Effects of Gln102Arg and Cys97Gly Mutations on the Structural Specificity and Stereospecificity of the L-Lactate Dehydrogenase from *Bacillus stearothermophilus*

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Abstract: The L-lactate dehydrogenase of *Bacillus stearothermophilus* (BSLDH) is a thermostable enzyme with considerable potential for applications in asymmetric synthesis. An understanding of the factors controlling its structural specificity and stereospecificity is therefore of interest. In this paper the effects of Gln102 → Arg and Cys97 → Gly mutations have been evaluated. In a survey of thirteen 2-keto acids, the Q102R mutation was found to reduce the activity of BSLDH toward the reduction of RCOCOOH substrates with small or hydrophilic R groups without affecting its activity toward those with larger, hydrophobic R substituents. In addition, the mutants have a high affinity for C_3- and C_4-2-keto dicarboxylates. The extent of fructose 1,6-diphosphate activation of the mutant enzymes was similar to its effect on wild-type BSLDH. The mutants also retained the synthetically desirable thermostability. As a probe of the commitment of BSLDH to L stereospecificity, the Q102R mutation was introduced to allow the new 102R site to compete with Arg171 for binding of the COO^- groups of the RCOCOOH substrates, which would reverse the normal RCOCOOH orientation at the active site and thereby open up the possibility of the formation of a D-2-hydroxy acid in place of the natural L product. However, L stereospecificity in 2-keto acid reduction was strictly retained by the Q102R mutants. This was confirmed by preparative-scale reductions of pyruvate and phenylpyruvate to give the corresponding L-hydroxy acids in enantiomerically pure form and by comparison of the kinetics of oxidation of L- and D-lactate and L- and D-phenyl lactate. No evidence for substrate activity for the D enantiomers of either of these was seen with WT or mutant enzymes. Some catalysis of D-malate oxidation by both WT and mutant BSLDH was observed, but the L enantiomer was still preferred to approximately the same degree in both cases. That the inability of BSLDH and its 102R mutants to catalyze D-2-hydroxy acid oxidations was not simply due to the failure of the D enantiomers to bind at the active site was established by a comparison of competitive inhibition constants for the above L- and D-hydroxy acids. CD spectroscopy showed that the Gln102 → Arg mutations were not benign but induced significant structural perturbations. Electrostatic potential contours suggest that the structural changes are partly due to long-range interactions of the positive charge of the guanidinium group of Arg102 with several other residues that form an area of negative potential adjacent to the active site. The Cys97 → Gly mutation, while inadvertent, was of interest because of the potential specificity effects arising from its location adjacent to the hinge of the flexible 98-110 loop. However, its effects on BSLDH specificity were minor.

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

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

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

substrate is pyruvic acid (**1a**), but reductions of other, structurally varied, 2-keto acids to the corresponding 2-hydroxy acids have been reported.[3-10] 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.[11-18] As a consequence, organic synthetic applications of LDH's are expanding.[7,8,10]

So far, only natural LDH's have been exploited synthetically. However, they suffer from the disadvantage that their structural specificities are generally too narrow to accept all 2-keto acid structures of organic chemical interest. Such shortcomings in enzyme specificity are potentially redressable by controlled structural modifications by site-directed mutagenesis, and there is an increasing number of reports of progress in this direction for several enzymes.[9,19-26]

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(1) Abbreviations used: LDH, lactate dehydrogenase; BSLDH, lactate dehydrogenase from *Bacillus stearothermophilus*; DMLDH, lactate dehydrogenase from dogfish muscle; NAD^+ and NADH, oxidized and reduced form of nicotineamide 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; C97G, cysteine 97 to glycine mutation; *E. coli*, *Escherichia coli*; ES, enzyme substrate.

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Table I. Apparent Michaelis–Menten Kinetic Constants for the Reduction of 2-Keto Carboxylic Acids Catalyzed by WT-BSLDH, Single-Mutant Gln102 → Arg, and Double-Mutant Gln102 → Arg/Cys97 → Gly

R(CO)COO⁻, R =	k_{cat}, s^{-1}			K_M, mM			k_{cat}/K_M, M^{-1} s^{-1}		
	WT	Q102R	Q102R/C97G	WT	Q102R	Q102R/C97G	WT	Q102R	Q102R/C97G
1a, CH_3	188[b]	2.45 ± 0.07	1.7 ± 0.1	0.04[b]	1.2 ± 0.1	0.8 ± 0.1	4.8×10^6	2.0×10^3	2.1×10^3
1b, CH_3CH_2	155[b]	2.60 ± 0.04	1.58 ± 0.01	0.34[b]	1.2 ± 0.1	1.22 ± 0.01	4.6×10^5	2.2×10^3	1.3×10^3
1c, $CH_3(CH_2)_2$	44[b]	1.56 ± 0.01	1.42 ± 0.03	2.38[b]	1.88 ± 0.03	1.5 ± 0.1	1.9×10^4	8.3×10^2	9.7×10^2
1d, $CH_3(CH_2)_5$	32[b]	33 ± 1	30 ± 1	0.35[b]	0.34 ± 0.03	0.15 ± 0.02	9.1×10^4	9.7×10^4	2.0×10^5
1e, $(CH_3)_2CH$	0.49[b]	0.0099 ± 0.0003	<0.05	17.3[b]	13 ± 1	n.d.	2.8×10^1	7.6×10^{-1}	n.d.
1f, $(CH_3)_2CHCH_2$	0.51[b]	0.26 ± 0.01	0.19 ± 0.01	4.9[b]	3.1 ± 0.2	1.6 ± 0.1	1.0×10^2	8.1×10^1	1.2×10^2
1g, $C_6H_{11}CH_2$	9.6 ± 0.4	6.2 ± 0.1	14 ± 2	1.2 ± 0.2	1.08 ± 0.04	1.9 ± 0.5	8.0×10^3	5.7×10^3	7.4×10^3
1h, $C_6H_5CH_2$	81[b]	89 ± 2	83 ± 2	0.67[b]	0.81 ± 0.04	0.60 ± 0.06	1.2×10^5	1.1×10^5	1.4×10^5
1i, ^-OOC	21 ± 1	238 ± 7	195 ± 4	1.0 ± 0.1	1.2 ± 0.1	0.98 ± 0.06	2.1×10^4	2.0×10^5	2.0×10^5
1l, $^-OOCCH_2$	6.0[c]	205 ± 5	59 ± 5	1.5[c]	0.019 ± 0.01	0.06 ± 0.01	4.0×10^3	1.1×10^7	1.1×10^6
1k, $^-OOC(CH_2)_2$	4.1 ± 0.1	0.667 ± 0.003	0.44 ± 0.01	3.9 ± 0.1	0.165 ± 0.003	0.10 ± 0.01	1.1×10^3	4.1×10^3	4.4×10^3
1l, $HOCH_2$	125[b]	4.77 ± 0.05	3.02 ± 0.04	0.16[b]	2.2 ± 0.1	2.5 ± 0.1	7.8×10^5	2.2×10^3	1.2×10^3
1m, $BrCH_2$	208 ± 12	61 ± 1	82 ± 1	0.50 ± 0.06	3.3 ± 0.1	2.3 ± 0.1	4.1×10^5	1.9×10^4	3.6×10^4

[a] At pH 6.0, 25 °C, 0.2 mM NADH, 5 mM FDP. [b] From refs 8 and 9. [c] From ref 34.

From our work in applying enzymes in organic synthesis,[27,28] we became interested in investigating and identifying the factors that control the structural specificity and stereospecificity of synthetically useful enzymes such as LDH's to optimize their synthetic potential. The target enzyme in this study was the L-LDH (BSLDH) from the thermophile *Bacillus stearothermophilus*. BSLDH is attractive for synthetic and specificity-modification purposes for several reasons. It is a very stable, mechanistically well-studied enzyme[29,30] of known X-ray structure[31] and of proven preparative-scale value.[8] Also, BSLDH has been cloned and overexpressed[32,33] and has been shown to be amenable to site-directed mutagenesis-induced changes in its specificity.[9,25,26,30,34]

In this paper we continue our exploration of the factors determining the structural specificity and L stereospecificity of BSLDH. Among the methods of probing the factors controlling enzyme stereospecificity, evaluating how effectively an enzyme resists attempts to change this capability is potentially one of the most powerful. Identification in this way of the factors contributing to L stereospecificity of BSLDH will thereby help to provide a better understanding of the stereospecificity determinants of all enzymes. This is of particular importance for the best exploitation of enzymes as chiral catalysts in asymmetric syntheses. In a previous study on this topic,[25] it was found that replacing Arg171, with whose guanidinium group a substrate's COO^- function forms a key binding interaction in LDH-catalyzed reactions,[2] by Trp or Tyr did not affect BSLDH's ability to produce L-hydroxy acids, with the Arg171 → Trp/Tyr mutants retaining full L stereospecificity.[25] The current study further probes the factors controlling BSLDH stereospecificity by an examination of the effects of the Gln102 → Arg mutation. The consequences of this, and of an inadvertent Cys97 → Gly mutation, on the structural specificity of the enzyme were also determined.

Results

The Gln102 → Arg mutation was generated from the cloned wild-type gene according to the method of Slilaty.[35] The required double base-pair change was created by use of the mutagenic oligonucleotide primer 5′-pGGC GCC AAC *CGG* AAA CCG GG-3′, in which the central codon (underlined) was changed to CGG from CAA in the wild-type gene. The protein was purified to homogeneity as judged by SDS-polyacrylamide gel electrophoresis and used for kinetic and preparative studies.

The Michaelis–Menten kinetic parameters for BSLDH Q102R with the 2-keto acid substrates **1a–m** were determined under steady-state conditions. The data are recorded in Table I, together with the corresponding values for the wild-type enzyme. Most substrates, particularly those with low K_M values, exhibited substrate inhibition at higher concentrations.

During gene sequencing, one *E. coli* clone used for expression of a Q102R mutant enzyme was found to contain a Cys97 → Gly mutation in addition to the desired Q102R replacement. This was due to an inadvertent change from *T*GC to *G*GC in the codon for amino acid 97, which is located 3 bases upstream from the 5′-end of the mutagenic primer annealing site. The presence of only one instead of two cysteine residues in the expressed protein was verified by titration with Ellman's reagent[36] and by amino acid analysis of the performic acid-oxidized protein.[37] This mutant was expressed and purified, and its substrate specificity was compared with that of the authentic Q102R single mutant (Table I).

The allosteric activator FDP sharply decreases the K_M values for pyruvate with the WT and mutant enzymes (50-fold for WT, 12-fold for Q102R, and 28-fold for Q102R/C97G) without altering the k_{cat} values. Both mutants are still thermostable enzymes, although each mutation does result in a small decrease in stability. Complexation with FDP increases the stability in all cases. For example, in the presence of 5 mM FDP, the halving-of-activity times at 85 °C were 21.2 ± 0.5 (WT), 11.0 ± 0.6 (Q102R), and 4.9 ± 0.4 min (Q102R/C97G), whereas at 75 °C without FDP the half-activity times were only 2.9 ± 0.2 (WT), 1.7 ± 0.1 (Q102R), and 1.6 ± 0.2 min (Q102R/C97G).

CD spectroscopy was used to investigate the effects of mutations and FDP binding on the protein conformation. The spectra in

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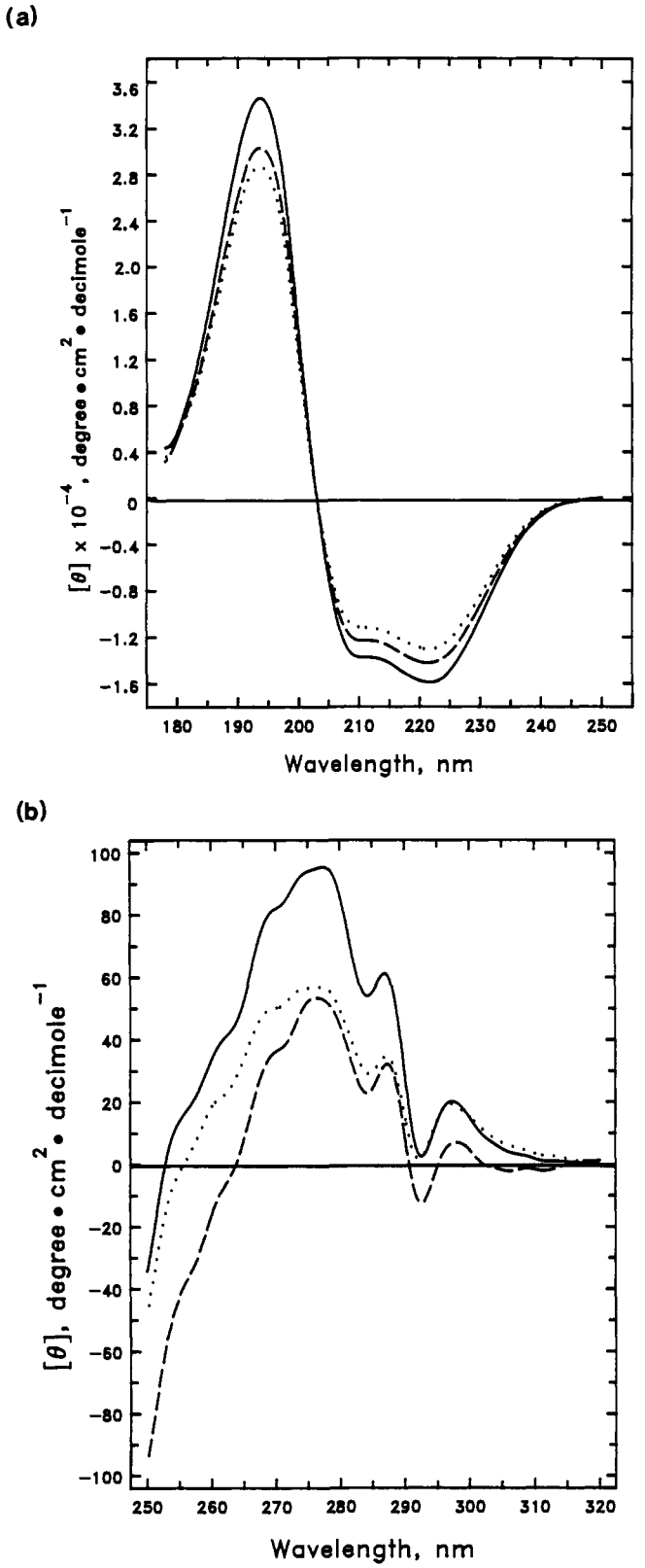

Figure 1. (a) Far-UV and (b) near-UV circular dichroism spectra of WT-BSLDH (—), BSLDH Q102R (···), and BSLDH Q102R/C97G (---). Spectra were recorded in the absence of coenzyme, substrate, or activator.

the absence of FDP are recorded in Figure 1. They are notably different for mutants and WT-BSLDH. The secondary structure analyses based on the Provencher and Gloeckner[38] protocol suggest decreases in α-helix and increases in β-sheet and β-turn content as a result of the mutations, more so for the single than for the

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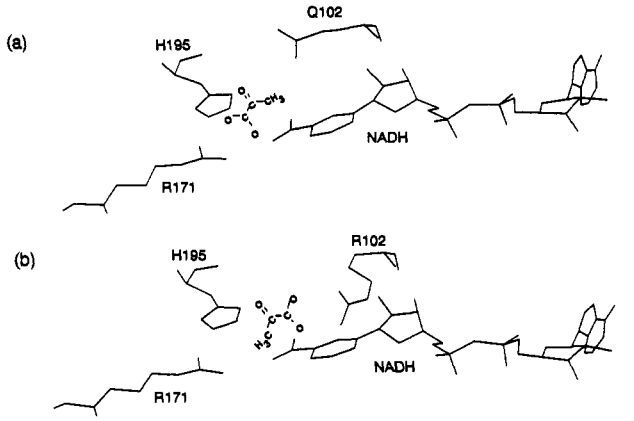

Figure 2. Computer graphics model of the partial active site (a) of WT-BSLDH and (b) of the Gln102 → Arg mutant. The position of pyruvate in (a) is based on the coordinates of the inhibitor oxamate in the original X-ray data. This is the natural binding mode that results in L-lactate formation. Part (b) illustrates the binding of pyruvate to Arg102 of a Q102R mutant in an orientation that would lead to D-lactate as the product.

double mutant. There was no significant change in the CD spectra when a saturating amount (5 mM[39]) of FDP was added.

To determine the stereospecificity of the Q102R/C97G mutant, preparative-scale enzyme-catalyzed reductions of pyruvate (**1a**) and phenylpyruvate (**1h**) were carried out. Only the (*S*)-2-hydroxy acids **2a** and **2h** were isolated, with each being enantiomerically pure within the detection limits of the NMR (>98% ee) and optical rotation (>95% ee) analytical methods applied. No traces of the D enantiomers were detected.

The commitment of BSLDH and its 102R mutants to L stereospecificity was further investigated by studying the kinetics of oxidation of both L and D enantiomers of the 2-hydroxy acids **2a,h,j** with the Q102R single mutant of BSLDH. The kinetic data obtained with both enantiomers of lactate (**2a**), phenyllactate (**2h**), and malate (**2j**) are summarized in Table II. Each L and D enantiomer of **2a,h,j** was also tested as an inhibitor of the WT-BSLDH and Q102R-catalyzed reductions of phenylpyruvate. The Grafit-calculated competitive inhibition constants are recorded in Table III.

Discussion

With regard to probing the stereospecificity of BSLDH, it should be emphasized that our goal was not necessarily to convert BSLDH into a D-lactate dehydrogenase but rather to use this enzyme as an example of one whose deep commitment to a particular stereospecificity can be exploited as an instrument for studying enzyme stereospecificity determinants in general. The natural L stereospecificity of L-LDH's is set by the fact that, in the active ES complex, pyruvate is oriented to receive the hydride equivalent from NADH on the *Re* face of the carbonyl group, as shown schematically in Figure 2a. One of the key interactions maintaining pyruvate in this L-lactic acid-producing position is that between Arg171 and the COO^- of pyruvate.[40] Reduction to D-lactic acid would require the enzyme to catalyze delivery of the hydride of NADH to the opposite (*Si*) face of the pyruvate carbonyl group. With L-LDH's like BSLDH, the simplest, least-motion way in which this could be accomplished would be via an active ES complex in which the binding of pyruvate was reversed, as depicted in Figure 2b. The legitimacy of Figure 2b-type ES complexes was supported by computer graphics analyses. In our previous study evaluating the contribution to L stereospecificity of the Arg171–COO^- interaction, it was shown that while replacing Arg171 by Trp to Tyr reduced the enzyme's activity, it did not disturb BSLDH's absolute commitment to L

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Table II. Apparent Michaelis–Menten Kinetic Parameters for the Oxidation of 2-Hydroxy Carboxylates Catalyzed by WT-BSLDH and BSLDH 102R[a]

substrate	k_{cat}, s^{-1}		K_M, mM		k_{cat}/K_M, M^{-1} s^{-1}	
	WT	102R	WT	102R	WT	102R
L-lactate	9.7 ± 0.3	0.28 ± 0.02	24 ± 2	472 ± 67	4.0×10^2	6.0×10^{-1}
D-lactate	n.d.	n.d.	n.d.	n.d.	$<10^{-3}$	$<10^{-3}$
L-3-phenyllactate	0.016 ± 0.001	0.0079 ± 0.0002	8 ± 3	7 ± 1	1.9×10^0	1.2×10^0
D-3-phenyllactate	n.d.	n.d.	n.d.	n.d.	$<10^{-3}$	$<10^{-3}$
L-malate	0.0020 ± 0.0005	1.3 ± 0.1	27 ± 2	3.4 ± 0.4	7.3×10^{-2}	4.0×10^2
D-malate	0.0014 ± 0.0003	1.0 ± 0.1	800 ± 300	79 ± 12	1.7×10^{-3}	1.3×10^1
hydroxymalonate	n.d.	0.0036 ± 0.0001	n.d.	54 ± 5	$<10^{-3}$	6.7×10^{-2}

[a] At pH 8.5, 25 °C, 10 mM NAD^+, 20 mM FDP.

Table III. Competitive Inhibition Constants for 2-Hydroxy Carboxylates as Inhibitors of the BSLDH-Catalyzed Phenylpyruvate (**2h**) Reduction[a]

inhibitor	inhibition constant (K_I), mM	
	WT	Q102R
L-lactate	54 ± 4	22 ± 2
D-lactate	130 ± 1	118 ± 2
L-3-phenyllactate	37 ± 4	16 ± 1
D-3-phenyllactate	3.4 ± 0.1	1.9 ± 0.1
L-malate	89 ± 7	6.8 ± 0.4
D-malate	500 ± 62	10.1 ± 0.5

[a] At pH 6.0, 25 °C. For substrate and inhibitor concentrations, see the Experimental Section.

stereochemistry.[25] In the present investigation, the Gln102 → Arg mutation was selected since it gives the pyruvate COO^- group two Arg sites with which to interact, one leading to L-lactic acid via the Figure 2a binding pattern and the other with the potential to yield the D enantiomer via the Figure 2b type of interaction. We were further encouraged in the feasibility of the Figure 2b binding orientation since it had been demonstrated earlier by the Holbrook group, in their successful conversion of BSLDH into a malate dehydrogenase-like enzyme,[34] that an Arg at position 102 was effective in binding COO^- functions.

For the site-directed mutagenesis, the BSLDH gene, including the *tac* promoter, was excised from its pKK223-3 plasmid[32] as a *Bam*H1 fragment and was cloned into a pTZ18R vector.[9] This plasmid was then used for overexpression of the protein in *E. coli* TG2 and for generating single-stranded template DNA in *E. coli* JM101 and M13 KO7 for sequencing. To select for the mutated gene, we initially used the mutagenesis method of Kunkel.[41] After transformation of the mutagenesis reaction products, one *E. coli* JM101 clone, in which the mutation had been identified by sequencing the mutated site, was used to isolate double-stranded plasmid DNA and to transform the expression strain TG2. Later this clone was found by extensive sequencing to contain an inadvertent Cys97 → Gly mutation, thus providing a double mutant of additional interest with regard to BSLDH specificity. The authentic Q102R single mutant was generated from wild-type template DNA and the same mutagenic primer. This was done according to a mutagenesis protocol[35] using the same selection method in *dut⁻/ung⁻ E. coli* strains, but with double-stranded rather than single-stranded template DNA. Neither method was without disadvantages. The Kunkel mutagenesis was hampered by difficulties in obtaining single-stranded pTZ-DNA carrying the BSLDH insert from *dut⁻/ung⁻* strains, whereas in the double-stranded approach a very poor mutagenesis yield (2%) of ampicillin resistant product clones was observed. This is probably due to weaker template binding of this particular mutagenic primer than of the closing oligonucleotide primer. Inefficient dideoxy sequencing using the same mutagenic primer and compressions regularly observed when sequencing the 102R site in the BSLDH gene support the hypothesis of inefficient primer annealing to this site.

The effects of Q102R mutation on the substrate specificity of BSLDH were also explored for the substrate range **1a–m**. Most

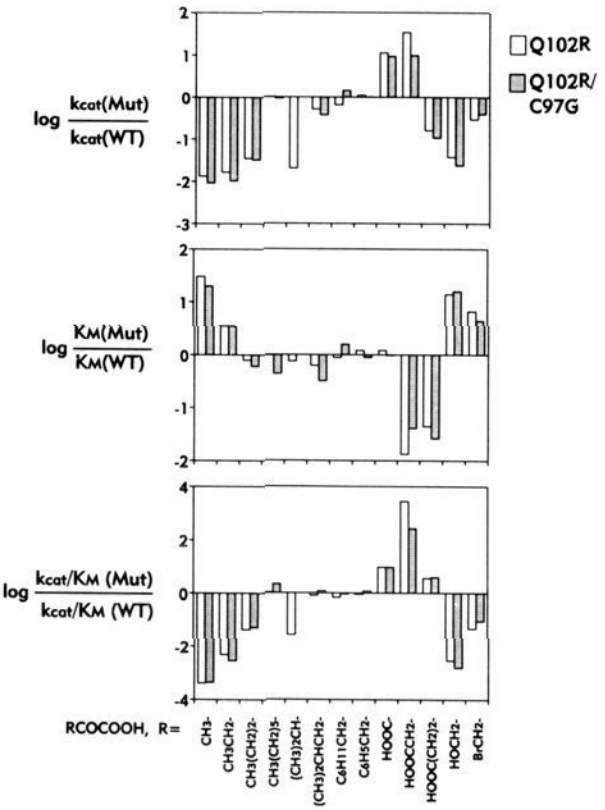

Figure 3. Ratios of kinetic parameters obtained for WT- and Q102R-BSLDH-catalyzed reductions of **1a–m**.

previous studies on 2-keto acid reduction by BSLDH have been carried out at pH 6.0 in triethanolamine–HCl.[8,9,25,29,42] However, triethanolamine-HCl is a poor buffer at this pH, and after extensive trials it was determined that kinetic evaluations of BSLDH reductions at pH 6.0 were best performed in 20 mM piperazine-HCl which, as a result of piperazine's pK_a of 6.2, possesses superior buffering capacity in this pH region. As Table I shows, both the Q102R and Q102R,C97G mutants displayed a broad, though somewhat altered from wild-type, substrate specificity while retaining a remarkably high catalytic efficiency. The relative changes in activities induced by the mutations are clearly evident in the comparisons of kinetic parameter ratios depicted in Figure 3. On the basis of these data, the structural specificity determinants of the 2-keto acid substrates **1a–m** fall into three categories:

1. Those with short, uncharged side chains, which are preferred by WT-BSLDH, are poor substrates for BSLDH Q102R. For this group, the Gln102 → Arg mutation decreases the k_{cat}'s for pyruvate (**1a**), 2-ketobutyrate (**1b**), and hydroxypyruvate (**1l**) by two to three orders of magnitude, while the respective K_M values increase by factors of 4 to 30. This indicates weak binding and inefficient orientation of these substrates by the mutant enzymes.

2. 2-Keto dicarboxylic acid substrates show an opposite trend to group 1 above with specificity constants one to three orders

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of magnitude higher for the mutants than for the wild-type. However, the three smallest homologues **1i–k** of this group behave inconsistently with regard to binding and reactivity. The catalytic constants for ketomalonate (**1i**) and oxalacetate (**1j**) are 10-fold higher for the mutants than for WT-BSLDH, whereas for the longer chain 2-ketoglutarate (**1k**) k_{cat} is decreased 4-fold upon mutation. On the other hand, for 2-ketoglutarate (**1k**) and oxalacetate (**1j**) the K_M's are much lower for the mutant catalyses, while for the smallest dicarboxylic acid substrate, ketomalonate (**1i**), the K_M values are virtually the same for all three enzymes.

Remarkably, in contrast to the irreversible reduction of ketomalonate (**1i**) catalyzed by mitochondrial malate dehydrogenase (attributed to the fact that the malate product **2i** binds preferentially to the protonated form of the enzyme[43]), BSLDH Q102R *does* catalyze **2i** oxidation, albeit at a comparatively slow rate (Table II). The reasons for this difference are not apparent, given that the functional active site residues are generally conserved between L-malate and L-lactate dehydrogenases and that residue 102 is arginine in both the malate[44] and Q102R enzymes.

3. The 2-keto acids with long hydrophobic side chains, exemplified by 2-ketooctanoate (**1d**) and phenylpyruvate (**1h**), are bound and turned over with similar efficiency by wild-type and mutant enzymes, resulting in almost equivalent specificity constants.

While the Cys97 → Gly replacement was inadvertent, it is of structural specificity interest in that it is adjacent to a structurally important loop region of the active site. This loop, composed of residues 98–110, is flexible and closes onto the active site after binding of the keto acid substrate and then re-opens to release the hydroxy acid product.[2] This loop movement is an essential element in LDH catalysis[45,46] and has been found to be rate-determining for BSLDH.[47,48] Cysteine 97 in the wild-type enzyme is at the C-terminal end of a β-sheet and is attached to the loop hinge[31] residue Ala98. The new Gly97 residue is thus in a position where increased flexibility could affect the enzyme's catalytic properties. However, as Figure 3 shows, the C97G mutation has essentially no effect on the kinetic parameters. It alters neither the structural specificity nor the maximum rates of the double mutant relative to the Q102R enzyme. In fact, for ketomalonate (**1i**) the k_{cat}'s for both mutants are virtually identical to that of WT-BSLDH with the natural substrate pyruvate (**1a**, Table I) and are close to the limiting rate of 250 s^{-1} determined by loop mobility.[48] The only distinct difference in kinetic behavior between the single Q102R and double Q102R/C97G mutants occurs with oxalacetate (**1j**) as a substrate, for which 3-fold differences in k_{cat} and K_M are evident (Tables I and II). These presumably reflect subtle structural changes induced by Gly97 that become significant only for the most perfectly oriented ES interactions that are required, combined with tight binding, to achieve the maximum rate.

The efficient activation of the mutants by FDP suggests that the active site of BSLDH can be altered without disturbing the adjacent activator binding site, even though residues in close proximity to the active site are involved.[31] FDP also has a significant stabilizing effect on the thermal stabilities of the enzymes, with the double mutant being least benefited. However, each of the mutations is seen to be deleterious to BSLDH's resistance to heat-inactivation, with the negative effects of Q102R and C97G changes in this regard being approximately additive.

With regard to ascertaining if changing Gln102 to Arg could induce some D stereospecificity via substrate binding in the Figure 2b orientation, the results showed that the natural L stereospecificity of BSLDH was not significantly disturbed by this mutation. This was established in two ways. With the Q102R/C97G enzyme, preparative-scale reductions were carried out of pyruvate (**1a**) to L-lactate (**2a**) and of phenylpyruvate (**1h**) to L-phenyllactate (**2h**). In each case the L products were, as far as we could judge, enantiomerically pure, with no vestige of D enantiomers evident. For the Q102R mutant, the even more sensitive stereospecificity test of comparing the rates of L- versus D-2-hydroxy acid oxidations was applied, using L- and D-**2a,h,j** as representative substrates, this time in triethanolamine–HCl buffer because the oxidation pH optimum is 8.5. The steady-state conditions applied permitted k_{cat}/K_M values as low as 10^{-3} M^{-1} s^{-1} to be reliably detected. As seen in Table II, the L enantiomers of **2a,h** were substrates for both the wild-type and mutant BSLDH, with the main difference being sharply reduced k_{cat} and raised K_M values for Q102R-catalyzed oxidation of L lactate. The constants for L-phenyllactate oxidation were similar for both enzymes. With D-lactate and D-phenyllactate as potential substrates, the wild-type and mutant BSLDH's exhibited no detectable activity. Each enzyme is active with L-malate as substrate, with the Q102R enzyme being much the superior catalyst as expected since, as noted earlier, this was a key change in inducing malate dehydrogenase activity into BSLDH.[34] Of greatest interest for L- and D-malate as substrates was the observation that, while a marked L-stereoselectivity preference remained, D-malate was also oxidized at significant rates by both WT and Q102R enzymes. At face value these data also indicate that the partiality toward L over D exhibited by WT-BSLDH is slightly reduced for Q102R catalyses. However, the errors involved in the kinetic parameters preclude any definite conclusion in this regard at this time. Nevertheless, this is the first hint that there may be scope for altering BSLDH stereospecificity with appropriate mutant and substrate structures.

Although the fact that the 2-keto dicarboxylic acids **1i–k** were good substrates for both mutant enzymes established that binding of a COO^- function at 102R as required for the "D"-orientation mode of Figure 2b was feasible, the possibility existed that the absence of D activity for **2a,h** was due to the inability of the D enantiomers to bind at the active site. This was probed by evaluating L- and D-lactate, L- and D-phenyllactate, and also L- and D-malate as inhibitors of WT- and Q102R BSLDH's. Interpretation of the kinetic data was not unequivocal, since the differences observed in apparent K_M and k_{cat} values were small and usually did not permit clear-cut decisions between competitive and noncompetitive inhibition. Table III records the K_I's calculated from the data on the basis of competitive inhibition. For lactate, neither the L nor the D enantiomer is a strong inhibitor of the WT- or Q102R-BSLDH, and the relative patterns of L versus D stereoselectivity remain the same with both enzymes. The same is true for the phenyllactate enantiomers, except that stronger inhibition than for L- and D-lactate is observed in each case. In fact, D-phenyllactate is an excellent inhibitor of both WT and mutant enzymes. Interestingly, neither L- nor D-malate bind effectively to WT-BSLDH. In contrast, they do bind well to Q102R BSLDH, but no L or D stereoselectivity is manifest in the K_I values. In this latter situation, it appears that the favored interaction of a dicarboxylate inhibitor with both Arg171 and the new Arg102 overrides any stereoselectivity of binding factors.

The Q102R mutation is located within the flexible active site loop composed of residues 98–110. Crystal structures[31,50] show

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(49) The optical purities of the L- and D-malate used were confirmed by their specific rotations; L-malate $[\alpha]^{23}_D$ −7.18 (disodium salt, *c* 15, H_2O, lit. $[\alpha]^{21}_D$ −7.0 under identical conditions, Aldrich catalog, Beil. 3, 424); D-malate $[\alpha]^{23}_D$ +7.21 (disodium salt, *c* 15, H_2O). The reactions observed with D-malate thus cannot be due to any significant contamination with L-malate.

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that in ternary enzyme–coenzyme–substrate–analogue complexes this loop folds over the substrate binding site in a specific conformation, whereas in the absence of bound substrate it is exposed to the solvent and remains highly flexible. The residue at position 102 should therefore be a less important contributor to the structure of the unliganded enzyme than residue 97, which is located at the end of a β-sheet in a densely packed region of the protein. However, as noted already, both the C97G and Q102R mutations have similarly deleterious effects on the thermostability of BSLDH.

To monitor the impact of the mutations on the overall structure of BSLDH, CD spectra of the WT, Q102R, and Q102R/97G apoenzymes were recorded. Although the three far-UV spectra in Figure 1a have virtually the same shape, ellipticity differences are noted, particularly at the two minima near 222 and 208 nm and the maximum near 194 nm, which indicate secondary structure changes. The near-UV CD spectra (Figure 1b) again reveal clear differences between the three enzymes, although the three spectra have basically the same general shape. The broad positive peaks near 297 and 288 nm probably represent contributions from some, or all, of the 3 Trp residues located in asymmetric centers. The broad maximum centered near 277 nm arises from Tyr residues located in asymmetric centers, but it is unusual in that the signal is positive in this region. This may imply strong interactions between some Tyr residues and α-helical regions. Phe contributions are found below 270 nm, down to 250 nm.

According to the CD data, and in agreement with the thermostability data, both mutations have an impact on the protein folding. To assist in deciphering these results a second computer model of BSLDH was created from the X-ray coordinates of the substrate-free enzyme with an open loop. The location of the side chain of Arg102 is such that no matter what conformation it adopts it cannot form short-range van der Waals or hydrophobic interactions with any structured part of the protein. However, electrostatic potential contours generated by the Delphi program for both enzymes show that in the Q102R mutant the positive charge of R102 is not compensated by adjacent negative potentials but is juxtaposed to a large negative potential surface covering the helices α1G, α2G, and the N-terminal end of α3G toward helix αH, including βH, βG, and the loop connecting these sheets. The Delphi contours suggest electrostatic interactions of R102 with these secondary structures and with residues D234, E241, E199, Y237, Y247, and Y248 in particular, which is consistent with the experimentally and spectroscopically observed changes in thermostability and secondary structure. Although mutations in flexible surface loops are considered to be of little importance for protein structure and stability,[51] this example illustrates the impact such a mutation might have when long-range interactions are induced.

The fact that Gln102 → Arg, or the previous Arg171 → Trp/Tyr,[25] mutations do not affect the L stereospecificity of BSLDH for 2-keto acid reduction provides an indication of the degree to which the enzyme's active site and catalytic machinery is committed to maintaining the natural stereochemical order. Clearly fail-safe determinants are present to take over when Arg171 is absent.[25] These same safety features may also play a role in offsetting COO^- binding when Q102R is introduced. However, the reduced stereoselectivity observed for L- and D-malate oxidations shows that the L stereospecificity preference of BSLDH need not be absolute for all mutant substrate structure combinations. Further studies probing this aspect are in progress.

Experimental Section

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. D-Phenyllactic acid was prepared according to a published procedure.[52] Cyclohexylpyruvic acid (3-cyclohexyl-2-oxopropanoic acid) was prepared in three steps from ethyl 1,3-dithiane-2-carboxylate following the method of Graham et al.[53] All other 2-keto carboxylic acids or the corresponding alkali salts and hydroxymalonic acid were bought from Sigma. Deoxyribonucleotides, Sepharose CL-4B, and Blue Sepharose were purchased from Pharmacia. Oligodeoxyribonucleotides were made by automated phosphoroamidite chemistry on an Applied Biosystems 380B DNA synthesizer.

WT-BSLDH was purified from *E. coli* TG2 cells as described previously.[8,9] For the purification of BSLDH mutants, the first two purification steps (ultrasonic cell disruption and heat treatment) were performed as for the wild-type enzyme. BSLDH was precipitated from the heated and clarified crude extract by 75% ammonium sulfate saturation. The precipitate was dissolved and dialyzed in buffer A (50 mM piperazine·HCl, pH 6.0) and loaded onto a Mono-Q HR10/10 column (Pharmacia) equilibrated with buffer A. Proteins were eluted by a 125-mL gradient from 0 to 200 mM sodium chloride in buffer A. Peak fractions containing LDH activity were pooled and used for enzyme kinetic measurements or for further purification. Aliquots of the Mono-Q pools were saturated to 25% with solid ammonium sulfate and loaded at room temperature onto a Phenyl-Superose HR5/5 column (Pharmacia), equilibrated with buffer B (30 mM piperazine·HCl, pH 6.0, 25% ammonium sulfate saturation). BSLDH was eluted in a 15-mL gradient from buffer B to buffer C (30 mM piperazine·HCl, pH 6.0). Active fractions were dialyzed against buffer D (30 mM sodium phosphate, pH 7.0) and used for spectroscopic purposes. Formate dehydrogenase was obtained from Boehringer Mannheim. Restriction enzymes, T4 DNA ligase, and Klenow fragment of DNA polymerase were bought from Bethesda Research Laboratories or Pharmacia.

E. coli strains used were the following: JM101, RZ1032, CJ236, and TG2 [*rec A*⁻ form of TG1].[54] The gene for lactate dehydrogenase from *Bacillus stearothermophilus*[32] was subcloned into a pTZ18R plasmid.[9] Growth medium for *E. coli* was YT(2×) (16 g of Bactotryptone, 10 g of yeast extract, 5 g of sodium chloride per litre).

Cloning and Mutagenesis. Cloning, sequencing, and mutagenesis of the BSLDH gene was either carried out as described previously,[9] except that the *E. coli* strain CJ236 was used instead of RZ1032, or according to the method of Slilaty.[35] The coding sequences of mutant BSLDH genes were verified by dideoxy-sequencing with a set of five oligonucleotide primers, equally distributed over the entire gene.

Kinetic Studies. Kinetics of 2-keto acid reductions were recorded at pH 6 at 25 °C on a Shimadzu UV-240/PR1, or a Pharmacia-LKB Ultrospec Plus spectrophotometer equipped with a temperature-controlled autofill unit, using the protocols described previously[25] but with 20 mM piperazine·HCl as buffer. Steady state rate assays with 2-hydroxy acids were done under published conditions.[55] The experimental data were evaluated by nonlinear regression analysis using the program "Grafit" (Erithacus Software Ltd., Staines, UK). If substrate inhibition was observed, the concentration for the regression was chosen such that no systematic deviation of the data points from the fitted curves was visible. For determining the inhibition constants, the rates of enzyme-catalyzed phenylpyruvate (**1h**) reduction were measured in 20 mM piperazine·HCl buffer at saturating concentrations of NADH (0.2 mM) and FDP (5 mM). Phenylpyruvate concentrations in the range between 0.1 and 3.0 mM and inhibitor concentrations within appropriate ranges to ensure strong inhibition were varied independently in the assays. The data were subjected to nonlinear regression analyses uses the "Grafit" program. All kinetic determinations were performed at least in duplicate.

Preparative Scale Reductions. 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.[25]

2(*S*)-Lactic Acid (2a). Sodium pyruvate (440 mg, 4 mmol), NAD^+ (22.5 mg, 0.032 mmol), sodium formate (408 mg, 6 mmol), FDP (55 mg, 0.1 mmol), dithiothreitol (1.5 mg, 0.01 mmol), lyophilized formate dehydrogenase (45 mg, 20 U), and BSLDH Q102R/C97G (4.7 mg) were dissolved in 25 mM piperazine·HCl buffer to a final volume of 20 mL and incubated overnight at pH 6.0 under pH-stat control to give 2(*S*)-lactic acid (163 mg, 1.81 mmol, 45% yield, >98% ee): $[\alpha]^{20}_D$ −13.1° (*c* 0.4, sodium salt in water, pH 11.5) (L-lactic acid (Sigma) $[\alpha]^{20}_D$ −14.4° under identical conditions). The ^{1}H-NMR spectra of the enzyme-derived and authentic L-lactic acids were identical.

2(*S*)-β-Phenyllactic Acid (2h). Sodium β-phenylpyruvate (301 mg, 1.48 mmol), NAD^+ (22.5 mg, 0.032 mmol), sodium formate (150 mg,

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2.2 mmol), FDP (55 mg, 0.1 mmol), dithiothreitol (1.5 mg, 0.01 mmol), lyophilized formate dehydrogenase (45 mg, 20 U), and BSLDH Q102R/C97G (1.5 mg) were dissolved in 25 mM piperazine·HCl buffer to a final volume of 20 mL and incubated overnight at pH 6.0 under pH-stat control. This reaction yielded 2(*S*)-β-phenyllactic acid (160 mg, 0.96 mmol, 65% yield, >95% optically pure) $[\alpha]^{20}_D$ −20.1° (*c* 1.9, in water), lit. (Beil. 10, IV, 653) $[\alpha]^{20}_D$ −21 ± 1°. The ^{1}H-NMR spectrum was as described previously.[8]

Determination of Enantiomeric Purities. The enantiomeric excess of 2(*S*)-lactic acid (**2a**) was determined from ^{1}H-NMR comparisons (using a Varian XL200 spectrometer) of the (*R*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetic acid (MTPA) esters of enzyme-derived and racemic **2a** as described previously.[8] The 2(*S*) absolute configuration of the enzyme-derived lactic acid was assigned from the sign of the optical rotation of authentic samples.[8] The enantiomeric purity of the enzyme-derived β-phenyllactic acid (**2h**) was calculated from comparison of its optical rotation with literature reference data (Beil. 10, IV, 653). The optical rotations were measured with a Perkin-Elmer 141 polarimeter.

Thermal Stability. Solutions containing 0.07 mg/mL of these enzymes in 50 mM piperazine·HCl buffer (pH 6.0) were heated to 85 °C in the presence of 5 mM FDP and to 75 °C in the absence of FDP. Aliquots of these solutions were removed at different time intervals and assayed for LDH activity. The same assay containing 20 mM piperazine·HCl (pH 6.0), 0.2 mM NADH, 5 mM FDP, and 5 mM phenylpyruvate was used for all enzymes. The times required to halve the activity were determined from the fit of a single exponential decay function to the experimental data using the GraFit program.

CD Spectroscopy. Circular dichroism (CD) measurements were obtained using a Jasco J-720 spectropolarimeter (Jasco Inc., Easton, MD) interfaced to an Epson Equity 386/25 computer. The instrument was controlled by software developed by Jasco. The cell was maintained at 25 °C with an RMS circulating water bath (Lauda, Westbury, NY) and 30 mM sodium phosphate buffer (pH 7.0) was used as a solvent. Near-UV (320–250 nm) scans were performed in a microcell of path length 1 cm (volume = 90 μL). In the wavelength range 255–190 nm, cells of path length 0.01 and 0.02 cm were used. The computer-averaged trace of ten scans was used in all calculations. Signal due to solvent was subtracted. The instrument was routinely calibrated with *d*-(+)-10-camphor sulfonic acid at 290 nm and with pantoyl lactone at 219 nm by following procedures outlined by the manufacturer. As a prerequisite to prevent distortion in the CD spectrum at low wavelengths, the high-tension voltage on the photomultiplier was never allowed to exceed the value indicated in the Jasco instruction manual. This was 500 V for the solvent and the path lengths described in this work. The data were normally plotted as mean residue weight ellipticity (expressed in deg·cm^2 per dmol) versus wavelength in nanometers. The mean residue weight was taken to be 109.98 for the wild-type protein, 110.11 for the Gln102 → Arg mutant, and 109.97 for the double mutant. The ellipticity-versus-wavelength data were analyzed by a computer program developed by Provencher and Gloeckner[38] which analyzes CD spectra as a sum of spectra of 16 proteins, the structures of which are known from X-ray crystallography. The input to the program was the mean residue ellipticities, in 1-nm intervals from 190 to 240 nm.

Graphics Analyses. The software package Insight II (Version 2.0.0, with Biopolymer and Delphi modules, Biosym Technologies Inc., San Diego, CA) was run on a Silicon Graphics Iris 4D240 workstation. For electrostatic potential calculations with Delphi on apo-BSLDH, the Brookhaven database file 2LDB was used, with all coordinates except for those of the protein deleted. Additional coordinates for the main chain and C_β atoms of residues not included in the database file were provided by the authors of this structure (Piontek, K., personal communication). Side chain coordinates for those residues were generated using Insight II default parameters. The following parameters were used for the Delphi calculations: interior dielectric constant = 2.0; exterior dielectric constant = 80.0; solvent radius = 1.8 Å; ionic radius = 2.0 Å; ionic strength = 0.145 M; full Coulombic boundary conditions; van der Waals radii and partial charges from the Insight II residue library for protein atoms.

Highly refined coordinates for the ternary complex of dogfish muscle LDH, NADH, and oxamate (Brookhaven data base entry 1LDM) were used to create an active site model on the computer graphics system. They were chosen because they represent the most accurate structural data available for an LDH active site. C_α coordinates for the BSLDH-NADH-FDP complex refined to 3.0 Å (Brookhaven entry 2LDB) were almost congruent with the dogfish muscle LDH data except for differences in the active site loop.[31] The Arg102 side chain of the mutants and the substrate ketomalonate (**1i**) were modeled into the dogfish muscle LDH–NADH–oxamate complex structure. For residue 102, the backbone atom and C_β coordinates were left unchanged. The ketomalonate model was created by maintaining the carboxylate and carbonyl group coordinates of oxamate in the X-ray data and replacing the amide nitrogen with a second carboxylate in coplanar conformation with respect to the carbonyl group. It was then possible to adjust the remaining side chain torsional angles of Arg102 to bring the terminal nitrogen atoms in close proximity to the carboxylate oxygens of the substrate, as close together as 2.6 and 3.0 Å for each N–O pair, in an almost coplanar conformation, resembling the strong ionic bond between the other substrate carboxylate and Arg171.[40]

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