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From Malate Dehydrogenase to Phenyllactate Dehydrogenase

INCORPORATION OF UNNATURAL AMINO ACIDS TO GENERATE AN IMPROVED ENZYME-CATALYZED ACTIVITY*

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Malate dehydrogenase (MDH) from *Escherichia coli* is highly specific for its keto acid substrate. The placement of the active site-binding groups in MDH effectively discriminates against both the shorter and the longer keto dicarboxylic acids that could potentially serve as alternative substrates. A notable exception to this specificity is the alternative substrate phenylpyruvate. This aromatic keto acid can be reduced by MDH, albeit at a somewhat slower rate and with greatly diminished affinity, despite the presence of several substrate-binding arginyl residues and the absence of a hydrophobic pocket in the active site. The specificity of MDH for phenylpyruvate has now been enhanced, and that for the physiological substrate oxaloacetate has been diminished, through the replacement of one of the binding arginyl residues with several unnatural alkyl and aryl amino acid analogs. This approach, called site-specific modulation, incorporates systematic structural variations at a site of interest. Molecular modeling studies have suggested a structural basis for the affinity of native MDH for phenylpyruvate and a rationale for the improved catalytic activity that is observed with these new, modified phenyllactate dehydrogenases.

An approach for the site-specific incorporation of any unnatural amino acid analog into an enzyme is being examined with a method called site-specific modulation that utilizes the inherent reactivity of cysteine to generate systematic structural variations at a site of interest. This approach combines the strength of site-directed mutagenesis (absolute specificity) with the wide range of structural analogs that can be accessed through chemical modifications. The earlier work in this area focused primarily on the regeneration of lysine analogs to restore activity in aspartase aminotranferase (1, 2) and ribulose bisphosphate carboxylase/oxygenase (3) or on arginine analogs to test the role of this functional group in the specificity of glutamine synthetase (4). Instead of the limited goal of replacing the target amino acid with an analog that mimics the natural amino acid, the aim of this approach is to systematically alter the enzyme active site groups that are responsible for substrate recognition and binding. In this way new catalysts can be generated with specificities that are designed for reactions of interest.

Malate dehydrogenase catalyzes the oxidation of malate to oxaloacetate (OAA)[1] with the concomitant reduction of NAD^+ to NADH. This enzyme is highly specific for its natural substrates, malate and OAA, and efficiently discriminates against both shorter and longer dicarboxylic acids. Examination of the crystal structure provides an explanation for the substrate selectivity that is observed with this enzyme (5). Both malate and OAA are proposed to be bound at each end through electrostatic interactions between the two substrate carboxyl groups and two arginyl residues (Arg-81 and Arg-153) that are positioned to interact precisely with a four-carbon dicarboxylic acid (Fig. 1).

Lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) are very similar 2-keto acid dehydrogenases that catalyze related reactions by an equivalent mechanism (5, 6). Despite the overall structural similarities the sequence homology between these functionally related enzyme families is quite low. However, the functional active site amino acids are highly conserved between these enzyme families (7). To examine the specificity of LDH the active site, which is normally specific for pyruvate, has been redesigned to use phenylpyruvate as a substrate by mutagenically switching sections of a mobile loop that is responsible for substrate specificity (8). Mutagenic replacement of an important active site residue in LDH (Gln-102) has also lead to an enzyme form that will utilize phenylpyruvate, with the k_{cat}/K_m for phenylpyruvate improved by a factor of 100 over that of the native enzyme (9). Native MDH has also been shown to be capable of catalyzing the reduction of phenylpyruvate to phenyllactate, although at a rate that is substantially reduced when compared with its physiological substrate OAA (10).

With the observation that MDH can use phenylpyruvate as an alternative substrate, and starting with the mutagenic studies on LDH, our goal was to expand the redesign options that are available by selectively incorporating unnatural amino acids that could potentially enhance the binding and catalysis of phenylpyruvate, using malate dehydrogenase as a structural scaffold. We report here the use of site-specific modulation to convert malate dehydrogenase into an efficient phenyllactate dehydrogenase.

EXPERIMENTAL PROCEDURES

Materials—The pEM 6 plasmid containing the *mdh* gene was a gift from Dr. N.C. Furumo (Eastern Illinois University). All restriction endonucleases and molecular weight markers were purchased from New England Biolabs. Electrophoresis grade agarose was purchased from SeaChem. Primers were obtained from Integrated DNA Technologies, Inc. Native *Pfu* DNA polymerase and the polymerase chain reaction optimization kit were purchased from Stratagene. Affi-blue resin

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[1] The abbreviations used are: OAA, oxaloacetate; LDH, lactate dehydrogenase; MDH, malate dehydrogenase.

was purchased from Bio-Rad. Phenyllactate, 2-bromopropane, 1-bromo-2-methylpropane, benzyl bromide, and 4-fluorobenzyl bromide were obtained from Aldrich. 4-Aminobenzyl alcohol was from Lancaster, and all other chemicals were purchased from Sigma. Purification of MDH was performed according to a previously published scheme (11).

Enzyme and Protein Assays—The activity of malate dehydrogenase was determined spectrophotometrically by measuring either the formation or the disappearance of NADH (E_{340} = 6.22 mM^{-1} cm^{-1}). Standard assay conditions were as follows: 50 mM buffer, 2 mM EDTA, 100 mM KCl, and variable concentrations of the hydroxy or keto acid substrates in the presence of saturating levels of coenzyme (either 0.15 mM NADH or 7 mM NAD$^+$) (12). Each reaction was initiated by the addition of enzyme, and the kinetic data were fitted by using an Enzyme Kinetics software package, adapted from the programs of Cleland (13), to obtain the kinetic parameters. Protein concentration was determined by the Bradford method (14) using bovine serum albumin as a standard.

Mutagenic Method—Site-directed mutagenesis was carried out by using recombinant circle polymerase chain reaction (15, 16), which utilizes four primers (two mutagenic and two nonmutagenic) that are designed to generate double-stranded, linear DNA molecules with blunt ends. Once combined, denatured, and reannealed, this linear DNA produces double-stranded DNA with discrete cohesive single-stranded ends, in addition to the previously made blunt ends. However, only the former polymerase chain reaction product containing the mutation in each strand will anneal to form recombinant circles of DNA that can effectively mimic circular DNA that is necessary to be transformed into TG-1 cells. This method was successfully utilized for the production of the linear DNA, which then allowed the creation of a R81S mutagenic site (*Sna*BI) within the malate dehydrogenase gene, where the mismatched bases to create the mutagenic replacements are shown in lowercase and the newly introduced restriction endonuclease site is underlined: R81S, CG TAC Gta GT; and R81C, CG TAC Gtt GT. The process was then repeated using the R81S DNA as the template, with any subsequent mutations at position 81 destroying the newly created *Sna*BI site. Initial screening for the presence of mutagenic colonies was accomplished by restriction enzyme mapping and was further verified by DNA sequencing.

Chemical Modulation—Chemical modification of R81C MDH with the various amino acid analog reagents was carried out in 250 mM borate buffer, pH 8.75, using an enzyme concentration of 4 mg/ml and a reagent concentration of 40 mM. The reagents were either obtained as the halides or were converted to the halides as described previously (17). The enzyme was typically incubated with each of the reagents for about 8 h at 4 °C. The extent of modification was followed by examining the free cysteine content, determined by the method of Ellman (18) or by several alternative fluorescent or enzyme-linked methods (19). In each case one (±0.2) cysteine was modified in the R81C mutant, with no cysteine modification observed in the native enzyme. After completion of the modification, the reaction was terminated by the removal of excess reagent by Amicon Centricon-30 filtration, with three successive washes with the standard assay buffer.

Molecular Modeling—Molecular modeling studies were carried out on a Silicon Graphics O_2 computer using the Sybyl (version 6.5) software package (Tripos, Inc.). Coordinates of native MDH were obtained as a Protein Data Bank file and the program Flexidock was used to position the substrates into the enzyme active site before energy minimization. This algorithm fixes the position of the protein backbone atoms and bonds and allows flexibility in the side chain and substrate atoms and bonds. The force field calculations take into account van der Waals', electrostatic, torsional, and constraint energy terms.

RESULTS

Production of R81C MDH—To screen colonies for mutated MDH, the production of the R81C mutant was carried out in two stages. Initially, a serine was introduced at position 81 in place of the native arginine. This was accomplished as described under "Experimental Procedures" by a two base change that also created an *Sna*BI restriction endonuclease site. After isolation of the R81S mutant a second round of mutagenesis was used to replace this serine with a cysteine, thus destroying the newly created *Sna*BI site. Restriction mapping for the presence and then the absence of this site was used to identify mutagenic colonies. Subsequent DNA sequencing, covering at least 800 bases centered around this position, confirmed the mutations that were made.

TABLE I
Substrate specificity of native and R81C MDH

Substrate	Kinetic parameters	Native	R81C
oxaloacetate	k_{cat} (s^{-1})	780 ± 125	250 ± 40
	K_m (μM)	42 ± 7	40 ± 8
	k_{cat}/K_m (M^{-1}s^{-1})	1.9 x 10^7	6.3 x 10^6
hydroxypyruvate	k_{cat} (s^{-1})	220 ± 51	130 ± 38
	K_m (mM)	26 ± 4	3.9 ± 0.7
	k_{cat}/K_m (M^{-1}s^{-1})	8.5 x 10^3	3.3 x 10^4
phenylpyruvate	k_{cat} (s^{-1})	200 ± 36	120 ± 28
	K_m (mM)	23 ± 1	3.7 ± 0.5
	k_{cat}/K_m (M^{-1}s^{-1})	8.7 x 10^3	3.2 x 10^4
2-ketoglutarate	k_{cat} (s^{-1})	18 ± 3	----[a]
	K_m (mM)	8 ± 0.8	----
	k_{cat}/K_m (M^{-1}s^{-1})	2.3 x 10^3	----

[a] No activity detected.

Kinetic Characterization of Arg-81 MDH Mutants—The R81C mutant was purified by using the standard purification procedure (11). The kinetic parameters were determined for this mutant and compared with those of the native enzyme (Table I). Replacement of Arg-81 with Cys results in a 3-fold decrease in k_{cat}, whereas essentially no change is observed in the Michaelis constant for OAA, leading to a modest 3-fold decrease in k_{cat}/K_m. Hydroxypyruvate, in which the β-carboxyl group of OAA is replaced by a hydroxyl group, is a poor alternative substrate for the native enzyme, with a k_{cat}/K_m value that is more than 3 orders of magnitude lower than that of OAA. Surprisingly, phenylpyruvate, in which the β-carboxyl group has been replaced with a phenyl ring, is also a substrate for MDH with a k_{cat} and a k_{cat}/K_m that are comparable with those of hydroxypyruvate. The R81C mutant has a k_{cat} with phenylpyruvate that is further decreased by a factor of 2 but has a lower K_m that leads to a k_{cat}/K_m that is improved by 4-fold compared with the native enzyme (Table I). The product of this reaction, phenyllactate, is also an alternative substrate in the reverse reaction; however, precise kinetic parameters are difficult to determine because of an initial time lag that is observed under all of the substrate concentrations and pH values that were examined.

Removal of the bulky arginyl side chain should provide an expanded binding pocket that should allow the accommodation of larger substrates into the active site. 2-Ketoglutarate, the 5-carbon homolog of OAA, is a poor alternative substrate for the native enzyme with a k_{cat} that is only 2% and a k_{cat}/K_m that is 4-orders of magnitude lower than OAA (Table I). However, instead of seeing an improvement with this substrate, the R81C mutant shows no detectable activity with 2-ketoglutarate nor with several other potential alternative substrates including 2-ketocaproate, the 6-carbon homolog.

In an attempt to provide an improved binding site and enhanced kinetics for phenylpyruvate as an alternative substrate the arginine at position 81 was replaced with a phenylalanine. The k_{cat} for phenylpyruvate decreases with the R81F mutant, but by less than a factor of 2, whereas the K_m value for this substrate shows a 3-fold improvement, resulting in a 2-fold enhancement in k_{cat}/K_m for phenylpyruvate compared with the native enzyme. The introduction of an aromatic side chain at this position also results in a decrease in k_{cat} for the physiolog-

TABLE II
Specificity of modulated malate dehydrogenases for oxaloacetate and phenylpyruvate

Enzyme forms	Oxaloacetate			Phenylpyruvate			OAA/phenylpyruvate ratio	
	k_{cat}[a] (s^{-1})	K_m (μM)	k_{cat}/K_m[a] (M^{-1} s^{-1})	k_{cat}[a] (s^{-1})	K_m (mM)	k_{cat}/K_m[a] (M^{-1} s^{-1})	k_{cat}	k_{cat}/K_m
Native	780	42 ± 7	1.9×10^7	200	23 ± 1	8.7×10^3	3.9	2200
R81C	250	40 ± 8	6.3×10^6	120	3.7 ± 0.5	3.2×10^4	2.1	200
R81F	56	31 ± 4	1.8×10^6	133	7.2 ± 0.7	1.8×10^4	0.4	100
Alkyl modulation								
R81C-Pr[b]	88	83 ± 16	1.1×10^6	110	7.5 ± 1.1	1.5×10^4	0.8	73
R81C-MePr[b]	51	58 ± 6	8.8×10^5	140	12 ± 0.7	1.2×10^4	0.4	73
R81C-Ch[b]	13	139 ± 6	9.4×10^4	98	6.1 ± 1.0	1.6×10^4	0.1	5.9
Aryl modulation								
R81C-AmBz[b]	87	68 ± 8	1.3×10^6	25	27 ± 7	9.3×10^2	3.5	1400
R81C-FBz[b]	136	62 ± 5	2.2×10^6	57	7.1 ± 1.7	8.0×10^3	2.4	280
R81C-NBz[b]	6	64 ± 8	9.4×10^4	30	5.3 ± 1.0	5.7×10^3	0.2	16
R81C-Bz[b]	58	37 ± 4	1.6×10^6	450	4.9 ± 0.4	9.2×10^4	0.1	17

[a] Standard errors on k_{cat} and k_{cat}/K_m are ±10% or less.
[b] Pr, propyl; MePr, 2-methylpropyl; Ch, cyclohexyl; Bz, benzyl; FBz, 4-fluorobenzyl; NBz, 4-nitrobenzyl; AmBz, 4-aminobenzyl.

ical substrate OAA to about 7% that of the native enzyme. However the K_m value for OAA is not affected by this mutation (Table II).

Chemical Modulation—To create a significant alteration in the specificity of MDH, it appears necessary to introduce a wider range of new functional groups into the active site to modify the substrate specificity. To this end the R81C mutant was chemically modulated by using several different hydrophobic aliphatic and aromatic amino acid analog reagents. In each case, through the time course of the modification reaction, there is an observed decrease of approximately one free cysteine/enzyme subunit. This decrease is consistent with the selective modification of the introduced thiolate at position 81. The native enzyme, which contains three cysteines/subunit, is not modified to any significant extent by these reagents under the reaction conditions. This lack of reactivity was anticipated because these cysteines are nearly completely buried in the native MDH structure (5). The kinetic parameters for these modified enzymes were determined and were compared with both the native enzyme and to the unmodified mutant enzyme.

Modulation of R81C with either 2-bromopropane or 1-bromo-2-methylpropane leads to the selective incorporation of a propyl or a 2-methylpropyl group at position 81. The resulting modified enzymes have essentially the same k_{cat} with phenylpyruvate as the unmodified R81C mutant. However, the K_m values for phenylpyruvate with these modified enzymes increase by factors of 2 and 3, respectively (Table II). Treatment of R81C MDH with benzyl bromide introduces a benzyl group at position 81, and this modified enzyme has a k_{cat} with phenylpyruvate of 450 s^{-1} compared with 120 s^{-1} for the R81C mutant and 200 s^{-1} for the native enzyme. The benzyl-modified enzyme has a modest increase in K_m when compared with the R81C mutant but has a 5-fold decrease when compared with the native enzyme, resulting in an overall improvement in k_{cat}/K_m for phenylpyruvate by a factor of 10 (Table II). Introduction of either an alkyl or an aryl group at position 81 leads to a further decrease in both k_{cat} and k_{cat}/K_m for OAA when compared with the unmodified R81C mutant, to values that are more than 2 orders of magnitude lower than those with the native enzyme.

To examine the importance of the aromatic benzyl group in substrate recognition, the R81C mutant was modified with the related cyclohexyl derivative. A modest 4-fold decrease is observed in the K_m for phenylpyruvate with this modified enzyme; however, there is also a 2-fold decrease seen in k_{cat}. The k_{cat} for OAA with this cyclohexyl-modified enzyme is less than 2% that of the native enzyme, and the 3-fold increase in K_m leads to a greater than 200-fold decrease in k_{cat}/K_m for OAA. To further assess the role of an introduced aromatic group in substrate binding the R81C MDH mutant was modified with several substituted benzyl reagents. Introduction of either 4-fluoro- or 4-amino substituent on the benzyl group leads to a decrease in k_{cat}, with OAA as the substrate, to 10–20% of the native enzyme with no effect on K_m. Modification with 4-nitrobenzyl results in a more dramatic decrease in k_{cat}, to less than 1% that of the native enzyme. Each of these enzymes that have been modified with *p*-substituted benzyl groups have k_{cat} values for phenylpyruvate that are only 20–50% that of the unmodified R81C enzyme, with K_m values that are increased by up to a factor of 7 (Table II).

These modified enzymes are each fairly specific for phenylpyruvate; pyruvate is not a substrate. Neither medium chain alkyl keto acids (*e.g.* 2-ketocaproate) nor a homologous aryl keto acid (phenylglyoxalate) are substrates for these R81C-modified enzymes when examined at concentrations up to 100 mM.

DISCUSSION

Substrate Specificity—Replacement of the β-carboxyl group of OAA with a hydroxyl group (hydroxypyruvate) leads to a modest decrease in k_{cat} but a dramatic increase in K_m by nearly 3 orders of magnitude. Substitution of a phenyl group at this substrate position has no additional deleterious effects, with native MDH preferring OAA as a substrate over phenylpyruvate by a factor of 2200. Removal of the arginine (Arg-81) that is proposed to interact with the β-carboxyl group of OAA results in a decline in this substrate preference to 200-fold with a thiol at this position and to only 100-fold when an aromatic site chain (phenylalanine) is introduced by site-directed mutagenesis. The presence of a positively charged guanido group discriminates against the binding of an aromatic substrate like phenylpyruvate or even a polar functional group such as hydroxyl and its removal appears to relieve some of this discrimination. Replacement of this functional group with an appropriately selected alkyl or aryl residue has the potential to dramatically enhance the recognition of this alternative aromatic substrate.

Incorporation of either a straight chain or a branched chain alkyl group at this position by chemical modulation of the introduced thiolate leads to a further decrease in the selectivity for the physiological substrates and against the aromatic alternative. Modification of R81C with benzyl bromide results in an enzyme form that still favors OAA as a substrate but now only by a factor of 17, and the cyclohexyl-modified enzyme has only a 6-fold preference for OAA over phenylpyruvate as measured

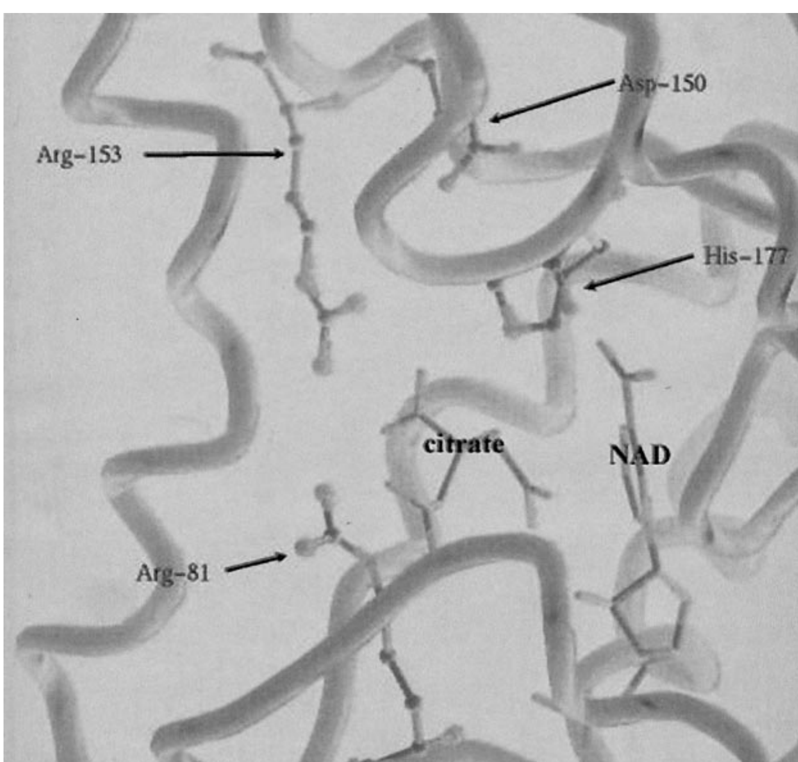


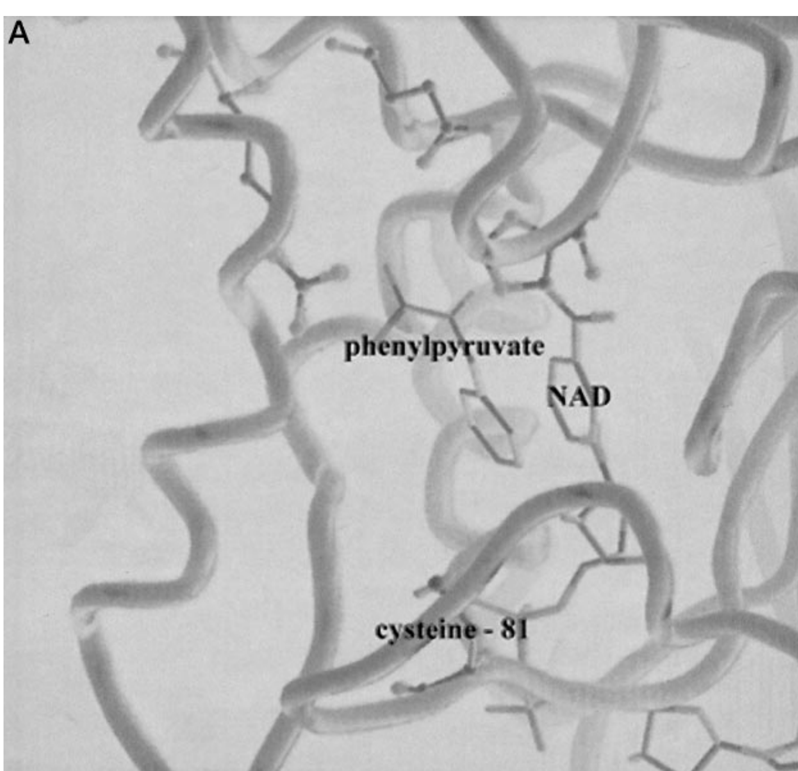
FIG. 1. **A picture of the active site of MDH.** The catalytic His-177 and Asp-150 are annotated, along with the substrate-binding residues Arg-81 and Arg-153. The structure was determined with NAD and citrate bound in the active site (5).

by k_{cat}/K_m criteria. This is a 370-fold decrease in the original substrate discrimination against phenylpyruvate by MDH. However, although the k_{cat}/K_m values for phenylpyruvate have improved relative to those for OAA, the k_{cat} values for this substrate have actually been reversed with the modified enzymes. When these substrates are compared by k_{cat} criteria, phenylpyruvate is now the better substrate by nearly 8-fold in both the cyclohexyl- and the benzyl-modified enzymes. By comparison, the replacement of an entire loop in the binding site in LDH was required to generate an altered enzyme in which phenylpyruvate is the preferred substrate. The best enzyme form that has been produced from LDH through the insertion of four additional amino acids and substitutions at several other positions in this mobile loop has a k_{cat}/K_m for phenylpyruvate that is 1700 times better than that for pyruvate (8). However, in this case, the reversal in specificity was achieved exclusively as a consequence of a dramatic decrease in k_{cat}/K_m for pyruvate, with no enhancement of the kinetics for phenylpyruvate. Despite the improvement in aromatic substrate selectivity, the k_{cat}/K_m for phenylpyruvate actually decreased by a factor of 2 in this altered LDH compared with the native enzyme. With MDH, the introduction of an aromatic benzyl group into the active site results in a 10-fold decrease in k_{cat}/K_m for OAA and a corresponding 10-fold increase for phenylpyruvate.

The improvement in k_{cat}/K_m that has been achieved for phenylpyruvate as a substrate upon modulation at a single position in MDH is unexpected in light of the more stringent substrate specificity of MDH compared with LDH. Unlike MDH, LDH can already utilize a range of straight and branched chain keto acids as reasonable alternative substrates, and this specificity can be further broadened by the mutagenic replacement of a single active site glutamine (20). Substitution of an arginine at this position leads to a shift of 7 orders of magnitude in the specificity of LDH from its physiological substrate pyruvate to OAA with both a dramatic decrease in the specificity for pyruvate and a corresponding increase in that for OAA (21). However, attempts to carry out the complimentary study, the conversion of MDH to an LDH, have been less successful. Replacement of the corresponding arginine in MDH with a glutamine results in a decrease of 5 orders of magnitude in k_{cat}/K_m for OAA, with some improvement but no reversal in the specificity for pyruvate (10).

Aromatic Substituent Effects—If the improvement in specificity for phenylpyruvate with MDH is a consequence of binding interactions between the introduced aromatic ring at position 81 and the substrate aromatic ring, then substrate binding should be sensitive to modulations at this introduced ring. To examine this potential interaction several substituents were introduced at the para-position of the benzyl ring. These included the electron withdrawing 4-fluoro and 4-nitro groups and the electron donating 4-amino group. The expectation was that if there is an interaction between these aromatic rings, then altering the electron density of the benzyl ring should affect this interaction. Some kinetic changes are observed with the introduction of these substituents. Modification of MDH with a 4-aminobenzyl group leads to a 10-fold decrease in k_{cat}/K_m both for OAA and for phenylpyruvate and, therefore, virtually no change in substrate selectivity. In contrast, substitution of a 4-fluorobenzyl group results in an 8-fold shift in preference toward phenylpyruvate, and placing a 4-nitrobenzyl group in this position leads to a greater than 100-fold shift in preference. These specificity shifts do not strictly correlate with changes in electron density of the introduced benzyl ring and must reflect additional steric and orientation effects that will require high resolution structural mapping to understand.

Structural Basis for the Specificity Changes—It is not immediately obvious from the structure of the native enzyme (5) how MDH can accommodate phenylpyruvate as an alternative substrate. In this structure the enzyme active site contains two arginyl residues (Arg-81 and Arg-153) that interact with two of the carboxyl groups of a bound citrate molecule (Fig. 1). It is proposed that these residues are the binding groups that the enzyme uses to orient OAA by electrostatic interactions with



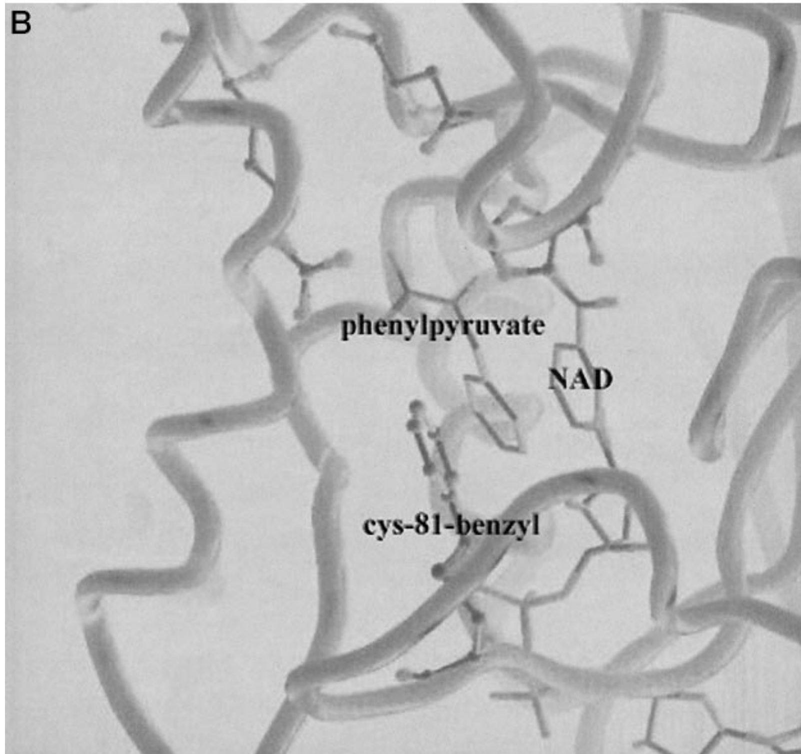


FIG. 2. **Molecular model of phenylpyruvate and NAD$^+$ bound at the active site of the R81C mutant of MDH (*A*) and the benzyl-modified R81C mutant (*B*).** The images were created by using Sybyl (version 6.5) and were energy minimized.

the α- and β-carboxyl groups of the substrate (5). Removal of the positively charged guanido group of Arg-81 by site-directed mutagenesis results in a 3–6-fold improvement in the K_m for phenylpyruvate. However, an examination of the active site structure of MDH does not reveal any hydrophobic pockets that the enzyme could use to accommodate the phenyl ring of this alternative substrate. To examine any potential interactions the substrates phenylpyruvate and NAD have been modeled into the MDH structure and energy minimized. This modeled structure shows a potential π-stacking interaction between the aromatic ring of phenylpyruvate and the nicotinamide ring of NAD that could account for some of the binding affinity for this alternative substrate (Fig. 2*A*). Interestingly, if the same ternary complex is modeled into the benzyl-modified R81C mutant, the introduced benzyl ring joins this π-stack to sandwich the substrate phenyl ring with the nicotinamide ring (Fig. 2*B*). This structure would be expected to further stabilize the binding of phenylpyruvate and could explain the 130-fold improvement in substrate specificity relative to OAA. Replacement of this aromatic functional group at position 81 with a cyclic alkyl

group leads to a 6-fold decrease in the specificity of this modified enzyme for phenylpyruvate, presumably because of the loss of aromatic stabilization in this enzyme form.

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Addendum—A preliminary structure of the R81F mutant of MDH has just been determined in the absence of substrates.[2] This structure is essentially identical to that of the native enzyme (5), except for a disordered loop that includes the site of mutation. This new structure confirms the local nature of any structural perturbations that might have occurred among this group of modified MDHs.

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