Designs for a Broad Substrate Specificity Keto Acid Dehydrogenase†

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ABSTRACT: Variations have been made to the structure of the nicotinamide adenine dinucleotide (NAD) dependent L-lactate dehydrogenase from *Bacillus stearothermophilus* at regions of the enzyme that we believe determine specificity toward different α-hydroxy acids ($RCHOHCOO^-$, R = CH_3, C_2H_5, etc.). Two regions of LDH that border the active site (but are not involved in the catalytic reaction) were altered in order to accommodate substrates with hydrophobic side chains larger than that of the naturally preferred substrate, pyruvate (R = CH_3). The mutations $^{102-105}$GlnLysPro → MetValSer and $^{236-237}$AlaAla → GlyGly were made to increase the tolerance for large hydrophobic substrate side chains. The triple and double mutants alone gave little improvement for branched-chain-substituted pyruvates. The five changes together produced a broader substrate specificity α-hydroxy acid dehydrogenase, with a 55-fold improved k_{cat} for α-ketoisocaproate to a value about $^1/_{14}$ that of the native enzyme for pyruvate. Rational protein engineering enabled coupled changes in enzyme structure to be obtained with greater probability of success than random mutagenesis.

One of the benefits that may result from protein engineering is the design and construction of enzymes with predetermined substrate specificity. Our long-term goal is to design a thermostable catalyst for the chiral reduction by NADH of any α-keto acid to the L-α-hydroxy acid. We have recently demonstrated the alteration of the substrate specificity of L-lactate dehydrogenase (LDH)[1] from *Bacillus stearothermophilus* to produce a specific, highly active malate dehydrogenase which retains the property of extreme thermostability (Wilks et al., 1988). In this paper we describe attempts to produce a thermophilic α-hydroxy acid dehydrogenase with broad specificity for neutral substrate side chains by redesigning the substrate binding site of NAD-dependent L-lactate dehydrogenase.

L-Lactate dehydrogenase has been shown to have high enantioselectivity, generating only L-lactate on reduction of pyruvate, and has also been shown to have high enantioselectivity toward unnatural substrates (Kim & Whitesides, 1988; Bur et al., 1989), and so it is a useful enzyme for the production of chiral α-hydroxy acids.

There is evidence that the specificity of LDH is determined by at least two regions of the enzyme which surround the side chain of the substrate. These two regions are a mobile loop of polypeptide (residues 98–112—the "coenzyme loop") which folds over the active site enclosing the substrate in the catalytic vacuole (Grau et al., 1981; Parker & Holbrook, 1977; Waldman et al., 1988) and a helix (G) onto which this loop folds (residues 220–242) (Figure 1).

The mammalian LDH isozymes from different tissues have different substrate specificities (Wilkinson & Withycombe, 1965). The LDH C_4 isozyme from mature testes is better able to catalyze the conversion of larger substrates such as 2-oxobutanoate and 2-oxopentanoate into their corresponding 2-hydroxy acids than either the A_4 (muscle) or B_4 (heart) LDH isozymes. Mouse LDH C_4 has been shown to present an even broader substrate specificity than the C isozymes from other species (Blanco et al., 1976). Because healthy adult human or mouse testes are not available in commercial quantities, we chose to try to obtain specificity for large substrates by incorporating motifs from the testicular enzyme structure into the readily available *B. stearothermophilus* enzyme (Barstow et al., 1986). There are at least seven differences out of sixteen residues of the coenzyme loop region which seem to increase the hydrophobicity of the mouse C subunit, compared to the A and B subunits which may be important in determining the unique catalytic properties of this isozyme (Pan et al., 1980).

A detailed understanding of the structure and mechanism of LDH is needed to enable the alteration of the substrate binding site of the enzyme while maintaining the elements essential for catalyzing the redox reaction. This is particularly difficult in an enzyme where many of the amino acid residues which are important for both catalysis and substrate specificity are carried on the same mobile loop of polypeptide chain. The main elements used in the design were a detailed understanding of the LDH catalytic mechanism (Holbrook et al., 1975; Clarke et al., 1987, 1988; Hart et al., 1987), the known sequences of both the *B. stearothermophilus* LDH gene (Barstow et al., 1986) and the mouse LDH C gene (Sakai et al., 1987; Wu et al., 1987), and the known three-dimensional structures of LDH in the apo, binary, ternary, and quaternary forms (dogfish: Adams et al., 1970; pig M_4: C. R. Dunn, H. Muirhead, and J. J. Holbrook, personal communication; *B. stearothermophilus*: D. B. Wigley, H. Muirhead, and J. J. Holbrook, personal communication).

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[1] Abbreviations: LDH, L-lactate dehydrogenase; FBP, fructose 1,6-bisphosphate; *B. stearothermophilus*, *Bacillus stearothermophilus*; *E. coli*, *Escherichia coli*.

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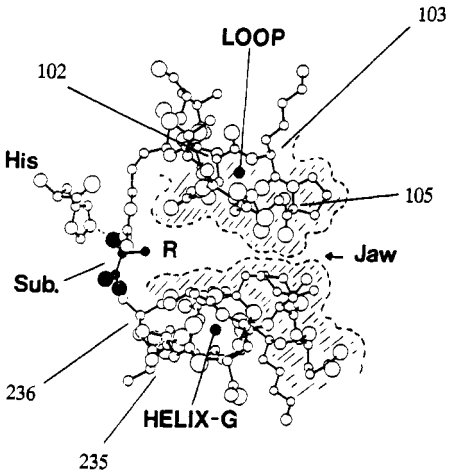

FIGURE 1: The lactate dehydrogenase catalytic vacuole. Oxidation of substrate (Sub.; black circles) is in an internal vacuole which is isolated from the solvent by the closure of a coenzyme loop (LOOP) onto HELIX-G. In this view the solvent is to the right and access to the vacuole is restricted by the Jaw region. This view was constructed by using partially refined coordinates of *B. stearothermophilus* LDH type IV crystals (Wigley et al., 1988) from PEG 6000 in the presence of NADH, sodium oxamate, and FBP (D. B. Wigley, H. Muirhead, and J. J. Holbrook, personal communication) when the refinement was at R factor 21.8% for 62 506 independent reflections at resolution 3–0.25 nm.

The designs that gave rise to the mutations were originally based on a model (J. J. Holbrook, unpublished) of the bacterial amino acid sequence built into the unrefined coordinates of mammalian LDHs available in the Brookhaven Database (Berstein et al., 1977) in 1984 [1LDX, 3LDH, 4LDH, and 5LDH (Grau et al., 1981)]. This did involve extrapolation around the coenzyme loop region (residues 98–110) and in particular a major rebuild required to insert a proline residue instead of a glutamine at position 105. There is now a published medium-resolution 3D structure for the *B. stearothermophilus* enzyme from ammonium sulfate (Piontek et al., 1990), but in this structure the coenzyme loop is disordered and not visible. The structure of crystals of *B. stearothermophilus* LDH in the presence of NADH, sodium oxamate, and FBP from PEG 6000 (Wigley et al., 1988) is being taken to high resolution (D. B. Wigley, H. Muirhead, and J. J. Holbrook, personal communication). Figure 1 was constructed from this, the only structure of the *B. stearothermophilus* enzyme showing the coenzyme loop at high resolution when the refinement had progressed to an *R* factor of 21.8% for 62 506 independent reflections at resolution 3–0.25 nm.

Materials and Methods

Mutagenesis. Mutants of the lactate dehydrogenase from *B. stearothermophilus* were generated by the oligonucleotide mismatch procedure (Winter et al., 1982) in M13 with the mutagenic oligonucleotide as the primer for in vitro chain extensions. To accommodate hydrophobic α-keto acids larger than pyruvate, the following mutations were made on separate genes: $^{102-105}$GlnLysPro → MetValSer (oligonucleotide sequence: $^{3'}$CCGCGGTTGTACCACAGCCCGCTCTGC$^{5'}$) and $^{235-236}$AlaAla → GlyGly (oligonucleotide sequence: $^{3'}$CGCGCTACCGCCGATGGTTTA$^{5'}$). In order to obtain a construction with both sets of mutations, the $^{102-105}$GlnLysPro → MetValSer gene was restricted with *Eco*RI and *Xho*I at sites on either side of the mutated region, and this gel-purified fragment was then ligated into gel-purified M13mp8 containing the $^{235-236}$AlaAla → GlyGly mutant gene from which the *Eco*RI/*Xho*I fragment had been removed (see Figure 2). The wild-type and mutant enzymes were expressed in the

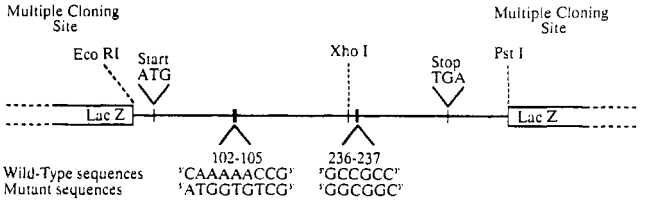

FIGURE 2: Map of the *B. stearothermophilus* lactate dehydrogenase gene in bacteriophage M13mp8. The diagram shows the two regions that were altered (indicated by the two ∧s) and the position of the *Xho*I restriction site between them which enabled the construction of a gene containing both mutated regions by fusion of the two genes which each contained one of the sets of mutations.

pKK223-3 plasmid in *Escherichia coli* as previously described (Barstow et al., 1986). Like the wild type, the level of expression of all of the enzymes was 25–30% of the soluble *E. coli* protein, indicating that the mutant enzymes will fold effectively and are resistant to *E. coli* proteases even with five amino acid substitutions.

Enzyme Purification. The wild-type enzyme was purified by affinity chromatography on oxamate-Sepharose (Clarke et al., 1985b), and the mutant enzymes were purified on Blue Sepharose-F3GA (Hart et al., 1987) owing to their poorer binding to oxamate–Sepharose. The mutant enzymes were found also to bind very poorly to the Blue Sepharose-F3GA column but were retarded sufficiently to separate them from most of the *E. coli* proteins. The fractions containing *B. stearothermophilus* LDH activity were then precipitated by the addition of ammonium sulfate (430 g/L). The precipitated protein was spun down (20 min at 10000*g*) and the pellet resuspended in, and dialysed against, 50 mM triethanolamine-HCl/NaOH, pH 7.5. The sample was then applied to a column (1.6 × 20 cm) of Q-Sepharose Fast Flow equilibrated in the above buffer and eluted with a gradient (0–0.35 M) of NaCl. *B. stearothermophilus* LDH eluted at a concentration of NaCl of about 0.2 M. The enzyme was estimated to be 98% pure by SDS–polyacrylamide gel electrophoresis on a Pharmacia Phast System.

Steady-State Kinetics. Steady-state measurements were made by following the decrease in absorbance at 340 nm in the NADH/NAD$^+$ conversion. All assays were at 25 °C and in 100 mM triethanolamine-HCl/NaOH buffer, pH 6. Determinations of k_{cat} and K_M for the various substrates were made at saturating NADH concentrations (0.2 mM) and in the presence of 5 mM FBP (unless otherwise stated). We define k_{cat} as the rate of turnover of the substrate at saturating coenzyme and substrate concentrations. The K_M for NADH was determined at saturating pyruvate concentrations (0.5 mM).

Primary Kinetic Isotope Effects. Deuterium isotope effects on k_{cat} in the pyruvate-to-lactate direction were determined in rapid-mixing, single-turnover experiments (Clarke et al., 1985a, 1986) using a Hi-Tech stopped-flow spectrometer (Hi-Tech, Salisbury, Wilts, U.K.) and measuring the decrease in A_{340} as NADH is converted to NAD$^+$. The rate constants were compared for reactions in which NADH and specifically deuterated NADH (NADD, [*nicotinamide*-4-^{2}H]NADH) were used as coenzyme. The conditions used were 100 mM triethanolamine-HCl/NaOH buffer, pH 6.0, 40 μM enzyme sites, 40 μM NADH, 5 mM FBP, and varying substrate concentrations. The measurements were made in transient rather than steady-state experiments owing to the insensitivity of the former to small amounts of tight-binding NADH breakdown products generated in the synthesis of the deuteriated coenzyme.

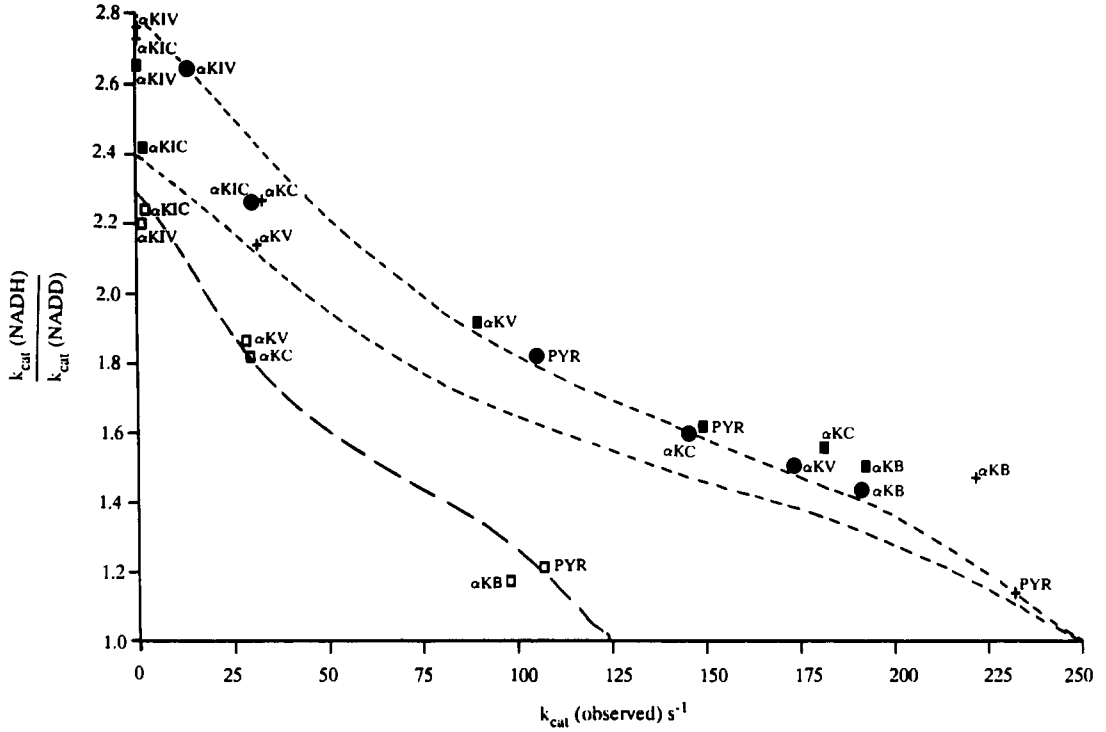

FIGURE 3: Variation in the primary deuterium isotope effect with the maximum velocity of the lactate dehydrogenase mutants. The ratio of the rates with NADH and with NADD was determined from stopped-flow experiments (see text) with saturating concentrations of pyruvate (PYR), α-ketobutyrate (αKB), α-ketovalerate (α-KV), α-ketocaproate (α-KC), α-ketoisovalerate (α-KIV), and α-ketoisocaproate (α-KIC). This ratio is plotted against the k_{cat} with NADH. The reaction was modeled as in Holbrook et al. (1975), as shown in eq 1. For wild-type

$$E^{pyruvate}_{NADH} \overset{k_{isom}}{\rightleftharpoons} E^{*pyruvate}_{NAD} \overset{k_{chem}}{\rightleftharpoons} E^{lactate}_{NAD} \rightarrow \text{products} \quad (1)$$

(+), $^{235\text{-}236}$GG (■), and $^{102\text{-}105}$MVS/$^{235\text{-}236}$GG (●), k_{isom} was 250 s^{-1}. The envelope lines (---) assume $k_{chem}(NADH)/k_{chem}(NADD)$ = 2.8–2.4. The line (--) models k_{isom} 125 s^{-1} and $k_{chem}(NADH)/k_{chem}(NADD)$ = 2.3 for the mutant $^{102\text{-}105}$MVS (□) with the various substrates.

Modeling Primary Kinetic Isotope Effects. Primary kinetic isotopes were modeled by using the FACSIMILE program (Chance et al., 1977). The model is a two-step reaction in which the first step is a conformational isomerization (coenzyme loop closure) and the second step is the redox reaction (Clarke et al., 1987). For the purpose of the model both steps are made essentially irreversible. The isotope effect is modeled by reducing the rate constants for the chemical step by the appropriate amount indicated on Figure 3 (the intrinsic isotope effect).

Thermal Stability. Thermal stability measurements were made at pH 6 over a range of temperatures from 40 to 100 °C. The concentration of protein in the incubations was 2 μM (subunits), and samples were incubated at each temperature for 20 min and stored on ice prior to assay.

Sequencing. For all three mutant enzymes the genes were sequenced by using the dideoxy chain termination method (Sanger et al., 1980) in order to check that the correct nucleotides had been incorporated and that no secondary mutations had occurred. Some sequences were determined by using a Du Pont Genesis 2000 automated sequencer.

RESULTS

Effects of Mutations on Steady-State Kinetic Constants. Table I shows the steady-state catalytic properties of the wild-type and mutant enzymes with various α-keto acids at saturating concentrations of coenzyme. The mutant enzymes are better able to tolerate substrates with bulky side chains than the wild-type enzyme.

With k_{cat} as a criterion of improved catalysis for larger substrates the enzyme containing $^{102\text{-}105}$MetValSer alone has generally reduced rates with smaller members of the series and marginal improvement with the two largest substrates tested. For all the substrates except pyruvate this mutant enzyme shows a generally improved (lower) K_M.

In the case of the $^{235\text{-}236}$GlyGly mutant enzyme k_{cat} is slightly worsened for the two smallest substrates but is increased for the larger members of the series. With the exception of pyruvate, the K_M values for all the substrates are only marginally altered. This is as expected if the mutation is facilitating the shape change of the helix rather than simply increasing volume available to side chains.

The mutant enzyme with both sets of changes ($^{102\text{-}105}$MetValSer/$^{235\text{-}236}$GlyGly) has improved k_{cat} for all of the substrates with the exception of pyruvate. The rates are improved by as much as 55-fold. Again, with the exception of pyruvate, the K_M values are hardly changed. This results in a higher catalytic efficiency (as measured by k_{cat}/K_M) than the wild-type enzyme for substrates larger than α-ketobutyrate.

Coenzyme Binding. The mutations have not adversely affected the binding properties of NADH in spite of the fact that the enzymes only bound poorly to the Blue Sepharose-F3GA column. Steady-state kinetics in the presence of 0.5 mM pyruvate show that the K_M for NADH binding is 18 μM for the wild-type and $^{235\text{-}236}$GlyGly mutant enzymes and 14.5 μM for the $^{102\text{-}105}$MetValSer and $^{102\text{-}105}$MetValSer/$^{235\text{-}236}$GlyGly mutant enzymes.

Primary Kinetic Isotope Effects. Figure 3 shows how the primary kinetic deuterium isotope effects vary with k_{cat} for all of the mutants with all of the substrates. The results of the modeling using FACSIMILE are represented by the dashed lines. The wild-type enzyme together with the $^{235\text{-}236}$GlyGly and $^{102\text{-}105}$MetValSer/$^{235\text{-}236}$GlyGly mutant enzymes all fit the model where the maximum rate of conformational change is

Table I: Steady-State Kinetic Properties of Wild-Type and Mutant Lactate Dehydrogenases with Various α-Keto Acids

		enzyme			
substrate	constant	wild type	$^{102-105}$MVS	$^{235-236}$CG	$^{102-105}$MVS/$^{235-236}$GG
pyruvate	k_{cat} (s^{-1})	250	66	167	32
	K_M (mM)	0.06	0.16	4	4
	k_{cat}/K_M (M^{-1} s^{-1})	4.2×10^6	4.1×10^5	4.2×10^4	8.0×10^3
α-ketobutyrate	k_{cat} (s^{-1})	186	31	92	205
	K_M (mM)	1.1	0.17	2.9	3.3
	k_{cat}/K_M (M^{-1} s^{-1})	1.7×10^5	1.8×10^5	3.2×10^4	6.2×10^4
α-ketovalerate	k_{cat} (s^{-1})	51	9.5	58	166
	K_M (mM)	8	1	8.3	4
	k_{cat}/K_M (M^{-1} s^{-1})	6.4×10^3	9.5×10^3	7.0×10^3	4.2×10^4
α-ketocaproate	k_{cat} (s^{-1})	29	20	240	185
	K_M (mM)	3.4	2.3	5.6	7.1
	k_{cat}/K_M (M^{-1} s^{-1})	8.5×10^3	8.7×10^3	4.3×10^4	2.6×10^4
α-ketoisovalerate	k_{cat} (s^{-1})	0.26	0.55	0.68	11.3
	K_M (mM)	22	8.2	14.3	22
	k_{cat}/K_M (M^{-1} s^{-1})	12	67	50	570
α-ketoisocaproate	k_{cat} (s^{-1})	0.33	0.67	1.74	18.5
	K_M (mM)	6.7	1.9	15.4	14.3
	k_{cat}/K_M (M^{-1} s^{-1})	50	353	110	1.3×10^3
pyruvate, no FBP	k_{cat} (s^{-1})	250	61		
	K_M (mM)	3.0	15	>50	>50
	k_{cat}/K_M (M^{-1} s^{-1})	8.3×10^4	4.1×10^3	665	190

250 s^{-1} and the intrinsic isotope effect is between 2.4 and 2.8. However, in the case of the mutant enzyme containing $^{102-105}$MetValSer the maximum rate of reaction is decreased and so fits the model where the maximum rate of conformational change is 125 s^{-1} and the intrinsic isotope effect is 2.3.

Thermal Stability. The mutant enzymes retain high thermal stability. The wild-type and mutant enzymes are all 100% active in an enzyme test after incubation at 60 °C for 20 min. The $^{102-105}$MetValSer mutant enzyme has the same thermal stability as the wild-type enzyme (50% of activity is lost at 77 °C during a 20-min incubation), while the other two mutant enzymes lose 50% activity at 72 °C during a 20-min incubation. The slight reduction in thermal stability observed for the $^{235-236}$GlyGly and $^{102-105}$MetValSer/$^{235-236}$GlyGly mutant enzymes is in accord with the design criterion that glycine residues would destabilize and mobilize the helix and generate the plasticity needed to envelope a range of different side chains (Bone et al., 1989).

Sequencing. The DNA sequences showed that the correct mutations had been incorporated and no additional mutations had occurred (data not shown).

Discussion

The environments of the two sets of mutations and their relationships to the substrate binding site of lactate dehydrogenase are shown in Figure 1. The $^{102-105}$GlnLysPro → MetValSer mutations are located on the coenzyme loop which closes over the active site after the substrate has bound. The $^{235-236}$AlaAla → GlyGly mutations are located on helix G onto which this mobile loop folds. Thus, we hypothesize that it is the "jaw" region, between the loop and helix G, where substrate side-chain recognition occurs.

The atomic structure of mouse testicular apo lactate dehydrogenase C_4 [which has broad substrate specificity (Blanco et al., 1976)] has been determined (Hogrefe et al., 1987) and was found to be "closed" and as such resembles the conformation found in ternary complexes rather than the "open" conformation found in dogfish apo-LDH A_4. This may be due to the increased hydrophobicity in the "jaw" region causing the coenzyme loop and helix G to stick together. This may explain why the mutant enzymes only bind poorly to Blue Sepharose-F3GA while steady-state coenzyme binding is virtually unaffected.

The introduction of methionine and valine into the coenzyme loop at 102–103 instead of glutamine and lysine has increased the hydrophobicity of this region of the protein so that substrates with large aliphatic side chains can bind to the enzyme more easily (giving rise to the observed improved K_Ms). The proline to serine mutation at 105 should give the loop more segmental flexibility and so allow larger substrates to access the active site.

The replacement of the two alanines at 235–236 with two glycines in the middle of helix G would have the effect of introducing a "break" or "kink" into the helix, like that which can be seen in the mouse LDH C_4 structure (Hogrefe et al., 1987). This may alter the conformation of the helix, enabling large substrates to bind in more favorable orientations, thus enhancing k_{cat}.

The kinetic analysis of the effect of deuterium substitution on the mechanism of both the wild-type enzyme and the mutant enzymes with different substrates revealed that with one exception the results could be represented by a simple model in which substrate binding to the enzyme–NADH complex resulted in a conformational change at 250 s^{-1}, to generate the catalytic vacuole, followed by a chemical bond breaking step at rates that varied with mutant and substrate (Clarke et al., 1986, 1988). Very fast enzymes were rate-limited by the shape change. As soon as either the changed substrate or the mutated enzyme reduced the rate of bond breaking significantly below 250 s^{-1}, then the shape change was no longer rate limiting. The model of Figure 3 illustrates that the mutant with $^{102-105}$MetValSer alone in the coenzyme loop region has an intrinsic rate of shape change of about 125 s^{-1}. This is the first time we have observed a "conformational" mutation in this lactate dehydrogenase—that is, one in which the rate of the shape change is reduced but without significantly decreasing the rate of chemical bond breaking. For any protein framework/substrate pair where k_{NADH}/k_{NADD} approaches the limit of about 3, the rate of isomerization is not determined by the experiments—other than that $k_{isom} > 5k_{cat}$. Thus there may be other shape-change mutant enzymes, but it is only with $^{102-105}$MetValSer with pyruvate and α-ketobutyrate when we observe reduced rates with no significant primary kinetic isotope effects that such a mutation can be identified with any confidence. It is perhaps not wholly surprising that the conformational mutation is in this mobile

coenzyme loop, the closure of which governs the maximal velocity of the wild-type enzyme. Figure 3 also shows that the rate of shape change increases again from 125 s^{-1} to 250 s^{-1} for the mutant enzyme in which the change in the structure of the coenzyme loop ($^{102-105}$MetValSer) is complemented by an increase in available volume at helix G due to the insertion of two glycine residues instead of alanines. This again suggests, in agreement with the results of Waldman et al. (1986), that the shape change is concerted, involving not only the movement of the coenzyme loop but also compensatory rearrangements of other structures around the active site (Atkinson et al., 1987).

These results show that it is possible to design and make a new α-hydroxy acid dehydrogenase with broad substrate specificity by making small changes to an existing lactate dehydrogenase framework. The resulting enzyme is one that retains high thermal stability and can catalyze reduction reactions at rates which are useful for chemical syntheses. For example, from Table I it can be seen that the enzyme containing both the helix G mutations and the coenzyme loop mutations (i.e., the $^{102-105}$MetValSer/$^{235-236}$GlyGly mutant enzyme) has k_{cat} values of 11.3 s^{-1} for α-ketoisovalerate and 18.5 s^{-1} for α-ketoisocaproate, which are both over 40-fold better than the k_{cat} values of the wild-type enzyme for these substrates. Neither of the two sets of mutations alone gave rise to such a dramatic improvement in k_{cat}. The maximum rate of conformational change is decreased in the mutant enzyme containing $^{102-105}$MetValSer, but this effect is reversed by the presence of $^{235-236}$GlyGly in the $^{102-105}$MetValSer/$^{235-236}$GlyGly mutant enzyme. This suggests that it is necessary to have both sets of mutations in order to produce a broad substrate specificity α-keto acid dehydrogenase in which the rate of loop movement is the same as in the wild-type enzyme. The chances of obtaining complementary mutations in both the coenzyme loop region and the helix region by random mutagenesis are very low. This object was obtained quickly in the present case and highlights the main advantage of rational protein design over random mutagenesis. However, once both the complementary surfaces are both redesigned, there is no reason why random mutagenesis followed by screening cannot be used for optimization.

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