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On the Effects of Replacing the Carboxylate-Binding Arginine-171 by Hydrophobic Tyrosine or Tryptophan Residues in the L-Lactate Dehydrogenase from *Bacillus stearothermophilus*†

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ABSTRACT: For L-lactate dehydrogenases (LDH's), the interaction of the guanidinium group of their Arg171 residue with the carboxylate group of an α-keto acid is of primary importance in orienting the substrate productively at the active site. LDH's such as that of *Bacillus stearothermophilus* (BSLDH) are of practical importance for the preparation of chiral 2-hydroxy acids used as synthons in asymmetric synthesis but would even be more valuable in this regard if their specificities were broader. With a view to tailoring the specificity of BSLDH toward carbonyl substrates that lack an α-carboxyl group such as ketones, site-directed mutagenesis has been applied to replace Arg171 by the approximately isosteric, but hydrophobic, amino acids Tyr and Trp. The mutant enzymes exhibit remarkably good catalytic activities toward representative α-keto acids RCOCOOH, where R = Me, Et, *n*-Pr, *n*-Bu, and CH_2OH, although for the mutant enzymes the k_{cat}/K_M's are lower by $\sim 10^3$–10^4-fold than those for native BSLDH. Surprisingly, the 171 → Tyr/Trp enzymes are significantly more active than 171 → Lys (Hart et al., 1987a), for which an interaction of a positively charged side chain with substrate COO^- is retained. Preparative-scale 171 → Trp catalyzed reduction of pyruvate gave optically pure L-lactate, showing that L stereospecificity of such LDH enzymes was unaffected by the loss of Arg171. The retention of L stereospecificity is attributed to secondary polar or hydrogen-bonding associations of Arg109 and Thr246, respectively, with the substrate COO^- function that are of sufficient magnitude to maintain "normal" substrate orientation. These "fail-safe" carboxylate-favoring interactions may also be responsible for the failure of the 171 mutant LDH's to accept the representative ketones acetone, methyl pyruvate, and butane-2,3-dione as substrates.

Lactate dehydrogenases (LDH's)[1] are well-documented, NAD/H-dependent enzymes of the oxidoreductase group that catalyze stereospecific C=O ⇌ CH(OH) transformations of the type shown in eq 1 (Holbrook et al., 1975). In addition

$$\underset{\mathbf{1}}{RCOCOOH} + NADH + H^+ \overset{LDH}{\rightleftharpoons} \underset{\mathbf{2}}{RCH(OH)COOH} + NAD^+ \quad (1)$$

a, R = CH_3 **d**, R = $(CH_2)_3CH_3$
b, R = CH_2CH_3 **e**, R = CH_2OH
c, R = $(CH_2)_2CH_3$

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to their natural pyruvate (**1a**) or lactate (**2a**) substrates, LDH's also catalyze the reductions of other structurally varied α-keto acids, still in a highly stereospecific manner, but usually with significantly reduced activity (Meister et al., 1952; Duncan et al., 1969; Lane & Dekker, 1969; Pensa et al., 1982; Kim & Whitesides, 1988; Luyten et al., 1989).

LDH's are among the enzymes that have been applied in organic synthesis (Akiyama et al., 1987; Wong & Whitesides, 1985; Jones & Beck, 1976; Jones, 1986; Klibanov, 1983, 1986; Schneider, 1986) for the preparation of valuable chiral synthons required in asymmetric synthesis of biologically important molecules. So far, only natural enzymes have been exploited for this purpose in this rapidly expanding field. However, it is already clear that the spectrum of wild-type enzymes currently available cannot hope to accommodate the enormous range of different substrate specificities that the structural diversity of the requirements of synthetic organic chemistry demand. This deficiency is potentially correctable by controlled modification of enzyme structure by site-directed mutagenesis, and several examples of progress in this direction have been reported (Benkovic et al., 1988; Graf et al., 1987; Russel et al., 1987; Wells et al., 1987; Cronin et al., 1987; Gardell et al., 1987). We selected the LDH group for protein engineering studies since, in contrast to most of the broad specificity enzymes in wide synthetic use, its members have relatively narrow tolerances of variations in substrate structures. Accordingly, we have begun attempts to broaden LDH specificity (Luyten et al., 1989), a goal with considerable practical potential since α-hydroxy acids are very valuable chiral synthons in organic syntheses (Hanessian, 1983; Mori, 1981; Seebach et al., 1980).

The target enzyme chosen was the L-LDH (BSLDH) from the thermophile *Bacillus stearothermophilus*. BSLDH was selected for several reasons. It is a very stable, mechanistically well-studied enzyme (Schär et al., 1979; Clarke et al., 1985, 1986a; Waldman et al., 1988) whose utility in preparative-scale reductions of α-keto acids has been established (Bur et al., 1989). In addition, being from a thermophile, BSLDH is tolerant of the high temperatures often applied in organic synthesis. This also simplifies its purification. Furthermore, BSLDH has been cloned and overexpressed (Barstow et al., 1986; Zülli et al., 1987) and established as an excellent candidate for controlled structure mutation and predetermined specificity change by site-directed mutagenesis (Hart et al., 1987a,b; Wigley et al., 1987a; Clarke et al., 1987a,b, 1988). Although the X-ray structure of BSLDH is not yet fully refined, it is known[2] to be similar to those of the LDH's from pig heart (PHLDH; Grau et al., 1981) and dogfish muscle [DMLDH, apo structure (Abad-Zapatero et al., 1987); ternary structure (Piontek and Rossmann, personal communication)]. The primary sequence of BSLDH corresponds closely to those of L-LDH's from many other species, with the amino acids around the active site and the coenzyme binding domain being particularly highly conserved (Stangl et al., 1987).

One of the major binding and orientation determinants of LDH specificity is the polar interaction of the positively charged guanidinium residue of Arg171 with the carboxylate anion of the substrate (Holbrook et al., 1975). This has been identified in all relevant X-ray crystal structures, including those of the benchmark PHLDH and DMLDH complexes. The Arg171 to COO^- binding, in conjunction with the association of the imidazolium residue of His195 with the carbonyl group, plays a key rôle in ensuring an optimal orientation of an α-keto acid substrate prior to the concerted stereospecific reduction of the carbonyl function by the hydride ion from the dihydronicotinamide moiety of NADH. The importance of Arg171 in this regard was verified by Hart et al. (1987a), who replaced it by the shorter lysine residue and found that binding efficiencies and catalytic rates were drastically reduced for α-keto acids **1a–c** with the 171 → Lys mutant BSLDH.

For broadened organic synthesis potential, it would be advantageous if BSLDH could accept carbonyl substrates, such as aldehydes and ketones, that lack the carboxyl group that has been mandatory up till now.[3] Toward this goal of making the enzyme less specific for carboxylic substrates, and to evaluate further the contribution of the protonated guanidinium group of Arg171 to substrate binding and catalysis, we have replaced this residue by the amino acids tyrosine and tryptophan. These are approximately isosteric with arginine, but have hydrophobic side chains. This paper reports the catalytic behavior of these mutants with the representative α-keto acid substrates **1a–e**.

Experimental Procedures

Materials and Strains. Deoxyoligoribonucleotides were made by automated phosphoramidite chemistry on a DNA synthesizer (Applied Biosystems 380B). Arg171 mutants were constructed with the following primers: Tyr (Y); GAT ACG GCG *TAC* TTC CGC; Trp (W); GAT ACG GCG *T*GG TTC CGC. Formate dehydrogenase (FDH, from yeast, lyophilized; batch 11123822-85), fructose 1,6-diphosphate (FDP), pyruvate, and NADH were purchased from Boehringer Mannheim and all the other α-keto acids from Sigma Chemical Co. Sodium formate was obtained from Fisher Scientific Co. The *Escherichia coli* strains used were JM101, RZ1032, and TG2 [*rec A* form of TG1 (Gibson, 1984)]. The BSLDH gene construct, obtained from J. J. Holbrook, was used in a pTZ-R18 vector as described (Luyten et al., 1989). The growth medium used was YT(2×) (16 g of bactotryptone, 10 g of yeast extract, and 5 g of NaCl per liter) supplemented with 70 μg/mL ampicillin.

Cloning and Mutagenesis. The mutant BSLDH genes were made by primer extension (Kunkel et al., 1987) as described previously (Luyten et al., 1989). Mutants were identified by colony hybridization with the ^{32}P-labeled mutagenic oligonucleotides following standard procedures (Wallace et al., 1979). The presence of the mutation was verified by dideoxy sequencing, and the whole coding sequence of the BSLDH gene was checked by sequencing with a set of four oligonucleotide primers, equally distributed over the whole gene.

Purification of Mutant BSLDH. The mutant genes were expressed in *E. coli* TG2 and purified as described earlier (Luyten et al., 1989) except that the enzyme was precipitated prior to hydrophobic interaction chromatography with ammonium sulfate (75% saturation) and redissolved in triethanolamine hydrochloride buffer (100 mM, pH 6.0), 25% saturated with ammonium sulfate. This yielded about 20 mg

[1] Abbreviations: BSLDH, lactate dehydrogenase from *Bacillus stearothermophilus*; LDH, lactate dehydrogenase; FDH, formate dehydrogenase; FDP, fructose 1,6-diphosphate; NADH, nicotinamide adenine dinucleotide (reduced).

[2] K. Piontek and M. G. Rossmann, personal communication. The X-ray structures of the binary BSLDH–NADH complex (M. G. Rossmann and K. Piontek, personal communication) and the ternary complex with BSLDH–oxamate–NADH (Wigley et al., 1988) are currently being refined.

[3] No BSLDH activity has yet been observed for any of the representative aldehydes or ketones surveyed so far (Luyten et al., unpublished results).

Table I: Kinetic Data of the Arg171 → Tyr/Trp/(Lys) Mutations in Lactate Dehydrogenase from *Bacillus stearothermophilus*[a]

	k_{cat} (s^{-1})				K_M (mM)				k_{cat}/K_M (M^{-1} s^{-1})			
R-COCOOH	WT[b]	Y	W	K[c]	WT	Y	W	K	WT	Y	W	K
1a	188	64	23	18	0.04	58	51	125	4.8E6	1.1E3	4.5E2	1.2E2
1b	155	6.4	5.6	0.3	0.34	117	175	55	4.6E5	5.4E1	3.2E1	5.5
1c	44	0.4	0.3	0.02	2.4	99	96	30	1.9E4	4.0	3.3	1.0
1d	25	1.0	0.8		1.5	91	54		1.7E4	1.1E1	1.4E1	
1e	125	30	13		0.16	56	90		7.8E5	5.5E2	1.5E2	

[a] Measured under steady-state conditions at 25 °C in triethanolamine hydrochloride buffer (100 mM), pH 6.0, NADH (0.2 mM), and FDP (5 mM). [b] Values for the wild-type enzyme from Bur et al. (1989) and Luyten et al. (1989), measured under the same conditions as in *a*, but in triethanolamine hydrochloride buffer (20 mM, pH 6). [c] Literature values from Hart et al. (1987a).

of LDH per 100 mL of overnight culture after the hydrophobic interaction chromatography (purity >95%, judged by SDS gel electrophoresis). In order to get a highly concentrated enzyme solution (200–450 μM), the BSLDH was then dialyzed against triethanolamine hydrochloride buffer (100 mM, pH 6.0), precipitated with ammonium sulfate (75% saturation), and redissolved in a small volume of triethanolamine hydrochloride buffer (100 mM, pH 6.0), containing EDTA (1 mM).

Kinetic Studies. The α-keto acids **1a–e** were used as received. Unless noted otherwise, all measurements were done under steady-state conditions at 25 °C in triethanolamine hydrochloride buffer (100 mM, pH 6.0) and with saturating concentrations of FDP (5 mM) and NADH (0.2 mM). Depending on the substrate, enzyme concentrations between 0.02 and 2.02 μM were used and the assay solutions incubated with FDP and NADH for 20 min prior to commencement of measurements. Enzyme concentrations were determined spectrophotometrically at 280 nm using extinction coefficients of 0.98 g^{-1} mL^{-1} for 171 → Tyr and 1.10 g^{-1} mL^{-1} for the 171 → Trp mutants. These values were calculated by using the total number of tyrosine and tryptophan residues (Cantor et al., 1980). The molecular mass of 34 862 daltons was calculated on the amino acid sequence on the basis of the nucleotide sequence for BSLDH (Barstow et al., 1986; Zülli et al., 1987). The reactions were initiated by addition of a substrate solution (0.5–2M) to give final concentrations of **1a–e** of 5–80 mM in a 1 mL, 1-cm path-length cuvette. The reactions were followed by recording the decrease in the NADH absorbance at 340 nm using a Shimadzu UV-240/PR1 spectrophotometer equipped with a constant-temperature cell holder. The kinetic parameters were calculated from single experiments [pyruvate (**1a**) in duplicate] with each individual data point being measured in triplicate. Michaelis constants (K_M) and turnover numbers (k_{cat}) were determined from Lineweaver–Burk plots and least-square analyses, the correlation coefficients being >0.995.

Preparative-Scale Reduction. The preparative-scale reduction was carried out under pH-stat (Metrohm 655) control (Bur et al., 1989); 10 mL of triethanolamine hydrochloride buffer (100 mM, pH 6.0) was degassed for 30 min by bubbling nitrogen, and then sodium pyruvate (**1a**, 550 mg, 5 mmol), FDP (27.5 mg, 50 μmol), sodium formate (374 mg, 5.5 mmol), dithiothreitol (1 μL of 100 mM solution), and NADH (10.2 mg, 14.3 μmol) were added. When all components were dissolved, the reaction was started by adding the mutant Arg171 → Trp BSLDH (3.13 mg, 90 nmol) and formate dehydrogenase (17 mg, 11.5 units). The reduction reaction was performed with gentle magnetic stirring under nitrogen at 22 °C, with the pH being maintained at 6.0 by pH-stat-controlled addition of HCl (0.5 M). After 45 h (9.44 mL, 4.72 mmol, 0.94 equiv of HCl consumed), the mixture was acidified with HCl (10 M) to pH 2.8, filtered through a Hyflo-SuperCel-packed sintered funnel, and extracted with ethyl acetate (3×). The combined organic phases were dried with magnesium sulfate and filtered, and the solvent was evaporated under reduced pressure. Kugelrohr distillation (110 °C, 1 mmHg) yielded an oil (259 mg, 56% yield) that solidified at room temperature: $[\alpha]^{22}_D$ −12.8° (*c* 1.01, 1.5 M NaOH), lit. (Beil., **4**, III, 263) sodium salt $[\alpha]^{22}_{546.1\text{Å}}$ −13.7° (*c* 4.0, water). The ^1H NMR spectrum was identical with that of authentic L-lactic acid.

Graphics Analyses. The graphics analyses on the wild-type enzyme and 171 → Trp/Tyr mutants were carried out for these BSLDH sequences modeled into the latest, highly refined, X-ray coordinates for the ternary complex of DMLDH–oxamate–NADH (Piontek and Rossmann, personal communication), using the MIDAS molecular modeling program (Ferrin et al., 1988) run on a Silicon Graphics Iris 2400 terminal.

Results

Purification of the enzyme was carried out as previously described using heat denaturation of the *E. coli* proteins at 65 °C (Fersht et al., 1988), followed by hydrophobic interaction chromatography on phenyl-Sepharose CL-4B (Bur et al., 1989). In control experiments, it was shown that at a temperature of 65 °C there was no loss in activity over a period of 80 min for the tyrosine mutant. In contrast, for the *E. coli* LDH isolated from TG2 cells which lacked the BSLDH plasmid, the total activity dropped by a factor of 2000 with this treatment. This *E. coli* activity after heating was insignificant compared with the total activity isolated from the same amount of cells harboring mutant BSLDH. The heat step ensured that all the activity recorded was due to the desired BSLDH, and not to inadvertent comtamination by *E. coli* LDH. This procedure resulted in proteins of >95% purity as judged by SDS gel electrophoresis. Further purification by chromatography on blue-Sepharose (Hart et al., 1987a; Luyten et al., 1989) gave >98% pure proteins but did not change the values of the kinetic constants. This extra step was therefore omitted for most of the protein preparations used in this study.

All the kinetic parameters were determined under steady-state conditions. They are recorded in Table I. While the two amino acid substitutions at position 171 each have a dramatic effect on catalytic performance, both mutant enzymes retain significant activity. In view of the drastic changes in 171 side-chain polarities of the wild-type and mutant enzymes, this is remarkable. In fact, it would not have been unexpected if such site-directed mutagenesis had resulted in proteins with no LDH activity. Binding efficiency, as reflected by K_M, is significantly reduced for both mutants for all the **1a–e** substrates, while the turnover numbers are lowered to lesser extents. In general, the K_M's for the tryptophan mutant are more seriously affected than for 171 → Tyr. With the natural substrate pyruvate, the k_{cat} for the tyrosine mutant remains surprisingly high, being only 3-fold lower than for the wild-type Arg171 protein. Progressive increases in the size of the alkyl side chains of the substrates have correspondingly

Table II: Fructose 1,6-Diphosphate Activation of Catalysis of Pyruvate (**1a**) Reduction by 171 Mutant LDH's

	k_{cat} (s^{-1})		K_M (mM)		k_{cat}/K_M (M^{-1} s^{-1})		
	+FDP[a]	−FDP[b]	+FDP	−FDP	+FDP	−FDP	activation (x-fold)
Tyr171 (Y)	64	62	58	227	1.1×10^3	2.7×10^2	4
Trp171 (W)	23	1.5	51	125	4.5×10^2	1.2×10	38
Lys171 (K)[c]	18	0.5	125	100	1.4×10^2	5	28
wild-type (R)[c]	250	250	0.04	2	6.3×10^6	1.3×10^5	50

[a] Measured under steady-state conditions at 25 °C in triethanolamine hydrochloride buffer (100 mM, pH 6.0), pyruvate (5–75 mM), NADH (0.2 mM), and FDP (5 mM). [b] Measured under the same conditions as in *a* but without FDP. [c] Literature constants from Hart et al. (1987a).

deleterious effects on the rates of catalysis for the first three members of the homologous substrates **1a–d**. In this series, the k_{cat}'s drop by factors of ≈10 per additional CH_2 group, reaching a minimum at 2-ketovalerate (**1c**). On addition of one further CH_2 group, to **1d**, the decrease in k_{cat} begins to recover. These overall trends are the same for both 171 mutant enzymes. The CH_2 effect is much less pronounced for the wild-type enzyme, where the k_{cat} differences between homologous α-keto acids are only 1.2–3.5-fold, with no reversal at **1d**.[4] The pattern in K_M behavior is just the opposite. The K_M values are similar, and consistently high, for both mutants with all the substrates, but in this case it is the K_M of the wild-type BSLDH that increases by a factor of ≈10 for each additional CH_2 group in the **1a–c** series. These opposing trends are normalized in the specificity constants, and the overall patterns of the decreases in catalytic efficiencies displayed by the k_{cat}/K_M values are the same for all three enzymes. Hydroxypyruvate (**1e**) is the best unnatural substrate for each enzyme and exhibits similar behavior for mutant and wild-type BSLDH catalyses. However, for both the 171 mutants, the k_{cat} and K_M constants for **1e** are significantly improved over those for its isostere 2-ketobuturate (**1b**).

BSLDH is allosterically activated by FDP (Clarke et al., 1986a). With pyruvate as substrate, this activation is due to a reduction of K_M in the presence of FDP, while k_{cat} remains unaffected (Table II). However, the 171 mutants differ in their response to FDP (Table II). The behavior of the Arg171 → Tyr enzyme is similar to that of native BSLDH in that its k_{cat} remains the same, but the reduction in its K_M is only 4-fold, compared with a 50-fold decrease for the native enzyme. On the other hand, the K_M's for the 171 → Trp enzyme, and the Lys mutant described by Hart et al. (1987a), are almost unaffected when FDP is added but show strongly increased k_{cat}'s. Both 171 → Trp/Lys mutants exhibit overall FDP activation factors of the same order of magnitude, as reflected by the k_{cat}/K_M values, as the native enzyme.

Without arginine at position 171 to help maintain the α-keto acid substrates in the correct orientation for *re* face delivery of the NADH hydride to the carbonyl group, as required for 2*S*-hydroxy acid formation to occur, it was conceivable that some reversal of BSLDH's normal L stereospecifcity might be occurring in the 171 → Trp/Tyr mutant catalyses. The degree of enantioselectivity retained by the mutant enzymes was therefore evaluated by carrying out a preparative-scale reduction of pyruvate on the 5-mmol scale with 171 → Trp BSLDH. FDP activating conditions were used together with NADH recycling with the FDH/formate system (Kula & Wandrey, 1987; Kim & Whitesides, 1988) as described previously (Bur et al., 1989). The reaction proceeded readily to over 90% conversion in 30 h. The reaction then slowed significantly due to the combination of the high K_M value and low remaining concentration of **1a**. The workup and purification yielded L-lactic acid[5] in 56% yield.

This experiment demonstrates that, even with their reduced activities, the mutant enzymes are fully viable in preparative-scale reactions. Furthermore, the 171 → Trp BSLDH showed no sign of substrate or product inhibition, even at the very high substrate concentrations employed synthetically.

Regrettably, our hope that the Arg171 → Tyr/Trp mutations would permit non-carboxy substrates such as ketones to become substrates of BSLDH was not realized. No detectable catalysis of NADH-dependent reduction of the representative ketones acetone, methyl pyruvate, or 2,3-dioxobutane was observable with either of the mutant enzymes, nor in the control experiments performed with native BSLDH itself.

Discussion

The observation that the 171 → Tyr/Trp enzymes remain viable LDH's despite the elimination of the polar Arg171–substrate–COO^- interaction previously considered essential in LDH-catalyzed reductions is remarkable. Even more surprising is the fact that the Tyr and Trp mutants, in which the 171-position side chains are hydrophobic, are more active than the 171 → Lys enzyme (Hart et al., 1987a,b) in which a polar 171–COO^- interaction is maintained.

There are significant differences between the catalytic behaviors of the native and mutant 171 → Tyr, → Trp, or → Lys enzymes toward the various α-keto acids **1a–e**. For example, they cope differently with the bulkier substrates. From Table I, it is evident that whereas the wild-type enzyme is relatively insensitive to increasing chain length of the substrate, a drastic reduction in k_{cat} is manifest for each of the 171 mutants. On the other hand, the trends in binding efficiencies, as reflected by K_M's, are in the opposite direction. Here it is the mutants that are the relatively insensitive enzymes, while the K_M's for the native BSLDH respond strongly to increasing chain lengths of **1a–d**. The changes in k_{cat}/K_M's for all the LDH's follow very similar patterns. However, with 2-ketohexanoate (**1d**), the specific activity increases from the **1c** levels for the 171 → Tyr/Trp mutants but remains the same for the wild-type protein.

Despite the similarities of the catalytic properties of 171 → Tyr/Trp with those of the 171 → Lys enzyme studied by Hart et al. (1987), there are differences such as the decreases in K_M for the bulkier substrates **1b,c** and the extent of allosteric activation by FDP. The reasons for the variations between the the 171 → Tyr enzyme, on the one hand, and 171 → Trp/Lys and native BSLDH, on the other, in their responses to allosteric activation by FDP (Table II) are not clear. In the wild-type enzyme, FDP is known to bind to Arg173 (Clarke et al., 1987a), which lies on the same α2F helix as Arg171 (Abad-Zapatero et al., 1987). It is therefore possible that slight structural differences between the 171 → Tyr

[4] The reversal for wild-type enzyme begins at 2-ketoheptanoic acid.

[5] Within the limits of experimental error, this material appeared optically pure. Since it was clear that the 171 → Trp enzyme retained its L stereospecificity, no direct measurements of percent enantiomeric excess were deemed necessary.

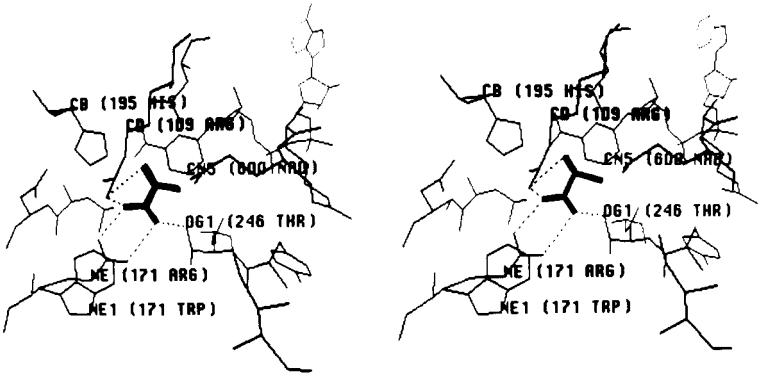

FIGURE 1: Graphics analysis of the mutant 171 → Trp BSLDH structure overlaid onto that of the wild-type 171Arg enzyme, with pyruvate (**1a**) at the active site. The BSLDH active-site structures were modeled on that of the 2.1-Å (R = 17.3) data of the oxamate–NADH–DMLDH ternary complex[2] using the MIDAS program (Ferrin et al., 1988). All of the amino acid residues displayed are identical for both DMLDH and BSLDH. The orientation of **1a** (drawn as heavy lines) depicted was modeled-in by using the known oxamate position and replacing the oxamate NH_2 group by CH_3. The key Arg171 guanidinium to COO^- binding interactions [shown as dotted lines, corresponding distances (d) of 2.79 and 3.12 Å] were retained. Additional interactions of **1a** with an NH_2 of Arg109 to the COO^- group (d = 2.86 Å) and to the carbonyl function (d = 3.21 Å) and of the OH of Thr246 to the COO^- of pyruvate (d = 2.52 Å) are also shown as dotted lines. These may be responsible for maintaining productive α-keto acid complex orientation in the mutant enzyme catalysis and for the retention of L stereospecificity by the 171 → Trp enzyme in its catalysis of **1a** to **2a**.

mutant and the other Table II LDH's are responsible for its unusually low degree of allosteric activation with FDP.

The lowered K_M effect of the 171 → Lys mutation was unanticipated and was attributed (Hart et al., 1987a) to nonproductive ES complex formation due to binding of carboxylate groups of **1b,c** by the Arg109 residue of the 171 → Lys mutant. It was suggested that this substrate-orienting competition between 171 → Lys and Arg109 was responsible for the unexpected reductions in the observed K_M values of **1b,c** relative to pyruvate (**1a**). In support of this, the existence of Arg109–COO^- binding has been identified in an X-ray structure with oxalate as a substrate analogue, where Arg171 and Arg109 each bind to one of the carboxylate functions of oxalate (White et al., 1976).

However, if this were the only reason, an even larger decrease in K_M's would be predicted for the 171 → Tyr/Trp mutants for which no strong 171 to COO^- interaction to ameliorate such Arg109 substrate binding is possible. In fact, the specificity constants for the Tyr/Trp mutants (Table I) are 3–10-fold higher than for 171 → Lys. This suggests that Arg109-induced nonproductive binding of α-keto acid substrates does not play a major rôle in the **1a–d** series.

In contrast, graphics analysis of wild-type and 171 mutant BSLDH's indicates that Arg109 can play a positive rôle in productive orientation of α-keto acid substrates. This is illustrated for the 171 → Trp enzyme in Figure 1, where one of the guanidinium NH_2's of Arg109 is suitably positioned to interact with the COO^- of **1a** and with that of the COO^- of oxamate in the most recent, highly refined, ternary structure of DMLDH–NADH–oxamate (Piontek & Rossmann, personal communication). In addition, further stabilization of this orientation is provided by the Thr246–COO^- hydrogen bonding shown.

The Arg109– and Thr246–COO^- interactions depicted in Figure 1 are the kinds of secondary functional group associations that may be responsible for the complete retention of L stereospecificity of the 171 → Trp enzyme in its preparative scale reduction of pyruvate to lactic acid. The existence of "fail-safe" interactions of this type for maintaining preferred ES alignments could be one of nature's devices to protect against major, inadvertent, changes in the specificities of key enzymes. If so, it will have to be taken into account whenever major alterations in specificity by protein engineering are contemplated. The predilection of the active-site Arg109 and Thr246 residues toward COO^- binding may also explain the inability of the 171 → Tyr/Trp enzymes to catalyze the reduction of the ketone substrate analogues evaluated, for which operation of any such carboxylate bias is precluded.

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Registry No. **1a**, 127-17-3; **1b**, 600-18-0; **1c**, 1821-02-9; **1d**, 2492-75-3; **1e**, 1113-60-6; FDP, 488-69-7; LDH, 9001-60-9; Arg, 74-79-3; Tyr, 60-18-4; Trp, 73-22-3.

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