may, in large part, arise from the hydrophobic contribution derived from the partial burial of the hydrophobic surface of the alanine methyl rather than from any conformational bias (see above).

In this paper (and the preceding paper[9]), we have reconsidered the partitioning of free energy contributions important in bimolecular associations and in ligand extension studies and present a self-consistent approach that may prove useful in the semiquantitative estimation of binding constants. Much work remains to firmly establish the thermodynamic basis of many of the interactions found in biologically important associations. In this regard we have shown that model systems, including crystals (as analogues of tight-binding complexes), can provide useful insights that guide estimates of entropy changes in molecular recognition phenomena.

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On the Factors Controlling the Structural Specificity and Stereospecificity of the L-Lactate Dehydrogenase from *Bacillus stearothermophilus*: Effects of Gln102→Arg and Arg171→Trp/Tyr Double Mutations

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Abstract: The factors determining the L-stereospecificity of the L-lactate dehydrogenase from *Bacillus stearothermophilus* have been probed by introducing Arg171Trp/Tyr and Gln102Arg mutations. These changes preclude normal 2-keto acid substrate binding via an Arg171–COO^- electrostatic interaction and are positioned to induce a reversal of the natural substrate binding mode, thereby leading to D-2-hydroxy acid formation. However, the L-stereospecificities of the mutant enzymes remain unchanged, showing that there are important fail-safe stereospecificity determinants that take over when the key Arg171–COO^- binding interaction is removed. The effects of the mutations on structural specificity are approximately additive, resulting in the broad 2-keto acid specificity of the wild-type enzyme being changed to give catalysts highly selective for the dicarboxylic substrate oxalacetate.

Lactate dehydrogenases (LDHs)[1] catalyze C=O ⇄ CH(OH) transformations of the type shown in eq 1.[2] The natural keto

$$RCOCOOH + NADH + H^+ \underset{\longleftarrow}{\overset{BSLDH}{\longrightarrow}} RCH(OH)COOH + NAD^+ \quad (1)$$

1	2
a. R=CH_3-	h. R=$C_6H_{11}CH_2$-
b. R=CH_3CH_2-	i, R=$C_6H_5CH_2$-
c. R=$CH_3(CH_2)_2$-	j, R=HOOC-
d. R=$CH_3(CH_2)_3$-	k, R=$HOOCCH_2$-
e, R=$CH_3(CH_2)_5$-	l. R=$HOOC(CH_2)_2$-
f. R=$(CH_3)_2CH$-	m. R=$HOCH_2$-
g. R=$(CH_3)_2CHCH_2$-	n. R=$BrCH_2$-

acid substrate is pyruvic acid (**1a**), but reductions of other structurally varied 2-keto acids to the corresponding 2-hydroxy acids have been reported.[3] Because such LDH-catalyzed reductions are stereospecific, each 2-hydroxy acid product is enantiomerically pure. This is of considerable practical importance since 2-hydroxy acids are valuable chiral synthons in asymmetric syntheses of biologically important molecules.[4-15] As a consequence, organic synthetic applications of LDHs are expanding.[3,16-18]

A remarkable feature of LDHs, as well as of most other dehydrogenases, is their high fidelity with regard to the stereose-

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(1) Abbreviations used: LDH, lactate dehydrogenase; BSLDH, LDH from *Bacillus stearothermophilus*; DMLDH, LDH from spiny dogfish muscle; NAD^+ and NADH, oxidized and reduced forms of nicotinamide adenine dinucleotide, respectively; FDP, fructose 1,6-diphosphate; MTPA, (*R*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl; CD, circular dichroism; WT, wild-type; Q102R, glutamine 102 to arginine mutation; R171Y and R171W, arginine 171 to tyrosine or tryptophan mutation, respectively; *E. coli*, *Escherichia coli*; ES, enzyme–substrate; K_M, Michaelis constant; k_{cat}, catalytic constant.

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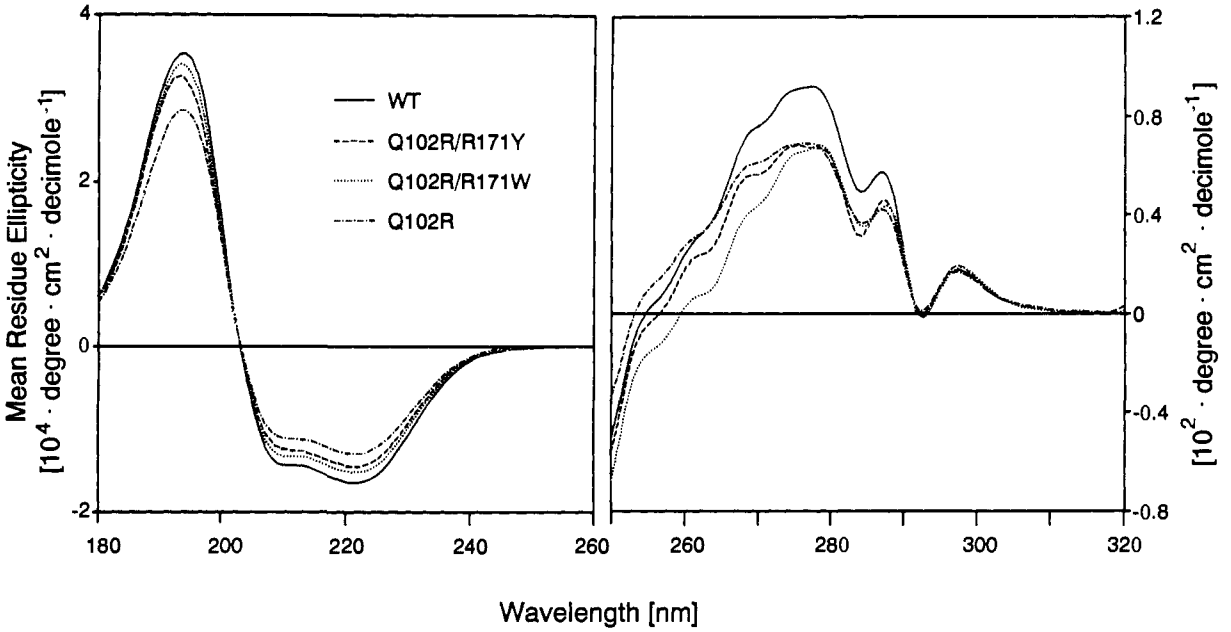

Figure 1. Far-UV (180–260 nm) and near-UV (250–320 nm) circular dichroism spectra of WT-BSLDH and mutants Q102R, Q102R/R171Y, and Q102R/R171W. Spectra were recorded in 30 mM sodium phosphate buffer at pH 7.2, without coenzyme, substrate, and activator. Data for the single mutant Q102R are from ref 26.

lectivity of oxidoreductions of both substrates[3,19] and coenzymes.[20,21] On the other hand, their structural specificities are not as strictly prescribed, and many LDHs tolerate some variation in substrate[3,16,17,22] and coenzyme[23-25] structures. However, the substrate structure tolerances of the natural enzymes are not broad enough to accept all 2-keto acids of organic chemical interest. In this regard, attempts to remove these synthetic shortcomings in structural specificity by controlled alterations in an LDH active site by site-directed mutagenesis have shown promise.[26-31] Furthermore, the specificity improvements achieved generally support the validities of the graphics and structural analyses applied in selecting the mutations made, thereby giving hope that a rationale basis will emerge for controlling LDH specificity by protein engineering methods. In contrast, while aspects of coenzyme stereospecificity have been addressed,[20,32,33] the molecular interactions determining the stereospecificity of lactate dehydrogenase-catalyzed reductions of its 2-keto acid substrates have not been explored experimentally to any significant degree. This paper further addresses the issues involved in determining both the structural specificity and stereospecificity of LDH substrates.

Our previous work on the factors controlling the structural specificity and stereospecificity of oxidoreductases was carried out using the L-LDH (BSLDH) from the thermophile *Bacillus stearothermophilus* as the target enzyme. BSLDH is an attractive vehicle for this purpose since it is synthetically useful[3] and is a very stable, mechanistically well-studied enzyme[34,35] of known X-ray structure[36,37] which has been cloned and overexpressed.[38,39] Crystal structure data[36] indicate the involvement of several active-site amino acid residues in enzyme–substrate (ES) interactions. One of the most obvious of the substrate binding forces not directly involved in catalysis, which has been considered a major contributor to substrate orientation in the L-stereospecificity mode, is the exceptionally strong[40] electrostatic bond that the side chain of Arg171 forms with the substrate's carboxylate group.[2] In a previous study,[41] the question of whether Arg171 was, in fact, crucial to L-stereospecificity was explored by introducing hydrophobic tyrosine and tryptophan residues at the 171 position. The disruption of the key R171–carboxylate interaction in the ES complexes for the R171Y,W mutant BSLDHs was manifest in diminished binding and catalysis for all substrate structures surveyed. However, the enzyme retained virtually complete L-stereospecificity with respect to pyruvate reduction. In a separate stereospecificity determinant study, we also generated a Glu102Arg mutant,[26] first described by Holbrook and co-workers[31] in their elegant introduction of malate dehydrogenase activity into BSLDH. As expected, this mutation provided an alternative substrate carboxylate binding site, as demonstrated by the high

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affinity of the Q102R BSLDH mutant for 2-keto dicarboxylic acids. It also changed the structural specificity of the enzyme. However, once again the L-stereospecificity of BSLDH was unaffected.[26]

In the present work, the effects of further perturbation of the electrostatic substrate-orienting forces induced by R171W,Y/Q102R double mutations on the L-stereospecificity and on the structural specificity of BSLDH are evaluated, together with the results on triple mutants in which a Cys97Gly replacement is also present. While the latter mutation was an inadvertant introduction, it is of interest due to its location in the "hinge" region of the 100–110 loop and because of its proximity to the enzyme's active site.[26]

Results

Generation of Mutant Enzymes. The genes for the BSLDH mutants Q102R and Q102R/C97G were changed in the codons for amino acid residue 171 to replace the arginine of the wild-type (WT) sequence by either tryptophan or tyrosine, using the mutagenic oligonucleotides 5′-pGATACGGCGTGGTTCCGC-3′ or 5′-pGATACGGCGTACTTCCGC-3′, respectively (mismatched bases are underlines). Screening of *E. coli* colonies after transformation with the products of the mutagenesis reactions by sequencing their plasmid DNA over the codons for amino acid 171 in the BSLDH genes revealed mutagenesis yields between 70 and 100%. R171Y mutants were also identified by *RsaI* restriction mapping due to the increase in the number of *RsaI* sites in the vector from 4 to 5. The absence of inadvertant mutations was verified by sequencing the entire coding regions of all four mutants. Positively identified single colonies were used to inoculate the shaking-flask cultures. After 15 h of incubation, 8 g/L of wet *E. coli* cells were harvested, yielding 107 mg/L of the mutant BSLDH in the crude extract after ultrasonic cell disruption and heat treatment. These values represent the average numbers from four cultures.

Circular Dichroism Data. The CD spectra were measured in order to evaluate the effects of the mutations on enzyme secondary structure. The data, which are recorded in Figure 1, show that the mutant proteins fold to native-like structures. Furthermore, the far-UV spectra of both the Q102R/R171Y and Q102R/R171W BSLDHs are of the shape characteristic of a helical protein. Fractional values for the occurrence of different secondary structures obtined by analysis of the far-UV CD data with the Contin program[42] indicate that the conformation of Q102R/R171Y is similar to that of the single mutant Q102R. In addition, the Q102R/R171Y data denote only a small decrease in helical content compared to WT-BSLDH, while for Q102R/R171W the spectra are even closer to the WT values. The CD spectra did not change when FDP was added. In the aromatic region between 250 and 320 nm, the spectrum of Q102R/R171Y is similar to that of the single mutant Q102R. The difference spectrum (not shown), using WT as reference, has a negative minimum at 275 nm and a negative shoulder at 283 nm. This is typical for the normal form of tyrosine. On the other hand, when the single mutant Q102R is used as reference, there is very little spectral difference above 270 nm. Thus, the addition of tyrosine at 171 does not contribute significantly to the CD spectrum. The spectrum of the mutant Q102R/R171W is also similar to that of Q102R between 320 and 275 nm. However, in the difference spectrum, there is a negative band around 265 nm, while the bands above 280 nm do not appear.

Stereospecificity Effects. These were first evaluated using phenylpyruvate (**1i**) as a representative substrate for preparative-scale reductions catalyzed by the mutant BSLDHs Q102R/R171Y, Q102R/R171W, Q102R/C97G/R171Y, and Q102R/C97G/R171W. Despite the lowering of the mutant's k_{cat} values to approximately 1% of the WT value, the mutant enzymes remain preparatively viable, with reductions of phenylpyruvate on a 2-mM scale affording 60–70% yields of (2*S*)-phenyllactate within 2–3 days, using enzyme amounts that are easily prepared in benchtop-scale fermentations. The phenyllactate (**2i**) samples isolated from the catalyses with each mutant and with WT for reference purposes were individually converted to the Mosher esters of their methyl esters. The ^{1}H-NMR spectra of these Mosher esters revealed that in all four cases the (2*S*)-phenyllactic acid products of the enzyme-catalyzed reactions were of >98% ee.

Numerous attempts were also made to use the best mutant substrate, oxalacetate (**1k**), for preparative-scale reductions. However, none of these was successful due to the rapid decarboxylation of **1k** to pyruvate under pH 6.0 reaction conditions, giving the maximum enzymic rate. At higher pHs, where decarboxylation to pyruvate was slower, the rates of reduction were also slower. As a result, only lactate products of pyruvate reduction were observed. All attempts to isolate and detect any malate (or its dimethyl ester after treatment with diazomethane) from the reaction mixtures were unsuccessful. Instead, the alternative approach of examining the rates of the reverse reactions, the oxidations of (*S*)- and (*R*)-malate, with NAD^+ as coenzyme and catalyzed by the Q102R/R171Y and Q102R/R171W enzymes, was explored. Both mutants did catalyze the oxidation of (*S*)-malate, with rates linearly increasing with substrate concentration. In the presence of 10 mM NAD^+, the highest turnover rates observed were 0.11 s^{-1} for Q102R/R171Y and 0.07 s^{-1} for Q102R/R171W, both at 1 M (*S*)-malate. With (*R*)-malate, no reaction was observed with either mutant in the concentration range 0.1–1.2 M at a turnover detection limit of 0.001 s^{-1}. Unfortunately, high assay-solution absorbance problems prohibited the use of higher malate concentrations in the photometric rate assays, and no saturation of the enzymes could be observed within the accessible malate concentration range. Consequently, k_{cat} and K_M values could not be determined. However, from the v vs [S] curves obtained, it was calculated[43] that the enantiomeric selectivities, as defined by the ratio $[k_{cat}/K_M((S)\text{-malate})]/[k_{cat}/K_M((R)\text{-malate})]$, are >110 and >70 for Q102R/R171Y and Q102R/R171W, respectively.

Structural Specificity Effects. Apparent Michaelis–Menten kinetic parameters were determined under steady-state conditions for the Q102R/R171Y- and Q102R/R171W-catalyzed reductions of the 2-oxo acid substrates **1a–n**. The results are summarized in Table I. With one exception (**1k**), the catalytic constants of both double mutants were reduced by 3–4 orders of magnitude compared to WT-BSLDH and were accompanied by increases in K_M values of similar magnitudes. However, for oxalacetate (**1k**), the k_{cat} and K_M values improved relative to WT to the extent that oxalacetate is now the best substrate for the mutant enzymes. The observation that the mutations narrowed the normally broad substrate specificity of WT-BSLDH while concomitantly improving the specificity toward the naturally disfavored oxalacetate was unexpected. Keto acids with large, hydrophobic side chains (**1d–i**) were better substrates for the Q102R/R171Y,W mutants than less hydrophobic keto acids (**1a,b,m**), the latter being the preferred substrates for WT-BSLDH. Branched-chain 2-keto acids such as **1f,g** are poorly accepted by both WT and mutant enzymes. In cases, such as for **1g,h**, in which kinetic parameters could be determined with sufficient reliability, the 171Trp mutant appears to accommodate the branched side chains better than the 171Tyr enzyme. Otherwise, the effects of Tyr and Trp substitution at the 171 position were approximately the same, except for the weak but consistent trend to higher k_{cat} and K_m values for the tyrosine mutant. However, this does not significantly affect the apparent second-order rate constants k_{cat}/K_M. For the triple mutant catalyses, the additional C97G substitution did not significantly alter the k_{cat}s nor the K_M values relative to the Q102R/R171Y,W mutant catalyses for any of the substrates assayed, as shown in the comparative plots in Figure 2.

Discussion

For the site-directed mutagenesis, the same pTZ18R vector carrying the previously mutated[26] BSLDH gene[39] was used as in

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Table I. Apparent Michaelis-Menten Kinetic Constants for the Reduction of 2-Keto Carboxylic Acids Catalyzed by WT-BSLDH and Mutants Q102R/R171Y and Q102R/R171W[a]

	k_{cat} (s^{-1})			K_M (mM)			k_{cat}/K_M (M^{-1} s^{-1})		
R(CO)COO⁻	WT	Q102R/ R171Y	Q102R/ R171W	WT	Q102R/ R171Y	Q102R/ R171W	WT	Q102R/ R171Y	Q102R/ R171W
1a, CH_3^-	188[b]	0.18 ± 0.01	0.062 ± 0.004	0.04[b]	38 ± 4	45 ± 5	4.8×10^6	4.7	1.4
1b, $CH_3CH_2^-$	155[b]	0.022 ± 0.007	0.039 ± 0.004	0.34[b]	47 ± 29	71 ± 14	4.6×10^5	0.47	0.57
1c, $CH_3(CH_2)_2^-$	44[b]	0.011 ± 0.002	0.013 ± 0.002	2.38[b]	111 ± 25	58 ± 19	1.9×10^4	0.11	0.22
1d, $CH_3(CH_2)_3^-$	25[b]	0.080 ± 0.008	0.051 ± 0.002	1.5[b]	76 ± 11	19 ± 2	1.7×10^4	1.0	2.7
1e, $CH_3(CH_2)_5^-$	32[b]	0.339 ± 0.008	0.22 ± 0.01	0.35[b]	34 ± 1	14 ± 1	9.1×10^4	10	16
1f, $(CH_3)_2CH^-$	0.49[b]	<0.001[d]	<0.001[d]	17.3[b]	nd[d]	nd[d]	2.8×10^1	nd[d]	nd[d]
1g, $(CH_3)_2CHCH_2^-$	0.51[b]	<0.001[d]	0.0018 ± 0.0001	4.9[b]	nd[d]	8 ± 3	1.0×10^2	nd[d]	0.23
1h, $C_6H_{11}CH_2^-$	9.6 ± 0.4	0.040 ± 0.002	0.042 ± 0.004	1.2 ± 0.2	49 ± 5	8 ± 2	8.0×10^3	0.82	5.3
1i, $C_6H_5CH_2^-$	81[b]	1.23 ± 0.03	0.34 ± 0.03	0.67[b]	34 ± 1	12 ± 2	1.2×10^5	36	28
1j, $^-OCC^-$	21 ± 1	nd[e]	0.143 ± 0.005	1.0 ± 0.1	nd[e]	5.7 ± 0.6	2.1×10^4	nd[e]	25
1k, $^-OOCCH_2^-$	6.0[c]	151 ± 3	49 ± 2	1.5[c]	2.3 ± 0.2	2.0 ± 0.2	4.0×10^3	6.6×10^4	1.6×10^4
1l, $^-OOC(CH_2)_2^-$	4.1 ± 0.1	0.0097 ± 0.0005	0.0139 ± 0.0005	3.9 ± 0.1	25 ± 3	21 ± 2	1.1×10^3	0.39	0.66
1m, $HOCH_2^-$	125[b]	0.086 ± 0.006	0.077 ± 0.004	0.16[b]	43 ± 6	61 ± 7	7.8×10^5	2.0	1.3
1n, $BrCH_2^-$	208 ± 12	0.5 ± 0.1	nd[f]	0.50 ± 0.06	99 ± 31	nd[f]	4.1×10^5	5.5	nd[f]

[a] At pH 6.0, 25 °C, 0.2 mM NADH, 5 mM FDP. [b] From ref 3 and 27. [c] From ref 31. [d] Maximum rates observed with up to 100 mM keto acid concentration were less than 0.0005 s^{-1}. [e] A reaction was observed, but due to low solubility of keto malonic acid, saturation kinetic parameters could not be determined. [f] A very slow reaction was observed, but no saturation kinetics curve could be fitted to the data.

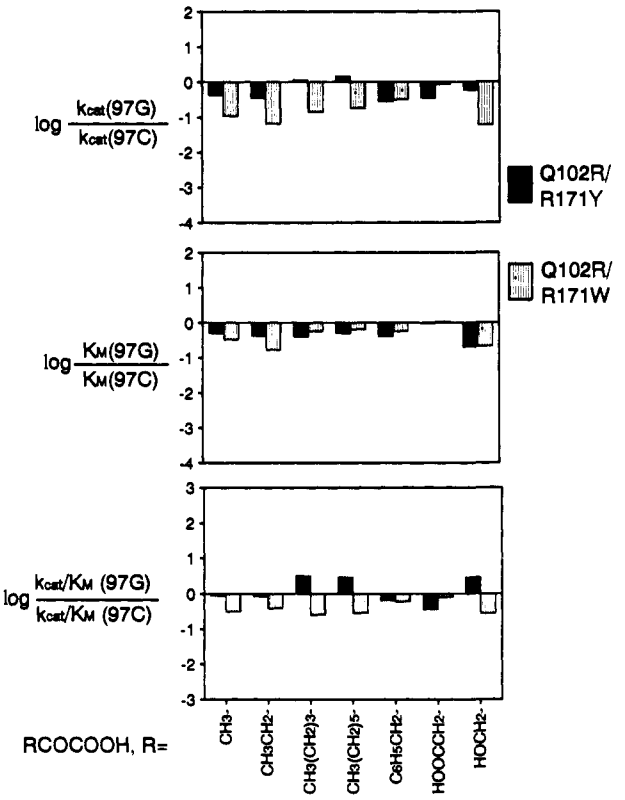

Figure 2. Effects of the C97G substitution on the catalytic properties of the R171Y/Q102R and R171W/Q102R mutant BSLDHs for substrates **1a**,**b**,**d**,**e**,**i**,**k**,**m**.[26,41]

our previous studies.[27] The additional DNA-base substitutions were achieved in a straightforward manner using the Eckstein method.[44] The Arg171Tyr/Trp mutations represent nonconservative replacements and can be regarded as helix breakers[45] in the central part of helix α2F.[37] However, the efficient in vivo protein synthesis, the significant enzymic activities, and the WT-like CD spectra demonstrate the generally benign structural influence of these mutations.

The kinetic data of Table I show that the structural specificity consequences of the 171 and 102 mutations are approximately additive. The characteristic structural specificity profile of the Gln102Arg single mutant[26] is retained for both double mutants, except, as expected,[41] the removal of the key Arg171-COO^- interaction of the WT enzyme engenders a massive drop in activity, with k_{cat} being reduced and K_M raised concomitantly for all substrates except oxaloacetate (**1k**). The double mutants are clearly severely crippled as catalysts but remain sufficiently active to be of preparative value for phenylpyruvate (**1i**) reductions. Oxaloacetate (**1k**) is the best substrate, but its preparative-scale reduction via double mutant catalysis could not be achieved due to the facility with which it decarboxylated to pyruvate during the prolonged reaction times needed. The strong preference for the β-keto dicarboxylic acid **1k**, compared with the α-keto dicarboxylic acid (ketomalonic acid, **1j**) and the γ-keto dicarboxylic acid (ketoglutaric acid, **1l**) may reflect a stringent distance requirement between the ω-COO^- anchor and the reactive carbonyl or hydroxyl group. However, no substrate activity was detected with 3-oxobutyrate, for either WT or mutant enzymes, thereby confirming the earlier finding[3,41] that an α-keto carboxylate moiety is an imperative for catalysis. Other carbonyl compounds such as methyl and ethyl pyruvate, acetone, butyraldehyde, butane-2,3-dione, pentane-2,3-dione, and hexafluoroacetone were also nonsubstrates.

After mutagenesis to generate the gene coding for BSLDH Q102R, one *E. coli* clone was identified by DNA sequencing that contained an inadvertant mutation, coding for an additional C97G substitution.[26] This amino acid replacement is of structural specificity interest in that it is adjacent to the structurally important 98–110 loop region of the active site. This loop is flexible, and its closing onto the active site after binding of the keto acid substrate is an essential element of the LDH-catalytic process.[46,47] It is also the rate-limiting step.[48,49] The loop then reopens to release the hydroxy acid product.[2] In a recent reevaluation of LDH X-ray structures, with and without bound ligands, a loop structure consisting of two hinges connected by a rigid turn has been proposed.[50] The carboxy-terminal hinge comprises a large part of the protein, whereas the amino-terminal hinge extends only over residues 98–100, and large changes occur in two torsional angles only. It was, therefore, of some interest to ascertain whether extension of the latter hinge by the introduction of the conformationally more flexible Gly in position 97 would affect catalysis

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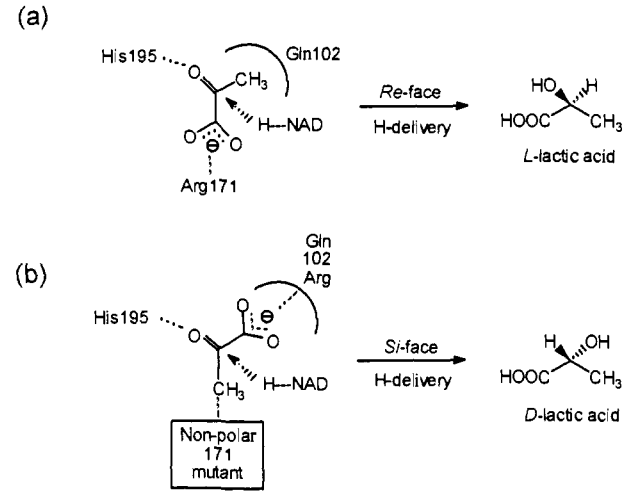

Figure 3. Schematic representation of pyruvate bound at the active site of BSLDH in orientations leading to L- or D-lactic acid formation. Part a represents the natural binding mode that results in L-lactate formation. Part b illustrates a reversal of the natural binding orientation of pyruvate (**1a**) that might be induced by a Q102R/R171Y,W mutant BSLDH and that would lead to D-lactate (**2a**) as the product.

by the inadvertant mutant. As Figure 2 shows, the effects of this mutation on the kinetic parameters of the Q102R/R171Y,W mutants are negligible compared to those of the 102 and 171 substitutions. Moreover, there are no apparent trends in structural selectivity or maximum rate differences upon C97G replacement. These data, in combination with those for the Q102R/C97G mutant BSLDH, which exhibited maximum apparent first-order rate constants similar to the WT enzyme,[26] suggest either that the mobility of the amino-terminal loop hinge is not a rate-limiting factor in BSLDH catalysis or that hinge movement is not limited by backbone conformational freedom.

The widespread adoption of enzymes as catalysts in asymmetric synthesis is a consequence of their abilities to promote stereospecific transformations. It is, therefore, of considerable interest to identify the factors controlling enzyme stereospecificity so that their asymmetric synthetic applications can be optimized, and it is in this context that the current work on applying protein engineering methods to probe the determinants of BSLDH stereospecificity was undertaken.

So far, there are relatively few examples of enzyme stereospecificity being somewhat influenced by site-directed, mutagenesis-induced changes of an enzyme's structure.[51,57] Furthermore, the data available do not help to identify the factors involved in conferring very high stereospecificity on an enzyme. For example, the relaxations of stereospecificity documented so far are the result of catalytic mechanism changes that permit nonspecific reactions with solvent[51] or are for enzymes not particularly evolved for stereospecific catalysis because they operate naturally on achiral substrates.[54,55] Small reductions in stereospecificity have also been recorded for mutant-enzyme catalyses of multifunctional substrates such as phospholipids[52,53] and NADH.[57] In these cases, mutations provide more conformational freedom of the reactive moieties of the substrates, while leaving the binding of the remainder of the molecules undisturbed. None of these situations applies to an enzyme such as BSLDH, whose stereospecificity is so firmly dedicated to producing L-2-hydroxy acids.

The natural L-stereospecificity of BSLDH is determined by the orientation of 2-keto acids, such as pyruvate (**1a**), in the ES complex such that the hydride equivalent from NADH is delivered to the *Re*-face of the carbonyl group, thereby yielding L-lactic acid (**2a**). This is depicted schematically in Figure 3a. An important interaction helping to maintain this pyruvate orientation is that between the substrate's COO^- and Arg171. As one measure of BSLDH's commitment to the L-pathway, we elected to evaluate its resistance to being induced to catalyze D-lactate formation. Reduction of pyruvate to D-lactate requires delivery of the NADH-"hydride" to the *si* face of pyruvate. One of the ways that can be invisaged of inducing this *si* face attack would be via an ES complex in which the orientation of pyruvate was reversed, as illustrated in Figure 3b.[58] In previous studies on this topic, we began to probe the viability of such a "reversed" ES complex by using site-directed mutagenesis to delete amino acid residues perceived as important L-determinants and to introduce new binding interactions that might favor the Figure 3b situation over that in Figure 3a. Toward this goal, Arg171 was replaced by Tyr and Trp.[41] In a subsequent study, the WT-Arg171 was retained but a second COO^--binding Arg residue was introduced in place of Gln102, thereby providing a competitive possibility between the Figures 3a and b pyruvate orientations.[26] None of these mutations disturbed the L-proclivity of BSLDH in any preparatively significant way.[26,41] In the current study, the above mutations were combined in order to eliminate the possibility of the COO^-–Arg171 interaction and at the same time to provide the opportunity for a reversal of pyruvate orientation via COO^-–102Arg binding. The Gln102 residue was selected for replacement by Arg, since when the 98–110 loop closes over the substrate[46,50] prior to formation of the active ES complex, graphics analysis identifies the 102 residue as the one best positioned for Arg involvement in the formation of the Figure3b-type complex required for D-lactate formation. Furthermore, the formation of the natural Arg171-directed Figure 3a-type complex is now clearly impossible for the Arg171Trp/Tyr;Gln102Arg double mutants.

That the 171 and 102 position mutations did not induce any significant reversal of 2-ketoacid binding from the Figure 3a → 3b orientation was established by the preparative-scale reduction of phenylpyruvate (**1i**). With each of 171Tyr/102Arg and 171Trp/102Arg and the corresponding 97Gly triple mutants, the phenyllactate products **2i** were each of L-configuration and of >98% enantiomeric purity.

In our previous study on the Gln102Arg mutant, considerable relaxation of stereospecificity was manifest in L- and D-malate (**2k**) oxidation and binding.[26] Unfortunately, a similar comparison of oxidation rates of L- vs D-**2k** by the current double and triple mutant BSLDHs could not be achieved due to the low substrate activity of each malate enantiomer, which precluded kinetic measurements under saturating substrate conditions. However, from the linear rate vs [S] plots for L- and D-malate oxidations by the 171Tyr/102Arg and 171Trp/102Arg enzymes, the limits of the k_{cat}/K_M ratios for L vs D oxidation are calculated to be >110 and >70, respectively. These minimum degrees of L-preference are in accord with the >98% ee precision levels of the NMR analytical procedure for the preparative-scale experiments. Thus, while the double mutations clearly cripple BSLDH with respect to the rates of 2-keto acid reduction, the overall control of stereospecificity remains unchanged.

While Arg171, due to its known function as a primary substrate-binding group,[2,40] is clearly a very important stereospecificity as well as catalytic rate determinant, the overall complete fidelity of L-products generated by the 171Trp/Tyr mutant enzymes shows

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(58) Alternative designs for reversing the C=O face attack, such as changing the position of His195, require more massive protein engineering in that Arg109 and Asp108, which are part of the charge-relay system and are in independent secondary structure regions, would also have to be relocated.

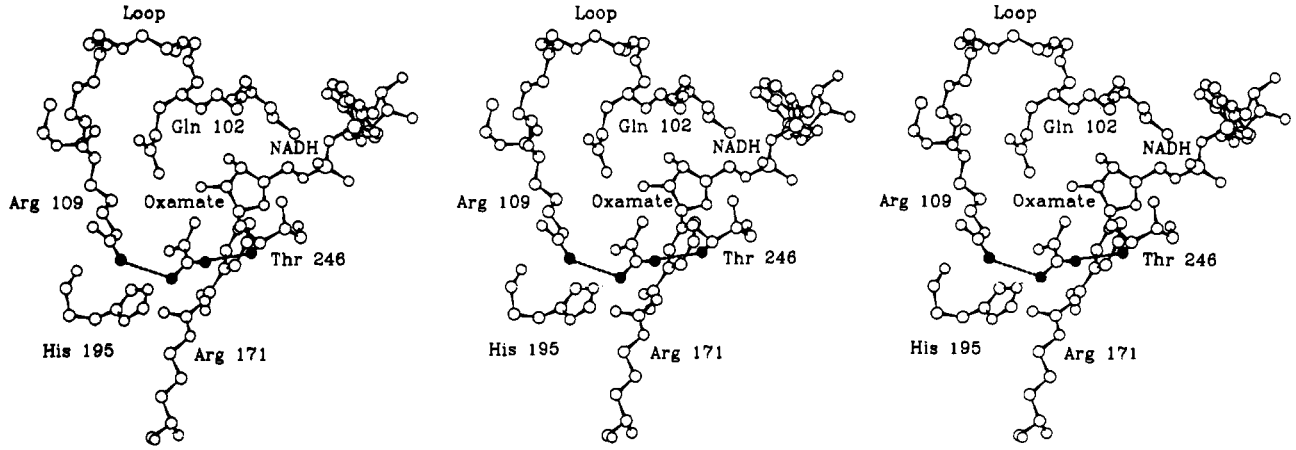

Figure 4. Stereoimages (left pair cross-eyed, right pair parallel) of the substrate analog oxamate and the coenzyme NADH bound to the active site of BSLDH, illustrating the proximity of the side chains of Arg109 and Thr246 to the COO^- group of the substrate-equivalent oxamate. This figure is based on the X-ray coordinates determined by Wigley et al.[36] The dashed lines show representative interactions between residues Arg109 and Thr246 and the carboxylate group of oxamate. The actual interatomic distances between the carboxylate oxygens of oxamate and the closest heavy atoms in BSLDH are as follows: Arg171:Nη1 3.04 Å; Arg171:Nη2 3.17 Å; Arg109;Nη1 2.93 Å; Thr246:Oγ 2.54 Å. The shortest distances between the carbonyl oxygen of oxamate and protein heavy atoms are Arg109:Nϵ 3.01 Å and His195:Nϵ 2.52 Å.

that there are fail-safe L-directing interactions that take over when Arg171 is not present. Two candidates for these fail-safe functions are Arg109 and Thr246. The X-ray data[36] indicate that side chains of both of these residues are close enough to the substrate carboxylate function to form effective electrostatic or hydrogen bonds, respectively. Also, the methyl group of Thr246 is in van der Waals contact with the (*R*)-group of substrates **1**. These interactions are identified in Figure 4, and experiments to evaluate their possible role as fail-safe binding forces that promote substrate orientation in a natural, Figure 3a-like ES complex are in progress. In this regard, it is recognized that nonproductive ES complexes involving substrate-COO^--to-102Arg interactions may well be important components of the overall reaction mixtures. The question of how such secondary interactions can override carboxylate binding to 102Arg-containing mutants in the productive ES complexes is also an intriguing one. This is particularly true for small substrates such as pyruvates, in which, other than the carbonyl group being reduced, the carboxylate group is the only function capable of binding strongly to the enzyme. Arg109[59] and Thr246[60] are also essential to high catalytic activity, and it may be that high catalytic activity and high stereospecificity are synergistically linked. The X-ray structure of the 171Trp/102Arg/97Gly triple mutant that is now being refined[61] should provide new insights into these questions. Already it is clear that Arg171Trp replacement is not wholly benign and that the 171Trp side chain shifts out of the active site.[62] It is also noteworthy that L-LDHs are not homologous with their D-specific counterparts but seem to have developed independently from unrelated protein scaffolds.[63-65] This is a further consideration in evaluating the multiplicity of interactions that nature has built in to highly stereospecific enzymes to aid them in their assistance to interference with the absolute configurations of their catalytic pathway intermediates and transition states.

Experimental Section

Proton-NMR spectra were recorded on a Varian XL200 spectrometer, and enzyme kinetics were followed with a Shimadzu UV-240/PR1 spectrophotometer. SDS polyacrylamide gel electrophoresis was carried out on a Pharmacia PHAST system using 8-25% gradient or 12.5% homogeneous gels and Coomassie Blue detection. For liquid chromatography purification, a Pharmacia FPLC system was used.

Materials, Enzymes, and *E. coli* Strains. Fructose 1,6-diphosphate, sodium pyruvate, and NADH were obtained from Boehringer Mannheim, (*R*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetic acid and piperazine from Aldrich, and triethanolamine from Fisher Scientific. Ketomalonic acid and bromopyruvic acid were purchased from Fluka. Cyclohexylpyruvic acid (3-cyclohexyl-2-oxopropanoic acid) was prepared in three steps from ethyl 1,3-dithiane-2-carboxylate according to the method of Graham et al.[66] All other 2-keto carboxylic acids or the corresponding alkali salts were brought from Sigma, and deoxyribonucleotides and liquid chromatography media were brought from Pharmacia. Oligodeoxyribonucleotides were prepared by automated phosphoroamidite chemistry on an Applied Biosystems 380B DNA synthesizer.

WT-BSLDH[3,27] and mutant enzymes[26] were purified from *E. coli* TG2 cells as described previously,[26] except that for the mutant enzymes the ammonium sulfate precipitation step was omitted and after the heat-treatment step the crude extracts were diluted 4-fold before being loaded onto the Mono-Q column. Formate dehydrogenase was obtained from Boehringer Mannheim, and restriction enzymes, T4 DNA ligase, and Klenow fragment of DNA polymerase were obtained from Bethesda Research Laboratories or Pharmacia.

The *E. coli* strain TG2 [*rec A*$^-$ form of TG1][67] was used as a host for expression, mutagenesis, and sequencing of the BSLDH gene,[39] which was subcloned into a pTZ18R plasmid.[27] Growth medium for *E. coli* was YT(2x) (16 g of Bactotryptone, 10 g of yeast extract, and 5 g of sodium chloride per liter). Cultures were grown at 37 °C for 15 h in 2-L Erlenmeyer flasks containing 500 mL of medium each in a shaking incubator at the highest shaking rate possible without excessive foam formation (approximately 150–200 rpm).

Mutagenesis. The mutants Q102R and Q102R/C97G were obtained by site-directed mutagenesis procedure as described previously.[26] Starting from these two mutant genes, the double and triple mutants containing the R171Y and R171W substitutions were generated by the method of Eckstein and co-workers,[44] using a reagent kit supplied by Amersham. However, *E. coli* TG2 was used as host strain throughout the procedure, and the product DNA was reintroduced into the cells by electroporation with Bio-Rad Gene Pulser and Pulse Controller instruments according to the manufacturer's suggestions for transformation of *E. coli*.

Enzyme Kinetics. Kinetics of 2-keto acid **1a–n** reductions were recorded at pH 6.0 at 25 °C on a Pharmacia-LKB Ultrospec Plus spectrophotometer equipped with a temperature-controlled autofill unit, using the protocols described previously[41] but using 20 mM piperazine·HCl (pH 6.0) as buffer.[26] Steady-state rate assays with 2-hydroxy acids were done under published conditions.[68] The experimental data were evaluated by

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nonlinear regression analysis using the program Grafit (Erithacus Software Ltd., Staines, UK). All kinetic determinations were performed at least in duplicate. The results are recorded in Table I.

Preparative-scale reductions of 2-keto carboxylic acids and isolation of the 2-hydroxy acid products were carried out by the method of Luyten et al.,[41] as follows:

(2*S*)-β-Phenyllactic Acid (2i). Sodium β-phenylpyruvate (1i, 400 mg, 2 mmol), NADH (57 mg, 0.08 mmol), sodium formate (340 mg, 5 mmol), FDP trisodium salt (138 mg, 0.25 mmol), dithiothreitol (1.5 mg, 0.01 mmol), lyophilized formate dehydrogenase (40 mg, 18 U), and mutant BSLDH (27-50 mg) were dissolved in 20 mM piperazine·HCl buffer (pH 6.0) to a final volume of 50 mL and incubated at pH 6.0 under pH-stat control until no further acid uptake was recorded. The reactions yielded (2*S*)-β-phenyllactic acid (2i, 240-280 mg, 1.2-1.4 mmol, 60-70% yield) with ^{1}H-NMR spectra identical with authentic samples.[3] The enantiomeric excesses of the (2*S*)-3-phenyllactic acids (2i) were determined as >98% on their Mosher esters as described previously[3] and their 2*S* absolute configurations from the chemical shifts of the ^{1}H resonances relative to tetramethylsilane as internal standard, with reference to those of authentic samples.[3]

CD spectroscopy on the mutant BSLDHs was performed as described previously for WT, Q102R, and Q102R/C97G BSLDHs.[26] The spectra are shown in Figure 1.

Graphics Analyses of the Q102R/R171Y,W mutants were performed by the general strategy outlined previously.[26] However, the current analyses were based on new X-ray coordinates, at 2.5-Å resolution, of the quaternary complex of BSLDH, oxamate, NADH, and FDP.[36]

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Registry No. Gln, 56-85-9; Arg, 74-79-3; Tyr, 60-18-4; Trp, 73-22-3; L-LDH, 9001-60-9; oxalacetic acid, 328-42-7.

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Consequence of Phosphorus Stereochemistry upon the Postinhibitory Reaction Kinetics of Acetylcholinesterase Poisoned by Phosphorothiolates

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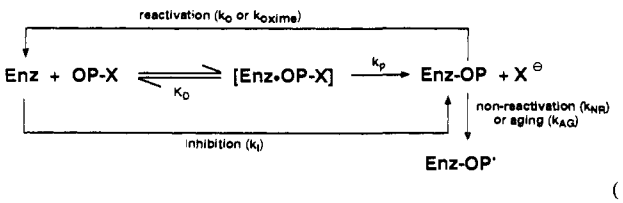
Abstract: Reaction of the chiral isomers of isoparathion methyl with solubilized rat brain cholinesterase (RBAChE) produced O,S-dimethylphosphorothiolated forms of the inhibited enzyme. The inhibited enzymes were evaluated for their individual rates of spontaneous reactivation, oxime-mediated reactivation, and non-reactivatability, and those rates were compared to those of RBAChE inhibited by the racemate. The rate constants for spontaneous reactivation were 1.13×10^{-2} min^{-1}, 1.02×10^{-2} min^{-1}, and 0.35×10^{-2} min^{-1}; for oxime-mediated reactivation were 4.10×10^{-2} min^{-1}, 4.57×10^{-2} min^{-1}, and 1.16×10^{-2} min^{-1}; and for non-reactivatability were 1.42×10^{-2} min^{-1}, 1.37×10^{-2} min^{-1}, and 3.07×10^{-2} min^{-1} for RBAChE inhibited with (-)-, (±)-, and (+)-isoparathion methyl, respectively. RBAChE inhibited by (-)-isoparathion methyl underwent spontaneous reactivation 3.23-times faster and oxime-mediated reactivation 3.53-times faster than RBAChE with the (+)-isomer. In contrast, RBAChE inhibited with (+)-isoparathion methyl underwent a time-dependent decrease in reactivatability twice that of the (-)-isomer. The postinhibitory rate constants obtained from RBAChE inhibited with racemic isoparathion methyl were similar to those obtained for inhibition with (-)-isoparathion methyl. These data correlate with the ability of the (-)-stereoisomer to inhibit the enzyme approximately 8-times faster than the (+)-isomer, leading to a larger mole fraction of RBAChE inhibited by (-)-isoparathion methyl. Chemical model studies investigating the mode of non-reactivatability by O,S-dimethylphosphorothiolated RBAChE suggest that the mechanism probably involves hydrolysis rather than dealkylation.

Introduction

Certain organophosphates (OP) inactivate acetylcholinesterases[1] and other related proteases by reaction at a nucleophilic serine hydroxyl to form a covalently modified enzyme (ENZ-OP) concomitant with the ejection of a leaving group (X^-) according to eq 1.[2] The inhibited enzyme is then incapable of hydrolysis, leading to an abnormal persistence and accumulation of the neurotransmitter acetylcholine in cholinergic nerve synapses and neuromuscular junctions.[3] The biological effects, which range from hyperexcitability to respiratory collapse, may result from exposure to insecticides[4] or nerve gas agents.[5]



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