

# Alteration of the Specificity of Malate Dehydrogenase by Chemical Modulation of an Active Site Arginine\*

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**Malate dehydrogenase from *Escherichia coli* is highly specific for the oxidation of malate to oxaloacetate. The technique of site-specific modulation has been used to alter the substrate binding site of this enzyme. Introduction of a cysteine in place of the active site binding residue arginine 153 results in a mutant enzyme with diminished catalytic activity, but with  $K_m$  values for malate and oxaloacetate that are surprisingly unaffected. Reaction of this introduced cysteine with a series of amino acid analog reagents leads to the incorporation of a range of functional groups at the active site of malate dehydrogenase. The introduction of a positively charged group such as an amine or an amidine at this position results in improved affinity for several inhibitors over that observed with the native enzyme. However, the recovery of catalytic activity is less dramatic, with less than one third of the native activity achieved with the optimal reagents. These modified enzymes do have altered substrate specificity, with  $\alpha$ -ketoglutarate and hydroxypyruvate no longer functioning as alternative substrates.**

Malate dehydrogenase from *Escherichia coli* (eMDH)<sup>1</sup> catalyzes the interconversion of malate and oxaloacetate, a critical step in carbohydrate metabolism. In eukaryotes the production of oxaloacetate in the mitochondria is required to accept the acetyl-coenzyme A that is generated from the catabolism of carbohydrates, lipids, and certain amino acids. The cytosolic form of this enzyme is involved in the malate/aspartate shuttle that exchanges reducing equivalents across the mitochondrial membrane. As expected for the catalysis of this important metabolic reaction, malate dehydrogenase in both prokaryotes and eukaryotes is highly selective for malate and oxaloacetate, with strong discrimination against other 2-keto mono- and dicarboxylic acids (1). Examination of the crystal structure of malate dehydrogenase from *E. coli* (2) provides an explanation for the high substrate selectivity that is observed with this enzyme. The substrate appears to be bound at both ends through electrostatic interactions of its two carboxyl groups with complementary arginyl side chains that are precisely positioned to interact with a four carbon dicarboxylic acid (Fig. 1). Additional constraints are placed by the location of the cata-

lytic histidine and by the positioning of the nicotinamide coenzyme for hydride transfer.

Several attempts at altering the specificity of this highly selective enzyme have been made, with limited success (1, 3). It appears that significant changes in specificity will require the additional flexibility of chemical modification to incorporate new functional groups into the active site of eMDH. In this report we have examined the effect of introducing unnatural amino acids at a single active site position on the substrate specificity of eMDH.

## EXPERIMENTAL PROCEDURES

**Materials**—The pEM6 plasmid containing the *mdh* gene that encodes for eMDH was a gift from Dr. Norb Furumo (Eastern Illinois University, Charleston, IL). 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 PCR optimization kit were purchased from Stratagene. Affi-blue resin was purchased from Bio-Rad. Iodoacetamide and 2-chloroacetamide were obtained from Lancaster and all other chemicals were purchased from Sigma. Purification of the enzyme was performed according to the previously published scheme (4).

**Enzyme Assay**—The catalytic activity of malate dehydrogenase was determined spectrophotometrically by measuring either the formation or the utilization of NADH ( $E_{340} = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$ ). Standard assay conditions were as follows: 50 mM buffer, 2 mM EDTA, 100 mM KCl, and variable concentrations of the substrates NADH and oxaloacetic acid (OAA) or NAD and malate (5). Inhibition studies were performed in either the direction of malate oxidation or OAA reduction. Conditions for the malate oxidation direction were: 50 mM Taps, pH 9.0, saturating NAD (7 mM), 5.0 mM malate, and varying concentrations of malate analogs. For the OAA reduction direction, conditions were: 50 mM Hepes, pH 7.2, 0.15 mM NADH (saturating), 0.2 mM OAA, and varying concentrations of OAA analogs. Each reaction was initiated by the addition of 7 nM active sites of native enzyme. For examination of alternative substrates with modified eMDHs, the enzyme concentrations were increased, in some cases up to 0.7  $\mu\text{M}$ . The kinetic data were fitted by using an Enzyme Kinetics software package (SciTech International, Chicago, IL) to obtain the kinetic parameters. Inhibition constants were determined by a Dixon analysis (6) and are corrected for the higher levels of substrates relative to their  $K_m$  values. Protein concentration was determined by the Bradford method (7) using bovine serum albumin as a standard.

**Mutagenic Method**—Site-directed mutagenesis was carried out by using recombinant circle PCR. The recombinant circle-polymerase chain reaction (RC-PCR) method (8, 9) utilizes four primers (two mutagenic and two non-mutagenic) 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 end molecules. However, only the former PCR product will anneal to form recombinant circles of DNA that can effectively mimic the circular DNA that is necessary to be transformed into TG-1 cells. This method was successfully utilized for the production of the linear DNA that then allowed the creation of a mutagenic site within the malate dehydrogenase gene. The specific changes introduced were to alter the DNA sequence in the region of interest that codes for the amino acid at position 153 to ACA tAT GAT, where the mismatched bases to create the mutagenic arginine to cysteine replacement and the

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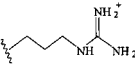
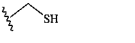
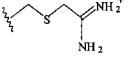
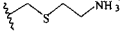
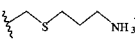
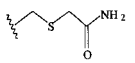
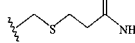
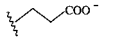
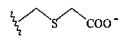
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TABLE I

## Chemical modulation of position 153 in malate dehydrogenase

The kinetic parameters are reported for the reaction in the OAA reduction direction. EAm, ethylamine; PAm, propylamine; AAd, acetamide; PAD, propylamide; AAn, acetamidine; Ac, acetate.

Enzyme form	Structure at position 153	$k_{cat}^a$ ( $s^{-1}$ )	$K_m$ ( $\mu M$ )	$k_{cat}/K_m$ ( $M^{-1}s^{-1}$ )
Native		780	42 ± 7	1.9 × 10 <sup>7</sup>
R153C		110	89 ± 14	1.2 × 10 <sup>6</sup>
R153C-AAn		65	17 ± 2	3.8 × 10 <sup>6</sup>
R153C-EAm		130	47 ± 3	2.8 × 10 <sup>6</sup>
R153C-PAm		232	68 ± 18	3.4 × 10 <sup>6</sup>
R153C-AAd		54	170 ± 25	3.2 × 10 <sup>5</sup>
R153C-PAd		190	51 ± 8	3.7 × 10 <sup>6</sup>
R153E		176	122 ± 9	1.4 × 10 <sup>6</sup>
R153C-Ac		63	82 ± 4	7.7 × 10 <sup>5</sup>

<sup>a</sup> The standard errors on  $k_{cat}$  are ±10% or less.

newly introduced *Nde*I restriction endonuclease site are shown in lowercase, with the restriction site underlined. For the R153E mutant, the sequence A TAT gga TTC was used to substitute a glutamate at this position and destroy the introduced *Nde*I restriction site. Initial screening for the presence of mutagenic colonies was accomplished by restriction enzyme mapping.

**Site-specific Modulation**—Chemical modulation of R153C eMDH with the amino acid analog reagents 2-chloroacetamidine, 2-bromoethylamine, 3-bromopropylamine, 2-chloroacetamide, 3-chloropropylamide, and 2-chloroacetic acid were performed in 250 mM borate buffer, pH 8.8, using an enzyme concentration of 4 mg/ml and reagent concentrations of 10–40 mM. The reactions were incubated for various times up to 12 h at 4 °C and were terminated by the removal of excess reagent by Amicon Centricon-30, followed by three successive washes with the standard assay buffer. The content of free cysteine was initially determined by the method of Ellman (10) using 2,2'-dithiobis(5-nitrobenzotriazole) (DTNB) at pH 7 and quantitating the product formed at 412 nm. The stoichiometry of cysteine modification by the amino acid analog reagents was determined by monitoring the decrease in the free cysteine content of the enzyme. When additional precision was required, the standard method of cysteine determination was supplemented (11) by using either fluorescent methods or by an enzyme amplification assay.

## RESULTS

**Characterization of the R153C Mutant**—The active site arginine (Arg-153) that interacts with the  $\alpha$ -carboxyl group of the substrate (Fig. 1) has been replaced with a cysteine by using the RC-PCR method. The resulting R153C mutant was purified using the purification procedure that was developed for the native enzyme (4). The kinetic parameters for this mutant were compared with those of native eMDH. Replacement of this arginine with a cysteine leads to an enzyme form with a  $k_{cat}$  that is about 7% that of the native enzyme. However, the  $K_m$  for malate (1.4 mM in the native enzyme) is essentially unaffected (1.9 mM in the R153C mutant) by the removal of this binding arginine residue, and the  $K_m$  for oxaloacetate increases but only by a factor of two (Table I).

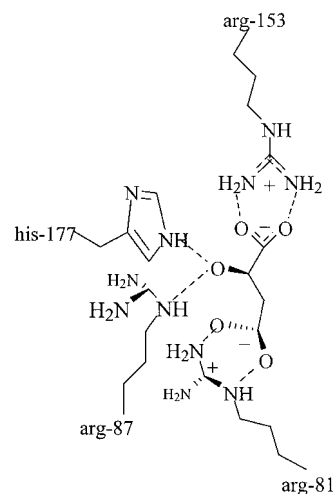


FIG. 1. Drawing of the active site structure of *Escherichia coli* malate dehydrogenase with bound oxaloacetate. Arginines 81, 87, and 153 provide substrate orientation, and histidine 177 is the active site catalyst. This representation is adapted from the citrate complex with eMDH determined by Hall *et al.* (2).

**Stoichiometry of Modification**—Native eMDH contains 3 cysteines/subunit. Treatment of the enzyme with DTNB under denaturing conditions (4 M guanidine HCl) leads to the modification of all three cysteines in the native enzyme and, in the R153C mutant, the fourth cysteine that was introduced is also modified under these conditions. However, when the titration with DTNB is conducted under non-denaturing conditions, a slow modification (>30 min) is observed of a single cysteine per subunit in the native enzyme. The introduction of a cysteine at position 153 places a reactive nucleophile within the active site of eMDH. Incubation of the R153C mutant with DTNB under non-denaturing conditions leads to the rapid (<5 min) modification of a single cysteine. If the reaction is allowed to proceed for an extended period, the slow modification of a second cysteine is observed. From the high reactivity of this first cysteine, the site of modification in the mutant eMDH appears to be at the introduced cysteine 153. To support this assignment, the rate of reaction of the slowly modified cysteine in the eMDH mutant was examined and was found to be indistinguishable from that of the single accessible cysteine that is modified in the native enzyme.

Treatment of the R153C mutant with moderate levels of most of the amino acid analog reagents results in the selective modification of only the most reactive cysteine (1.0 ± 0.2 cysteines modified/subunit) that has been assigned to the cysteine introduced at position 153. For the more reactive reagents, particularly those with bromide as the leaving group, both the introduced cysteine and the non-essential exposed cysteine can be modified (2.0 ± 0.2 cysteines modified/subunit) under these conditions at extended reaction times. However, even in these cases, the introduced cysteine shows significantly higher reactivity with these reagents than does the native cysteine. Modification of the native cysteine with DTNB, or with some of the more reactive amino acid analog reagents, has a negligible effect on the kinetics of native eMDH, with both  $k_{cat}$  and  $k_{cat}/K_m$  for the modified enzyme remaining at 80–90% of the values for the unmodified enzyme.

**Site-specific Modulation**—Based on these results, the R153C mutant can now be specifically chemically modulated by using various amino acid analog reagents to introduce a range of functional groups into the active site of eMDH. Modification of this introduced cysteine with 2-chloroacetamidine results in the incorporation of an acetamidine (AAn) group and thus the

TABLE II  
 Binding specificity ( $K_m$ , mM) of modified R153C malate dehydrogenases

Eam, ethylamine; PAm, propylamine; AAd, acetamide; PAd, propylamide; AAn, acetamidine; Ac, acetate.

Inhibitors	Native	R153C	R153C-AAn	R153C-EAm	R153C-PAm	R153C-AAd	R153C-PAd	R153E	R153C-Ac
Pyruvate [CH <sub>3</sub> C(=O)-COO <sup>-</sup> ]	25 ± 4	56 ± 5	15 ± 1	22 ± 1	34 ± 3	36 ± 8	25 ± 3	30 ± 5	22 ± 4
α-Ketoglutarate [ <sup>-</sup> OOC-(CH <sub>2</sub> ) <sub>2</sub> C(=O)-COO <sup>-</sup> ]	4.5 ± 0.7	12 ± 2	3.7 ± 0.4	8.8 ± 1.0	<b>20 ± 2<sup>a</sup></b>	<b>17 ± 4</b>	3.4 ± 0.3	12 ± 1	<b>20 ± 1</b>
β-Ketoglutarate [ <sup>-</sup> OOC-CH <sub>2</sub> C(=O)CH <sub>2</sub> -COO <sup>-</sup> ]	49 ± 9	31 ± 5	<b>13 ± 3</b>	45 ± 6	47 ± 11	<b>93 ± 7</b>	54 ± 3	30 ± 9	<b>127 ± 14</b>
L-Lactate [CH <sub>3</sub> CH(-OH)-COO <sup>-</sup> ]	9 ± 1	<b>190 ± 20</b>	20 ± 3	30 ± 7	29 ± 5	7.6 ± 1.4	25 ± 4	<b>340 ± 35</b>	16 ± 5
D-Lactate [CH <sub>3</sub> CH(-OH)-COO <sup>-</sup> ]	27 ± 2	50 ± 5	42 ± 6	63 ± 19	<b>NI<sup>b</sup></b>	31 ± 5	27 ± 6	<b>7.6 ± 1.7</b>	<b>8.6 ± 1.1</b>
Mercaptosuccinate [ <sup>-</sup> OOC-CH <sub>2</sub> CH(-SH)-COO <sup>-</sup> ]	14 ± 1	12 ± 1	<b>2.3 ± 0.5</b>	<b>1.9 ± 0.1</b>	ND <sup>c</sup>	ND	<b>4.6 ± 0.7</b>	ND	ND

<sup>a</sup> Significant improvements in binding affinities are shown in **bold**, whereas significant decreases in affinities are shown in **bold italics**.

<sup>b</sup> No inhibition is observed at concentrations up to 175 mM.

<sup>c</sup> Not determined.

generation of a homoarginine analog. This new active site functional group does not lead to significant recovery of activity; however, there is a 5-fold decrease in the  $K_m$  for oxaloacetate to a value that is nearly 3-fold lower than that of the native enzyme (Table I). Modifications at this position to incorporate either amine (lysine analogs) or amide (glutamine analogs) functional groups at this position lead to modest changes in the kinetic parameters. In each case the longer homolog results in the greater improvement, with the propylamide-modified enzyme having a  $k_{cat}$  that is about 25% of the native enzyme and the propylamine-modified enzyme at about 30% of the native  $k_{cat}$  value (Table I).

These modifications that have been introduced were aimed at generating reasonable structural analogs of the active site arginine that was removed. It is also possible to use this approach to characterize the role of an active site group by disrupting the enzyme-substrate interactions. Mutation of arginine 153 to glutamate places a negative charge in the vicinity of the substrate α-carboxyl binding site and should result in decreased affinity for the physiological substrates. As expected, the  $K_m$  for OAA increases but only by a factor of 3. The position of this carboxyl group can be extended by the modulation of cysteine 153 with a halocarboxylic acid. Introduction of an acetate group leads to a further decrease in  $k_{cat}$  and in  $k_{cat}/K_m$  to less than 10% of the values observed for the native enzyme (Table I).

**Inhibition Studies**—The changes that have been introduced into the active site as a result of these chemical modulations were next investigated through a series of inhibition studies aimed at focusing exclusively on alterations in binding affinity. Generation of the R153C mutation results in an increase in the inhibition constant for pyruvate (a 3-carbon substrate analog) and α-ketoglutarate (a 5-carbon substrate analog) of 2–3-fold compared with that with the native enzyme (Table II). Specific modifications of this introduced cysteine with a variety of amino acid analog reagents leads to further alterations in binding specificity. Modification of R153C through the introduction of an AAn group at this position leads to enhanced binding for each inhibitor that was examined when compared with the unmodified mutant enzyme. β-Ketoglutarate interacts with the AAn-modified enzyme 4-fold tighter than with the native enzyme, and the interaction with mercaptosuccinate is 6-fold tighter (both shown in bold in Table II). The ethylamine-modified enzyme shows a similar increase in affinity for mercaptosuccinate.

Introduction of a carboxyl group at this position, by mutation of arginine 153 to glutamate, does not affect the binding of either pyruvate or β-ketoglutarate, but does cause a 3-fold decrease in the affinity of the enzyme for α-ketoglutarate. Extending the carboxylate further into the binding pocket by modification of R153C with an acetate group leads to a 4-fold decrease in the affinity for both α- and β-ketoglutarate, but has

no effect on the binding of pyruvate when compared with the native enzyme.

For the hydroxyacid substrates, the affinity of R153C for L-lactate, the 3-carbon analog of malate, decreases by over 20-fold when compared with the native enzyme (Table II). Native eMDH shows a 3-fold stereochemical discrimination for the binding of L-lactate as an inhibitor over the D-isomer; however, this selectivity is reversed when arginine 153 is substituted by cysteine. There is a return to the stereochemical preference for binding the L-isomer of lactate over the D-isomer when this position is modified by the introduction of an acetamidine, an acetamide, or an ethylamine functional group (Table II). In the case of the propylamine-modified enzyme, absolute binding specificity for the L-isomer is observed, with no inhibition by D-lactate up to a concentration of 175 mM, while in contrast the corresponding propylamide-modified enzyme shows no discrimination in the binding of these isomers. The introduction of a carboxylate at this position in R153E leads to enhanced affinity for the binding of D-lactate, with over a 40-fold preference when compared with L-lactate. However, when the position of this carboxyl group is extended (R153C-Ac), the stereochemical selectivity for the binding of the D-isomer decreases to only a factor of 2.

**Alternative Substrates**—eMDH is highly selective for malate and oxaloacetate as substrates. However, when examined at sufficiently high concentrations, several alternative substrates have been reported (1). The reduction of α-ketoglutarate, the 5-carbon analog of OAA, is catalyzed at a rate that is only 2% that of the physiological substrate and has a  $k_{cat}/K_m$  that is decreased by about 10<sup>4</sup> (Table III). Both hydroxypyruvate and phenylpyruvate are also weak alternative substrates for eMDH, with similar  $k_{cat}/K_m$  and enhanced  $k_{cat}$  values compared with α-ketoglutarate. However, the  $k_{cat}$  values with these substrates are still only 15–25% that with OAA. A wide range of additional substrate structural analogs have been examined, including α-ketobutyrate, pyruvate, and acetoacetate, that show no activity with native eMDH even when examined at very high levels.

Removal of arginine 153, the enzyme functional group that interacts with the α-carboxyl group of the substrate, leads to a decrease in catalytic efficiency. Both phenylpyruvate and hydroxypyruvate remain weak substrates for the R153C mutant. The  $k_{cat}$  for phenylpyruvate decreases by a factor of 25 and an even greater decrease is seen for hydroxypyruvate compared with the native enzyme. The substrate activity of α-ketoglutarate can no longer be detected with the R153C mutant. However, unexpectedly, pyruvate is now found to be an alternative substrate with a  $k_{cat}$  that is comparable to that of the physiological substrate with this mutant, but with  $k_{cat}/K_m$  that is still over 200-fold lower than that of OAA.

Modification of R153C by the introduction of an acetamidine functional group does not result in recovery of the capability of

TABLE III  
 Alternative substrates for malate dehydrogenase

Substrate	Native enzyme		R153C		R153C-AA <sub>n</sub>	
	$k_{\text{cat}}^a$ $s^{-1}$	$k_{\text{cat}}/K_m$ $M^{-1} s^{-1}$	$k_{\text{cat}}^a$ $s^{-1}$	$k_{\text{cat}}/K_m$ $M^{-1} s^{-1}$	$k_{\text{cat}}^a$ $s^{-1}$	$k_{\text{cat}}/K_m$ $M^{-1} s^{-1}$
<b>Oxaloacetate<sup>b</sup></b>	<b>780</b>	<b><math>1.9 \times 10^7</math></b>	<b>110</b>	<b><math>1.2 \times 10^6</math></b>	<b>65</b>	<b><math>3.8 \times 10^6</math></b>
$\alpha$ -Ketoglutarate	18	$2.3 \times 10^3$	— <sup>c</sup>	—	—	—
Pyruvate	—	—	90	$5.7 \times 10^3$	—	—
Hydroxypyruvate	110	$1.0 \times 10^3$	<1 <sup>d</sup>	<1	—	—
Phenylpyruvate	200	$8.6 \times 10^3$	8	$9.6 \times 10^2$	120	$4.5 \times 10^3$

<sup>a</sup> The standard errors on  $k_{\text{cat}}$  are  $\pm 10\%$  or less.

<sup>b</sup> The physiological substrate is shown in **bold**.

<sup>c</sup> No activity observed at concentrations up to 100 mM.

<sup>d</sup> Very low activity that could not be quantitated.

the enzyme to reduce either  $\alpha$ -ketoglutarate or hydroxypyruvate. In addition, the catalysis of pyruvate that was obtained upon replacement of arginine 153 with cysteine is lost when this introduced cysteine is modified (Table III). Similar losses of activity are observed upon modification of cysteine 153 with either ethylamine or propylamine. However, the acetamidine-modified enzyme does retain the capability to reduce phenylpyruvate, with enhanced kinetic parameters to values that approach those observed with the native enzyme.

#### DISCUSSION

*Specificity of Modification*—Cysteines contain an inherently reactive sulfhydryl group that is the most potent nucleophile among the naturally occurring amino acids. However, in proteins, these functional groups are frequently found either in disulfide linkages or in the solvent-inaccessible interior (12). In most cases, treatment of proteins with thiol reagents under non-denaturing conditions will result in the modification of only the accessible surface cysteines. Only one of the three cysteines per subunit that are found in native eMDH reacts with the amino acid analog reagents that have been examined, and this modification reaction is very slow despite a wide range in the inherent reactivity of these reagents with sulfhydryl groups (13). An examination of the structure of eMDH shows that two of the cysteines in the native enzyme are buried in the protein interior, and only one, the presumed site of reaction, is partially solvent exposed. Furthermore, the modification of this single cysteine has been shown to have a negligible effect on the kinetics of the reaction catalyzed by eMDH. The R153C mutant contains an additional cysteine that is modified by these reagents, and this introduced cysteine is significantly more reactive than the partially exposed native cysteine. Incubation of R153C eMDH with any of the amino acid analog reagents for short periods of time under non-denaturing conditions results in the modification of only the introduced cysteine. From these observations, it appears that the modification of the introduced cysteine at position 153 is responsible for the kinetic changes that are observed.

*Alterations in Substrate Specificity*—Lactate and malate dehydrogenases are structurally and mechanistically related keto acid dehydrogenases (2, 15). The sequence identity between these related enzyme families is quite limited despite the overall structural similarities; however, many of the functional active site amino acids are conserved between these enzyme families (16). To demonstrate that these enzymes are functionally related, several of the active site amino acids in the lactate dehydrogenase framework have been altered to the corresponding malate dehydrogenase residues by site-directed mutagenesis. Replacement of the glutamine 102 of lactate dehydrogenase (LDH) with an arginine resulted in the reversal of specificity, with the conversion of LDH to an eMDH with high specificity for oxaloacetate (17). The corresponding replacement in eMDH resulted in an enzyme with broader specificity, but does not lead to

a reversal of specificity (1). Mutagenic replacements at several additional active site functional groups have confirmed the more stringent substrate specificity of eMDH over LDH (3).

Malate dehydrogenase is quite specific for the oxidation of L-malate and the reduction of OAA. No activity is observed with the 3-carbon keto acid analog pyruvate, and the activity with the 5-carbon analog  $\alpha$ -ketoglutarate is diminished by nearly 10,000-fold when compared with the physiological substrate. The introduction of a hydroxyl or a phenyl group in place of the  $\beta$ -carboxyl group of OAA does allow the enzyme-catalyzed reduction of these analogs, but with significantly decreased  $k_{\text{cat}}/K_m$  values that are comparable to those observed with  $\alpha$ -ketoglutarate.

*Binding Discrimination*—One of the goals of this chemical modulation approach is to introduce new catalytic activities into existing enzymes. Re-designing the active site of eMDH will require an examination of each of the putative substrate binding groups. Replacement of arginine 81, a group proposed to interact in a bidentate fashion with the substrate (Fig. 1), with several unnatural alkyl and aryl groups has resulted in the alteration of the specificity of eMDH (14). The introduction of a benzyl group at this position leads to a 10-fold decrease in the  $k_{\text{cat}}/K_m$  for OAA, and a corresponding 10-fold increase for phenylpyruvate as a substrate. The  $k_{\text{cat}}$  values for these substrates have actually been reversed for this benzyl-modified enzyme, with the  $k_{\text{cat}}$  for phenylpyruvate now 8 times greater than that for OAA and within a factor of 2 of the  $k_{\text{cat}}$  for OAA with native eMDH (14).

The arginine at position 153 was selected as the next target for modification because there appears, from an assessment of the high resolution structure, to be fewer direct interactions to the 1-carboxylate of the bound substrate than to the 4-carboxyl group. Removal of arginine 153 from malate dehydrogenase results in a greater than 10-fold decrease in catalytic activity. This loss of activity reflects the need for the placement of a binding group to correctly orient the substrate by interacting with the 1-carboxyl group. When the interaction of this mutant enzyme was examined with a variety of substrate structural analogs, some interesting binding patterns emerged. Replacement of arginine 153 with cysteine leads to a loss in binding affinity for pyruvate and lactate, the 3-carbon analogs of the physiological substrates that are missing the 4-carboxyl group. However, the increases in  $K_i$  values are surprisingly modest when considering that, for these analogs, the electrostatic interactions at both the 1-carboxyl end, by removal of arginine 153, and at the 4-carboxyl end, by elimination of the 4-carboxyl group, have been disrupted. These results clearly point to the presence of significant additional interactions between the enzyme and its keto or hydroxyacid substrate.

The introduction of a positive or a polar functional group at position 153 is sufficient to restore binding of these 3-carbon analogs to near native levels. However, alterations in the iden-

tity of the functionality at this position have little effect on the binding of the 5-carbon analogs  $\alpha$ - and  $\beta$ -ketoglutarate (Table II). The introduction of an amidine at this position does lead to a nearly 4-fold increase in the binding of  $\beta$ -ketoglutarate compared with that of the native enzyme. The decrease of one bond length in the side chain of the R153C-AA<sub>n</sub> enzyme, compared with the native enzyme, is apparently compensated for by the one carbon shift in the position of the carbonyl group in  $\beta$ -ketoglutarate. This shift would preserve the positioning in the active site of the carbonyl carbon that is the site of reduction. In contrast, the incorporation of a comparably positioned amide at position 153 results in decreased affinity for these substrate analogs. These results indicate the preference for a positively charged functional group at this position to form an electrostatic interaction with the substrate 1-carboxyl group.

The introduction of a negatively charged functional group at position 153 would be expected to disrupt the interaction between the enzyme and its substrate. The binding of the 5-carbon ketoglutarate analogs are weaker with the R153C-Ac enzyme than with any other modified enzyme that has been examined (Table II). These are not large effects but, even if fully extended, the introduced carboxyl group in R153C-Ac would still not be within Van der Waals contact of these substrates. Modeling studies suggest that the potential charge repulsion between the substrate carboxyl group and the carboxyl group introduced at position 153 could be avoided by a rotation of the side chain into an alternative conformation (18).

**Modulation of Catalytic Activity**—The introduction of a variety of arginine analogs has led to an enhancement of the R153C mutant enzyme's capability to bind its substrates and competitive inhibitors. In a number of cases (shown in bold in Table II), these introduced functional groups have resulted in improved affinity when compared with the native enzyme. Although similar variations are observed in catalytic activity among the modified enzymes, in no case does the activity exceed or even approach that of the native enzyme. The best recovery of activity was observed with the propylamine-modified enzyme, and even here  $k_{\text{cat}}$  for malate is less than 20% and for OAA is about 30% of the native enzyme. These results clearly show that more stringent requirements must be met

beyond the recovery of reasonable binding affinity in order to achieve enhanced catalysis.

For almost all of these modified enzymes, there is little difference between the decreases in the catalytic efficiency of the enzyme that are observed under saturating substrate conditions ( $k_{\text{cat}}$ ) and under limiting substrate conditions ( $k_{\text{cat}}/K_m$ ). Binding of the substrate at the enzyme active site is a necessary requirement for catalysis. However, efficient catalysis also requires precise positioning of the substrate in close proximity to the enzyme catalytic group and to the coenzyme for effective hydride transfer. These studies suggest that the primary role of the individual arginines at the active site of eMDH is not in substrate binding, but in substrate orientation.

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