear at this juncture. It is unlikely that it serves as the general base because of the following: (1) Y126 is too far away from the position of the C2 hydroxyl [>4 Å (*12*)]; (2) the general base has an intrinsic pK of 5 (*6*), and it is thus unlikely, given the hydrophobic nature of the active site, that the tyrosine pK could be perturbed by 5.5 pH units from its solution pK of 10.5; (3) the partition ratio for oxalacetate and the isotope effect on oxalacetate reduction are identical for the WT and Y126F mutant enzymes. Y126 likely has some effect on the localized structure and/or environment of the malic enzyme active site, but its exact function will have to await further experimentation.

ACKNOWLEDGMENT

We thank the reviewers for suggestions concerning the involvement of K199 in the conformation change to close the site and orient catalytic groups.

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BI000790P