An Evaluation of the Substrate Specificity, and of Its Modification by Site-Directed Mutagenesis, of the Cloned L-Lactate Dehydrogenase from *Bacillus stearothermophilus*

Marcel A. Luyten,[†] Daniel Bur,[†] Hla Wynn,[†] Wendy Parris,[‡] Marvin Gold,[‡] James D. Friesen,[‡] and J. Bryan Jones*,[†]

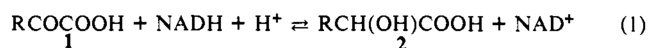
Contribution from the Departments of Chemistry and Medical Genetics, University of Toronto, Toronto, Ontario, Canada M5S 1A1. *Received May 19, 1988*

Abstract: The L-lactate dehydrogenase of *Bacillus stearothermophilus* (BSLDH) is a stable, thermophilic oxidoreductase. It has been selected as a model of enzymes with considerable future promise in asymmetric synthesis in that it has been cloned to ensure a plentiful and inexpensive supply and because of the potential for tailoring its specificity to accept unnatural substrate structures via the site-directed mutagenesis techniques of molecular biology. In this study, the specificity of BSLDH toward representative α-keto acids possessing straight- and branched-chain alkyl, cycloalkyl, or aromatic side chains has been evaluated. The results show that substrates that are sterically bulky in the region of the α-keto group to be reduced are poorly accepted by the enzyme. Graphics analyses indicated that the low activities of these hindered substrates might be partly due to a bad interaction of the active site residue Gln102 with large or branched substituents adjacent to the α-keto group. Accordingly, Gln102 has been replaced by the smaller Asn residue by site-directed mutagenesis in an attempt to expand the active site volume available to receive substrates larger than the natural pyruvate. However, the kinetic data show that bulky α-keto acids are only marginally better accommodated by the Gln102 → Asn mutant than by the wild-type enzyme.

Despite the numerous applications of enzymes for the generation of chiral synthons that have been documented,[1] the field continues to expand, with many new examples being reported each year. However, the number of enzymes of broad synthetic capability that are readily and inexpensively available is somewhat restricted at present. Fortunately, recent advances in molecular biology offer a solution to this limitation in that plentiful supplies of many useful enzymes can be assured by cloning and overexpressing their genes. Furthermore, the potential now exists for tailoring the specificities of such enzymes toward nonnatural substrates via the site-directed mutagenesis techniques of protein engineering.[2]

The L-lactate dehydrogenase of *Bacillus stearothermophilus* (BSLDH)[3] was selected as our target enzyme for exploring this potential. The LDH group of enzymes is one whose asymmetric synthon preparation efficacy has been demonstrated[4] and about which a considerable body of mechanistic,[5] specificity,[4a,6] and structural[7] information is available. BSLDH is a good candidate for evaluating the feasibility of tailoring enzyme specificity for organic synthetic purposes in a controlled manner for several reasons. It is a well-characterized thermophilic enzyme[8,9] whose gene has been cloned[8a,9a] and for which site-directed mutagenesis has already been shown to be feasible.[10] In fact, its amenability to predetermined specificity change has recently been demonstrated in a very elegant manner by Holbrook and his collaborators, who created a malate-favoring mutant by site-directed mutagenesis of the native lactate-selective BSLDH structure.[10a] Furthermore, the usefulness of BSLDH for the preparation of (2*S*)-hydroxy acid synthons by inducing enantiotopically face-specific reductions of 2-keto acids has been established.[11]

Lactate dehydrogenases are nicotinamide coenzyme dependent enzymes whose in vivo function[5] is to catalyze pyruvate (**1**)-lactate (**1a**) oxidoreductions as shown in eq 1.



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

1a, R = CH_3
1b, R = CH_2CH_3
1c, R = $(CH_2)_2CH_3$
1d, R = $(CH_2)_3CH_3$
1e, R = $(CH_2)_4CH_3$
1f, R = $(CH_2)_5CH_3$
1g, R = CH_2OH
1h, R = $CH(CH_3)_2$
1i, R = $CH(CH_3)CH_2CH_3$
1j, R = $CH_2CH(CH_3)_2$
1k, R = cyclopropyl
1l, R = cyclobutyl
1m, R = cyclopentyl
1n, R = cyclohexyl
1o, R = C_6H_5
1p, R = $CH_2C_6H_5$

LDH's from various mammalian and bacterial sources can tolerate some diversity in the structures of their 2-keto acid or 2-hydroxy acid substrates,[4,6] and a number of these enzymes, including BSLDH, have already been used in preparative-scale

Table I. Kinetic Parameters for BSLDH-Catalyzed Reductions of **1a-p**[a]

substrate	k_{cat} for BSLDH (s^{-1})	K_M for BSLDH (mM)	k_{cat}/K_M for BSLDH ($M^{-1}\,s^{-1}$)
1a	188 (203)[b]	0.04 (0.03)	4.70E6 (6.77E6)[f]
1b	155 (101)[b]	0.34 (0.43)	4.55E5 (2.35E5)
1c	44 (11)[b]	2.4 (1.5)	1.83E4 (7.33E3)
1d	25 (11)[c]	1.5 (1.9)	1.67E4 (5.79E3)
1e	23 (1.2)[d]	0.53 (1.05)	4.34E4 (1.14E3)
1f	32 (0.2)[d]	0.35 (0.68)	9.14E4 (2.94E2)
1g	125 (244)[d]	0.16 (0.17)	7.81E5 (1.44E6)
1h	0.49 (0.65)[b]	17 (3.9)	2.88E1 (1.67E2)
1i	0.03 (0.02)[c]	14 (5.2)	2.14E0 (3.85E0)
1j	0.51 (0.03)[c]	4.9 (3.6)	1.04E2 (8.30E0)
1k	5.0 (7.3)[b]	1.7 (1.6)	2.94E3 (4.56E3)
1l	1.6	12	1.33E2
1m	0.12	8.7	1.38E0
1n	*e*	*e*	<0.5
1o	0.96 (<0.02)[c]	14	6.43E1
1p	81 (5.4)[b]	0.67 (1.4)	1.21E5 (3.86E3)

[a] Values for BSLDH measured at 25 °C, with [E] = 0.2-5000 nM, [S] = $0.2K_M$ to $3.0K_M$, [NADH] = 0.2 mM, and [FDP] = 5.0 mM in TEA buffer (20 mM, pH 6.0); error values determined were ≤15%. (The constants in parentheses are for PHLDH.) [b] For PHLDH, from ref 11. [c] PHLDH values from ref 4a. [d] Measured for PHLDH at 25 °C in phosphate buffer (100 mM, pH 7.0) with [E], [S], and [NADH] as in footnote *a*. [e] No detectable reaction. [f] Exponential notation; i.e., 4.70E6 is 4.70 × 10^6.

[†] Department of Chemistry.
[‡] Department of Medical Genetics.

(1) (a) Jones, J. B.; Beck, J. F. *Tech. Chem. (N.Y.)*, **1976**, *10*, 107. Jones, J. B. *Tetrahedron* **1986**, *42*, 3351. (b) Wong, C. H.; Whitesides, G. M. *Angew. Chem., Int. Ed. Engl.* **1985**, *24*, 617. Akiyama, A.; Bednarski, M.; Kim, M. J.; Simon, E. S.; Waldmann, H.; Whitesides, G. M. *Chem. Br.* **1987**, *23*, 645. (c) Klibanov, A. M. *Science* **1983**, *219*, 722. Klibanov, A. M. *Chem. Tech.* **1986**, 354. (d) *Enzymes in Organic Synthesis*; Ciba Foundation Symposium 111; Porter, R.; Clark, S., Eds.; Pitman: London, 1985. (e) *Biocatalysis in Organic Synthesis*; Tramper, J.; von der Plas, H. C.; Linko, P., Eds.; Elsevier: Amsterdam, 1985. (f) *Enzymes as Catalysts in Organic Synthesis*; Schneider, M., Ed Reidel: Dordrecht, The Netherlands, 1986.

(2) (a) Gerlt, J. A. *Chem. Rev.* **1987**, *87*, 1079. (b) Shaw, W. V. *Biochem. J.* **1987**, *246*, 1. (c) Leatherbarrow, R. J.; Fersht, A. R. *Protein Eng.* **1986**, *1*, 7.

(3) Abbreviations: LDH, lactate dehydrogenase; NAD and NADH, oxidized and reduced forms, respectively, of nicotinamide adenine dinucleotide; BSLDH, *Bacillus stearothermophilus* LDH; PHLDH, pig heart LDH; DMLDH, dogfish muscle LDH; Gln, glutamine; Asn, asparagine; FDP, fructose 1,6-bisphosphate; TEA, triethanolamine hydrochloride; buffer A, TEA (20 mM, pH 6.0).

0002-7863/89/1511-6800$01.50/0 © 1989 American Chemical Society

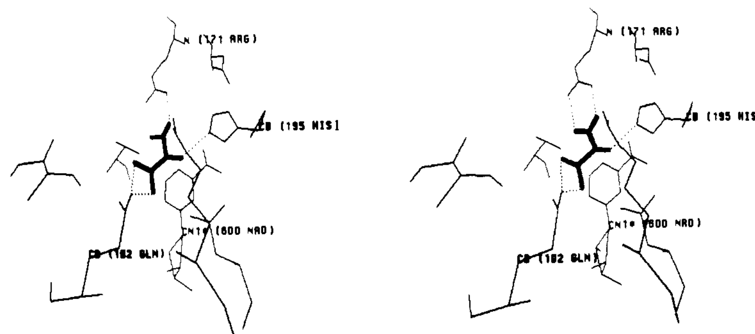


Figure 1. Graphics analysis, in stereo, of the interactions of the β-branched substrate 3-methyl-2-ketobutanoate (**1h**) at the active site of BSLDH, performed with the MIDAS program. The BSLDH active site structure was modeled on that of the 2.1-Å (R = 17.3) data of the oxamate–NADH–DMLDH ternary complex,[16a] all of the amino acid residues shown being identical for both enzymes. The orientation of **1h** (drawn in heavy lines) depicted was modeled in with the known oxamate pyruvate analogue position[16a] as a guide to locate it acceptably with respect to NADH and with the key Arg171 guanidinium to COO^- and His195 imidazolium to C=O binding interactions (shown in dotted lines) in place. In this binding mode, **1h** cannot avoid steric interactions of its two β-methyl groups with the terminal amide oxygen of the Gln102 side chain, indicated by the two remaining dotted lines, for which the atomic separations are 2.6 Å instead of the ~4 Å needed for a strain-free fit. This ES complex is therefore disfavored. The changes in orientation of **1h** induced by adverse interactions of this type result in a poor overall active site fit, in accord with the weak binding and low catalytic constants observed experimentally for this and other bulky substrates.

reactions, both with[4,11,12] and without[4c,d] recycling of the NADH cofactor. In order to further explore the range of substrate-structure acceptance of BSLDH, the enzyme has now been evaluated with the extended series of substrates **1a–p**. For reference and comparison purposes, their kinetic parameters have also been measured for mammalian pig heart LDH (PHLDH),[3] a well-documented oxidoreductase that is representative of the LDH's used preparatively so far. We also report an attempt to expand BSLDH specificity by site-directed mutagenesis.

Table II. Kinetic Parameters for Gln102 → Asn BSLDH Catalyzed Reductions of **1a–c, f–k, o, p**[a]

	k_{cat} (s^{-1})		K_M (mM)		k_{cat}/K_M (M^{-1} s^{-1})	
substrate	Gln102	Asn102	Gln102	Asn102	Gln102	Asn102
1a	188	123	0.04	0.23	4.70E6[b]	5.35E5
1b	155	105	0.34	0.25	4.56E5	4.20E5
1c	44	54	2.4	1.7	1.83E4	3.18E4
1f	32	39	0.35	0.27	9.14E4	1.44E5
1g	125	59	0.16	1.2	7.81E5	4.92E4
1h	0.49	0.81	17	20	2.88E1	4.05E1
1i	0.03	0.03	14	10	2.14E0	3.00E0
1j	0.51	0.57	4.9	5.2	1.04E2	1.10E2
1k	5.0	3.5	1.7	1.9	2.94E3	1.84E3
1o	0.96	1.5	14	12	6.43E1	1.25E2
1p	81	73	0.67	0.71	1.21E5	1.03E5

[a] Reaction conditions for wild-type (Gln102) enzyme, see footnote *a* in Table I. Values for the Gln102 → Asn enzyme were determined at 25 °C in TEA buffer (20 mM, pH 6.0), with [E] = 5–1000 nM, [S] = $0.1K_M$ to $3.0K_M$, [NADH] = 0.2 mM, and [FDP] = 5.0 mM; error values determined were ≤15%. [b] See footnote *f* in Table I.

Results

The 2-keto acid substrates **1a–j,o–p** and PHLDH were purchased. The cyclopropyl **1k**[13] and the other substrates **1l–n**[14] were prepared by two different, literature, methods. BSLDH was readily obtained following the procedure described previously.[11] The kinetic parameters for both enzymes were measured under steady-state conditions and are recorded in Table I.

The Table I data show that 2-keto acids with branched (**1h–j**) and alicyclic (**1k–n**) R groups are very poor substrates. From graphics analyses of these results, Gln102 was identified as an amino acid whose unfavorable side-chain interactions with branched 2-keto acids in the ES complexes preceding reduction (Figure 1) could be contributing to the low catalytic rates observed with bulky substrates. In order to try to reduce the magnitude of this interaction, the native Gln102 residue was changed to the smaller Asn by site-directed mutagenesis. This mutation maintains the hydrophilic nature of the amino acid residue at position 102, while potentially providing more room for bulky substrates due to the smaller $-CH_2CONH_2$ side chain than $-CH_2CH_2CONH_2$

(4) (a) Kim, M.-J.; Whitesides, G. M. *J. Am. Chem. Soc.* **1988**, *110*, 2959. (b) Hummel, W.; Schuette, H.; Kula, M.-R. *Eur. J. Appl. Microbiol. Biotechnol.* **1983**, *18*, 75. (c) Chen, Ch.-S.; Zhou, B.-N.; Girdaukas, G.; Shieh, W.-R.; VanMiddlesworth, F.; Gopalan, A. S.; Sih, Ch. *Bioorg. Chem.* **1984**, *12*, 98. (d) Nardini, M.; Kodama, H.; Ricci, G.; Federici, G.; Cavallini, D. *Biochem. Int.* **1985**, *11*, 789. (e) Chenault, H. K.; Kim, M.-J.; Akiyama, A.; Miyazawa, T.; Simon, E. S.; Whitesides, G. M. *J. Org. Chem.* **1987**, *52*, 2608. (f) Nonhebel, D. C.; Orszulik, S. T.; Suckling, C. J. *J. Chem. Soc., Chem. Commun.* **1982**, *1146*. (g) MacInnes, I.; Nonhebel, D. C.; Orszulik, S. T.; Suckling, C. J. *J. Chem. Soc., Perkin Trans. 1* **1983**, 2771.

(5) (a) Holbrook, J. J.; Liljas, A.; Steindal, S. J.; Rossmann, M. G. *Enzymes (3rd Ed.)* **1975**, Chapter 4. (b) Everse, J.; Kaplan, N. O. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1973**, *37*, 61. (c) Schwert, G. W.; Winer, A. D. *Enzymes, 2nd Ed.* **1963**, Chapter 6.

(6) (a) Duncan, R. J. S.; Tipton, K. F. *Eur. J. Biochem.* **1969**, *11*, 58. (b) Lane, R. S.; Dekker, E. E. *Biochemistry* **1969**, *8*, 2958. (c) Pensa, B.; Costa, M.; Colosimo, A.; Cavallini, D. *Mol. Cell Biochem.* **1982**, *44*, 107. (d) Meister, A. *J. Biol. Chem.* **1950**, *184*, 117. (e) Meister, A. *J. Biol. Chem.* **1952**, *197*, 309. (f) Martensson, J. *Anal. Biochem.* **1986**, *154*, 43.

(7) (a) Grau, A. M.; Trommer, W. E.; Rossmann, M. G. *J. Mol. Biol.* **1981**, *151*, 289. (b) Abad-Zapatero, C.; Griffith, J. P.; Sussman, J. L.; Rossmann, M. G. *J. Mol. Biol.* **1987**, *198*, 445.

(8) (a) Stangl, D.; Wiederkehr, F.; Suter, F.; Zuber, H. *Biol. Chem. Hoppe-Seyler* **1987**, *368*, 1157. (b) Wirz, B.; Suter, F.; Zuber, H. *Hoppe-Seyler's Z. Physiol. Chem.* **1983**, *364*, 893. (c) Tratschin, J. D.; Wirz, B.; Frank, G.; Zuber, H. *Hoppe-Seyler's Z. Physiol. Chem.* **1983**, *364*, 879. (d) Schaer, H.-P.; Zuber, H. *Hoppe-Seyler's Z. Physiol. Chem.* **1979**, *360*, 795.

(9) (a) Barstow, D. A.; Clarke, A. R.; Chia, W. N.; Wigley, D. B.; Sharman, A. S.; Holbrook, J. J.; Atkinson, T.; Mington, N. T. *Gene* **1986**, *46*, 47. (b) Clarke, A. R.; Atkinson, T.; Campbell, J. W.; Holbrook, J. J. *Biochim. Biophys. Acta* **1985**, *829*, 387. (c) Clarke, A. R.; Wilks, H. M.; Barstow, D. A.; Atkinson, T.; Chia, W. N.; Holbrook, J. J. *Biochemistry* **1988**, *27*, 1617. (d) Clarke, A. R.; Waldman, A. D. B.; Munro, I.; Holbrook, J. J. *Biochim. Biophys. Acta* **1985**, *828*, 375. (e) Clarke, A. R.; Evington, J. R. N.; Dunn, C. R.; Atkinson, T.; Holbrook, J. J. *Biochim. Biophys. Acta* **1986**, *870*, 112. (f) Clarke, A. R.; Wigley, D. B.; Chia, W. N.; Barstow, D.; Atkinson, T.; Holbrook, J. J. *Nature* **1986**, *324*, 699. (g) Clarke, A. R.; Wigley, D. B.; Barstow, D. A.; Chia, W. N.; Atkinson, T.; Holbrook, J. J. *Biochim. Biophys. Acta* **1987**, *913*, 72.

(10) (a) Clarke, A. R.; Smith, C. J.; Hart, K. W.; Wilks, H. M.; Chia, W. N.; Lee, T. V.; Birktoff, J. J.; Banaszak, L. J.; Barstow, D. A.; Atkinson, T.; Holbrook, J. J. *Biochem. Biophys. Res. Commun.* **1987**, *148*, 15. (b) Hart, K. W.; Clarke, A. R.; Wigley, D. B.; Waldman, A. D. B.; Chia, W. N.; Barstow, D. A.; Atkinson, T.; Jones, J. B.; Holbrook, J. J. *Biochim. Biophys. Acta* **1987**, *914*, 294. (c) Hart, K. W.; Clarke, A. R.; Wigley, D. B.; Chia, W. N.; Barstow, D. A.; Atkinson, T.; Holbrook, J. J. *Biochem. Biophys. Res. Commun.* **1987**, *146*, 346. (d) Wigley, D. B.; Clarke, A. R.; Dunn, C. R.; Barstow, D. A.; Atkinson, T.; Chia, W. N.; Muirhead, H.; Holbrook, J. J. *Biochim. Biophys. Acta* **1987**, *916*, 145.

(11) Bur, D.; Luyten, M. A.; Hla, W.; Provencher, L. R.; Gold, M.; Friesen, J. D.; Clarke, A. R.; Holbrook, J. J.; Jones, J. B. *Can. J. Chem.*, in press.

(12) (a) Abril, O.; Whitesides, G. M. *J. Am. Chem. Soc.* **1982**, *104*, 1552. (b) Mansson, M.-O.; Siegbahn, N.; Mosbach, K. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 1487. (c) Siegbahn, N.; Mansson, M.-O.; Mosbach, K. *Appl. Biochem. Biotechol.* **1986**, *12*, 91. (d) Chenault, H. K.; Whitesides, G. M. *Appl. Biochem. Biotechol.* **1987**, *141*, 147. (e) Kula, M. R.; Wandrey, C. *Methods Enzymol.* **1987**, *136*, 9. (f) Wichmann, R.; Wandrey, Ch.; Bueckmann, A.; Kula, M.-R. *Biotechol. Bioeng.* **1981**, *23*, 2789.

(13) Basnak, I.; Farkas, J. *Collect. Czech. Chem. Commun.* **1975**, *40*, 1038.

(14) Tanaka, K.; Nakai, T.; Ishikawa, N. *Tetrahedron Lett.* **1978**, 4809.

side chain of Asn and Gln, respectively. The effects of this Gln102 → Asn mutation on the enzyme's specificity are summarized in Table II.

Discussion

Specificity of BSLDH. BSLDH is a well-characterized NAD/H-dependent oxidoreductase composed of two (at low concentrations) or four (at high concentrations or when allosterically activated by FDP[9e]) identical subunits of molecular mass 34 862 daltons. It is a very stable enzyme of high specific activity that, being a thermophile,[8b,9e] can act at the elevated temperatures often applied in organic synthesis. Also, as a consequence of its cloning into the pKK223-3 vector and overexpression in *Escherichia coli* TG1,[9a] it is readily available in large amounts from small fermentation volumes.[11] The reduction pathway of the eq 1 reaction was selected for study since it is of more asymmetric interest than the reverse, oxidation, direction.

Previous data on BSLDH specificity[8e,10b,11] had indicated that the enzyme might accept broad structural variations in its substrate. This was confirmed by the Table I data on 2-keto acids of widely varying side-chain structures, ranging from linear alkyl (**1a–f**) to branched alkyl (**1h–j**), alicyclic (**1k–n**), and aromatic (**1o,p**). Although each of the artificial substrates **1b–p** showed measurable activity, none exhibited k_{cat} or k_{cat}/K_M values as high, nor K_M's as low, as those of the natural substrate pyruvate (**1a**), with the values for the 2-hydroxy derivative **1g** coming closest.

The k_{cat} values for the linear-alkyl substrates (**1a–g**) drop dramatically with the introduction of the first side-chain homologation (**1a** → **1b**) but then remain relatively unaffected by further $-CH_2$ additions, as for **1c–f**, with the k_{cat} values remaining fairly high overall even for the longest normal alkyl side chains. In contrast, ES binding within this series, as reflected by lowered K_M's, is worse than that for pyruvate for each of **1b–f**. The K_M's increase by ≈10-fold for the first two CH_2 group additions, **1a** → **1b** and **1b** → **1c**, respectively. This is equivalent to a loss of ≈1.5 kcal/mol in binding energy per $-CH_2$, in accord with previous findings.[10b,11] Binding then recovers progressively for the higher homologues **1d–f**, with the K_M of **1f** being only ≈10-fold higher than that of **1a**, presumably because of improved hydrophobic bonding effects.[15] The overall indicator of the catalytic efficiency, the specificity constant k_{cat}/K_M, decreases gradually from **1a** to **1d** and then increases steadily back up to **1f**.

The deleterious effects of branched-alkyl groups are manifest by the dramatically reduced k_{cat} and increased K_M values for **1h–j**, for which the specificity constants are reduced by up to $\approx 10^6$-fold relative to those of **1a**. The downward influence on k_{cat} is most extreme for **1i** and is significantly ameliorated in its isomer **1j** in which the branching methyl substituent at C-4 is one methylene group further removed from the α-keto function. This structural change also improves binding, with K_M decreasing by ≈3-fold for **1j** relative to that for **1i**. The combined k_{cat} and K_M improvements result in the specificity constant for **1j** being almost 100-fold larger than that of **1i**. This indicates the sensitivity of the enzyme to steric congestion in the immediate neighborhood of the keto group being reduced.

The unfavorable effects of nonlinear side chains are lessened remarkably when the branching is part of a very small ring, as for the cyclopropyl substrate **1k**. In this case, the reduced C–C–C bond angles of the cyclopropyl ring, relative to those of the isopropyl group of the analogous acyclic keto acid **1h**, result in ≈10-fold improvements in both k_{cat} and K_M and, hence, in a ≈100-fold higher specificity constant for **1k** over that for **1h**. In contrast, the increased steric influences in the neighborhood of the 2-keto group exerted by the larger ring substrates **1l–n** translate into progressive reductions in the k_{cat}/K_M values for the cyclobutyl and cyclopentyl substrates, respectively, with the most bulky 2-cyclohexyl-2-oxo acid **1n** being effectively a nonsubstrate.

The ring is better tolerated if it is planar, as in the 2-phenyl-2-keto substrate **1o**, for which the specificity constant of 63.1, while still very low, clearly represents a significant enhancement in substrate activity over that of its cyclohexyl analogue **1n**. Displacement of the phenyl ring from the keto group by a CH_2 group, as for the benzyl substrate **1p**, results in a dramatic improvement in both k_{cat} and K_M. In fact, the specificity constant of **1p** is only 39-fold lower than for the natural substrate pyruvate. The 5000-fold difference in specificity constant between **1o** and **1p** is not attributable solely to the steric relief achieved by displacing the branching from the C-3 to the C-4 position. It is also likely that electronic effects make some contribution. For example, conjugation of its phenyl ring with the 2-keto function will help to maintain **1o** in a fairly rigid conformation, whereas for **1p** the unconjugated aromatic ring of the benzyl group is free to rotate and find its most favorable orientation in the preferred ES complex.

```
          1   1   1       1       1      2    2    2
          0   0   1       7       9      3    4    4
          0   5   0       1       5      5    0    5

DMLDH  AGARQQEGESRL    DSARFR    EHGD   SAYEVIKLKGYTSW

PHLDH  AGVRQQEGESRL    DSARFR    EHGD   SAYEVIKLKGYTNW

BSLDH  AGANQKPGETRL    DTARFR    EHGD   AAYQIIEKKGATYY
```

Figure 2. Active site (Asp168, Arg171, His195) and loop region (residues 98–110 and 235–248) amino acid sequences of DMLDH,[7b] PHLDH,[7a] and BSLDH.[8a,9a] Residue 104 is missing in the LDH sequence numbering convention.[28]

Comparison of the BSLDH kinetic data with those of PHLDH, selected as the benchmark enzyme of the LDH's used in asymmetric synthesis up till now, reveals a number of similarities, and some differences, in the specificities of the two enzymes. BSLDH is a somewhat superior catalyst overall, particularly with respect to the longer straight-chain keto acids **1e,f**, the *sec*-butyl substrate **1j**, and the benzyl compound **1p**. This somewhat broader substrate tolerance, coupled with the much higher stability of BSLDH and the ease with which large amounts of enzyme can now be produced, makes it an attractive, low-cost candidate for routine, preparative-scale, LDH-catalyzed reactions in the near future. In this regard, we have estimated[11] that any α-keto acid with a $k_{cat}/K_M > 10$ is amenable to BSLDH-catalyzed preparative-scale reductions to the corresponding (2*S*)-hydroxy acids. Of the Table I substrates, 14 out of 16 meet this condition.

Graphics Analyses. These were performed in order to identify possible reasons for the differences in substrate activities of the 2-oxo acids **1a–p** and as a guide for making amino acid replacements in the active site region to improve the enzyme's acceptance of the poor substrates. The fully refined X-ray picture for BSLDH is not yet completed, but it is already clear[16a] that its structure and those of PHLDH[7a] and dogfish muscle LDH[7b] (DMLDH, the most highly refined LDH structure available[16] at present) are very similar. Furthermore, the amino acid sequences for all three enzymes exhibit virtually complete homology in the active site domain, particularly with respect to the key residues Asp168, Arg171, and His195 and the loop (residues 98–110) and broken helix (residues 235–248) regions which shield the active site from solvent, as shown in Figure 2.

Graphics analysis indicates that the reduction in specificity constant for **1h**, one of the poorest substrates, relative to that of pyruvate (**1a**) may be the result of interactions of the C-4 methyl groups of **1h** with the loop residue Gln102, as depicted in Figure 1. Only poor binding and orientation of **1h** would then be possible. This interpretation is in accord with its high K_M, with its low k_{cat} attributable to a less favorable, sterically induced change in substrate orientation in the reductively productive ES complex. In contrast, the proximity of Gln102 to the α-keto acid side chain becomes a positive factor in the case of **1g** since hydrogen bonding

(15) Jencks, W. P. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1975**, *43*, 219.

(16) (a) The DMLDH X-ray data used as the reference are those of a new structure of the oxamate–NADH ternary complex refined with 6.0–2.1-Å data to an *R* factor of 17.3% and containing 2542 protein atoms, 247 water oxygens, 1 NADH, and 2 oxamate molecules. The X-ray structure of the binary BSLDH–NADH complex currently being refined is similar (Rossmann, M. G.; Piontek, K., personal communication); (b) Schaer, H.-P.; Zuber, H.; Rossmann, M. G. *J. Mol. Biol.* **1982**, *154*, 349.

of its hydroxyl function with the terminal amide of Gln102 can now occur. This improves binding compared with that of its steric analogue **1b**, for which no such additional beneficial hydrogen-bonding interaction is possible, as reflected by the 2-fold lower K_M for **1g** over that of **1b**. For the cyclopropyl acid **1k**, the negative consequences of β-branching are somewhat diminished, in accord with reduced steric interactions with Gln102 resulting from the contraction of bond angles that occurs on incorporation of the carbon atoms into a three-membered ring. Making the ring planar and displacing it one CH_2 unit further from Gln102, as achieved with phenylpyruvate (**1p**), virtually eliminates the conflict with Gln102, and the specificity constant for **1p** reduction recovers almost to pyruvate levels (Table I).

Site-Directed Mutagenesis. The graphics analyses suggested that the adverse interactions of Gln102 with bulky, especially branched-chain, substrates could be diminished by reducing the size of the 102-position amino acid side chain (Figure 1). To test the validity of this conclusion, Gln102 was replaced by Asn, its lower homologue of similar hydrophobicity.

To obtain the Gln102 → Asn mutant, the BSLDH gene, including the tac promoter, was excised from its pKK223-3 plasmid[9a] as a *Bam*H1 fragment and was cloned into a pTZ-R18 vector. This construct was then used for the protein expression as well as for generating single-stranded DNA for site-directed mutagenesis and DNA sequencing. The site-directed mutation was made with a mutagenic 21-mer oligonucleotide following standard procedures for single-stranded DNA mutagenesis.[17] The DNA of the mutated gene was sequenced to verify the change and then overexpressed in *E. coli* TG2, and the altered protein was purified by a slight modification of the wild-type enzyme procedure.[11]

From the similarities in the kinetic parameters for the native and mutant enzymes (Table II), it is apparent that the Gln102 → Asn mutant protein has not undergone any major structural changes. As expected, the kinetic parameters for pyruvate (**1a**) are less satisfactory for the mutant enzyme, with K_M increased by 5-fold and k_{cat} reduced by about 2-fold. However, for the substrates with longer straight chains, **1b,c,f**, the Gln102 → Asn mutant has somewhat higher turnover numbers and smaller binding constants than for the native Gln102 catalyst.

The results for hydroxypyruvate (**1g**) are also in accord with the graphics analysis suggestions of a favorable hydrogen-bonding interaction of its $HOCH_2$ group with the Gln102 side chain amide function. This is not possible for the Gln102 → Asn mutant with its shorter side chain. This is manifest by the 8-fold increase in K_M for catalysis by the mutant. As a consequence of **1g** binding being no longer assisted by hydrogen bonding for the mutant enzyme, its specificity constant is now 9-fold lower than that for its isostere, 2-ketobutyrate (**1b**), instead of 2-fold higher as observed with the wild-type enzyme. The conjugated phenyl substrate **1o** shows a 2-fold gain in specificity constant with the Gln102 → Asn enzyme, reflecting its better accommodation in the enlarged active site. Catalysis of the reduction of the cyclopropyl compound **1k** is not improved by the Gln102 → Asn change. The most sterically demanding branched-chain substrates **1h–j** show virtually no improvement in specificity constants with the Gln102 → Asn enzyme. In retrospect, this is not too surprising since, for the representative graphics analysis depicted in Figure 1, any gain in active site volume on replacing Gln by Asn would be insufficient to fully relieve the unfavorable steric interaction incurred on changing the substrate structure from pyruvate (**1a**) to 3-methyl-2-ketobutyrate (**1h**). The volume difference between **1h** (isopropyl side chain) and **1a** (methyl substituent) is 50.2 $Å^3$, while only an additional 25.9 $Å^3$ can be gained by the Gln102 → Asn mutation.[18]

These initial studies on BSLDH already demonstrate that its specificity is broad enough to be of preparative value in (2*S*)-hydroxy acid chiron production. Although it is clear that the currently available methods of analyzing enzyme–substrate interactions, including graphics-based techniques, are not fully adequate, the activity and specificity observed for the mutant enzyme selected on these bases are encouraging, even though its improvement in this regard over the wild-type enzyme is only marginal. While full control over BSLDH specificity toward any α-keto acid structure will clearly not be reached quickly, it remains a potentially achievable goal, particularly in view of the progress being made in this direction for a number of enzymes.[10a,19]

Experimental Section

Melting points were measured in an electrothermal capillary apparatus and are uncorrected. Mass spectra were recorded on a VG-70/250S mass spectrometer. IR spectra were recorded on a Nicolet 5DX FT-IR system as liquid films or in KBr if solid. Proton NMR spectra were recorded in $CDCl_3$, unless noted otherwise, on a Varian T60, XL200, or XL400 instrument. The chemical shift values are given in ppm relative to the internal standard tetramethylsilane. Kinetics were measured on a Shimadzu UV-240/PR1 spectrophotometer equipped with a constant-temperature cell holder.

Chemicals, Enzymes, and *E. coli* Strains. Fructose 1,6-bisphosphate, NADH, and L-lactate dehydrogenase from pig heart H_4 (EC 1.1.1.27) (crystalline suspension in 1.9 M $(NH_4)_2SO_4$ solution; pH 6.0; batch 10525922-14) were obtained from Boehringer Mannheim, and 2,2,2-trifluoroethyl tosylate was from Aldrich. Restriction enzymes, T4 DNA ligase, and Klenow fragment of DNA polymerase I were purchased from Bethesda Research Laboratories. Deoxyribonucleotides, phenyl-Sepharose CL-4B, and blue Sepharose were obtained from Pharmacia. Oligodeoxyribonucleotides were made by automated phosporamidite chemistry on a DNA synthesizer (Applied Biosystem 380B). The Gln102 → Asn mutant was constructed with the following primer: Asn (N), GGC GCC AAC *AAT* AAA CCG GGC. *E. coli* strains used were JM101, RZ1032, and TG2 (*rec* A^- form of TG1).[20] The gene for lactate dehydrogenase from *B. stearothermophilus*, cloned into a pKK223-3 plasmid, was obtained from Dr. J. J. Holbrook.[9a] Growth medium used for *E. coli* was YT (2×) (16 g of Bactotryptone, 10 g of yeast extract, and 5 g of NaCl per liter).

Substrates. With the exception of **1e,k–n** the 2-keto acids were commercially available and used as received. Compound **1a** was purchased from Boeringer Mannheim, substrates **1b–d,f–g,h,j,p** were from Sigma Chemical Co., and the acids **1i** and **1o** were from Aldrich. The 2-cyclopropyl-2-oxoacetic acid (**1k**) was made as described previously.[13] The four 2-oxo acids **1e**, **1l–n** were prepared according to the general procedure of Tanaka et al.[14] as follows:

(a) **2-Cyclobutyl-2-oxoacetic Acid (1l).** Cyclobutanone (2 g, 28.57 mmol) was added to a mixture of 2,2,2-trifluoroethyl tosylate (8.7 g, 34.3 mmol) and LDA (60 mmol) in THF (40 mL) at −78 °C. The solution was warmed to 20 °C and stirred for an additional 2 h. An excess of cold (at 0 °C) 2 N HCl was added, and the resulting mixture was extracted with dichloromethane (3 × 40 mL). The extract was dried ($MgSO_4$) and concentrated to give an oil, which was redissolved in an excess of cold (0 °C) 98% H_2SO_4 and stirred for 20 min, and the mixture then poured into ice–water (100 mL). The resulting emulsion was extracted with ethyl acetate (3 × 50 mL). This extract was dried ($MgSO_4$) and concentrated to give an oil, which was purified by flash chromatography (silica gel; ethyl acetate/hexane 1:1) to give 2-cyclobutylidene-2-[(4-methylphenyl)sulfonyl]acetic acid (4 g, 50%): IR (film) 3561–2559, 1715 cm^{-1}; ^1H NMR δ 2.25 (2 H, m), 2.60 (3 H, s), 2.78–3.45 (4 H, m), 7.40–7.95 (4 H, m), 8.20 (1 H, s). This acetic acid derivative (1.2 g, 43 mmol) was dissolved in 10% NaOH (20 mL) and refluxed for 1 h. After cooling, ice-cold 1 N HCl was added to give a final pH of 2 and the solution extracted with ethyl acetate (3 × 20 mL). The extracts were dried ($MgSO_4$) and concentrated. Kugelrohr distillation of the crude product yielded 2-cyclobutyl-2-oxoacetic acid (**1l**, 300 mg, 55%): bp 60 °C (0.05 mmHg); IR (film) 3468–2632, 1715 cm^{-1}; ^1H NMR δ

(17) Kunkel, T. A.; Roberts, J. D.; Zakow, R. A. *Methods Enzymol.* **1987**, *154*, 367.
(18) Richards, F. M. *Annu. Rev. Biophys. Bioeng.* **1977**, *6*, 151.

(19) Some recent references in this already large field are as follows: (a) Benkovic, S. J.; Fierke, C. A.; Naylor, A. M. *Science* **1988**, *239*, 1105. (b) Blundell, T.; Carney, D.; Gardner, S.; Hayes, F.; Howlin, B.; Hubbard, T.; Overington, J.; Singh, D. A.; Sibanda, B. L.; Sutcliffe, M. *Eur. J. Biochem.* **1988**, *172*, 513. (c) Graf, L.; Craik, C. S.; Patthy, A.; Roczniak, S.; Fletterick, R. J.; Rutter, W. J. *Biochemistry* **1987**, *26*, 2616. (d) Russel, A. J.; Thomas, P. G.; Fersht, A. R. *J. Mol. Biol.* **1987**, *193*, 803. (e) Wells, J. A.; Cunningham, T. P.; Graycar, T. P.; Estell, D. A. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 5167. (f) Cronin, C. N.; Malcolm, B. A.; Kirsch, J. F. *J. Am. Chem. Soc.* **1987**, *109*, 2222. (g) Gardell, S. J.; Hilvert, D.; Barnett, J.; Kaiser, E. T.; Rutter, W. J. *J. Biol. Chem.* **1987**, *262*, 576. (h) Sampson, S. M.; Chapman, J. L.; Belagaje, R.; Queener, S. W.; Ingolia, T. D. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 5705. (i) Murali, C.; Creaser, E. H. *Protein Eng.* **1986**, *1*, 55.
(20) Gibson, T., Ph.D. Thesis, University of Cambridge, U.K., 1984.

2.00–2.40 (6 H, m), 3.90–4.00 (1 H, m), 7.90 (1 H, s); MS (CI, isobutane) 129.15 (M + 1) [calcd for $C_6H_8O_3$ 128 (M)]. (Elemental analysis was not possible due to the facility with which decarboxylation occurred.) The same procedure was used to prepare **1e,m,n**.

(b) 2-Cyclopentyl-2-oxoacetic Acid (1m). Cyclopentanone (1.25 g, 14.88 mmol) gave 2-cyclopentylidene-2-[(4-methylphenyl)sulfonyl]acetic acid (1.23 g, 28%): IR (film) 3369–2636, 1709 cm^{-1}; ^{1}H NMR δ 1.59–1.90 (4 H, m), 2.58 (3 H, s), 2.60–3.00 (4 H, m), 7.30–8.00 (4 H, m), 10.40 (1 H, s). This acetic acid derivative (1 g, 3.38 mmol) yielded **1m** (200 mg, 40%): mp 47–48 °C (lit.[21] mp 54 °C); IR (film of freshly distilled product) 3442–2632, 1728 cm^{-1}; ^{1}H NMR δ 1.50–2.30 (8 H, m), 3.20–3.85 (1 H, m), 9.45 (1 H, s).

(c) 2-Cyclohexyl-2-oxoacetic Acid (1n). Cyclohexanone (1.25 g, 12.76 mmol) afforded 2-hexylidene-2-[(4-methylphenyl)sulfonyl]acetic acid (2.37 g, 60%): IR (film) 3229–2625, 1742 cm^{-1}; ^{1}H NMR δ 1.50–2.00 (6 H, m), 2.40–3.00 (7 H, m), 7.40–7.90 (4 H, m), 8.80 (1 H, s). This acid (2 g, 6.45 mmol) was converted to **1n** (800 mg, 80%): mp 50–51 °C (lit.[21] mp 50 °C); IR (film of freshly distilled material) 3515–2585, 1722 cm^{-1}; ^{1}H NMR δ 1.40–2.40 (10 H, m), 3.00–3.50 (1 H, m), 8.45 (1 H, s).

(d) 2-Oxoheptanoic Acid (1e). Pentanal (2 g, 23 mmol) yielded 2-[(4-methylphenyl)sulfonyl]-2-heptenoic acid (2.2 g, 32%): IR (film) 3340–2488, 1728 cm^{-1}; ^{1}H NMR δ 0.82 (3 H, t, J = 7 Hz), 1.40–1.80 (6 H, m), 2.50 (3 H, m), 6.75 (1 H, t, J = 7 Hz), 7.20–7.75 (4 H, m), 10.40 (1 H, s). This acid (2 g, 6.71 mmol) gave **1e** (600 mg, 62%): bp 60 °C (1 mmHg) (lit.[22] bp 87 °C, 6 mmHg); IR (film) 3349–2638, 1722 cm^{-1}; ^{1}H NMR δ 0.90 (3 H, t, J = 6 Hz), 1.40–2.00 (6 H, m), 2.90 (2 H, t, J = 7 Hz), 6.95 (1 H, s).

Preparation of Gln102 → Asn Enzyme via Site-Directed Mutagenesis. The BSLDH gene, including its promoter, was excised from the pKK223-3 vector[9a] with *Bam*H1 and cloned into a pTZ-R18 plasmid. This construct was employed for the protein expression in *E. coli* TG2 and production of single-stranded DNA for sequencing (*E. coli* JM101) and site-directed mutagenesis (*E. coli* RZ1032). The Gln102 → Asn mutant was made according to the dut$^-$, ung$^-$ enrichment procedure.[17] Single-stranded DNA was grown in *E. coli* RZ1032 with the f1 helper phage R408[23] but without supplementing the growth medium with uridine. The mutation was made by the primer extension method with the above-described oligonucleotide, the Klenow fragment of DNA polymerase I, and T4 DNA ligase.[17] The in vitro synthesized double-stranded DNA was transfected into competent *E. coli* JM101, and mutants were identified by probing the colonies by hybridization with the ^{32}P-labeled mutagenic oligonucleotide following standard procedures. The coding sequence of the BSLDH gene was checked by dideoxy sequencing with a set of four oligonucleotide primers, equally distributed over the whole gene.

Isolation and Purification of Mutant BSLDH. The mutant LDH gene was expressed in *E. coli* TG2 in YT medium (2×) supplemented with ampicillin (70 μg/mL) for 16 h at 37 °C. Cells were harvested by centrifugation (10 min, 6000*g*), resuspended in triethanolamine hydrochloride (TEA) buffer (20 mM, pH 6.0; buffer A), and lysed by sonication. The cell debris was removed by centrifugation, the supernatant was heated for 60 min at 65 °C, and denatured proteins were eliminated by centrifugation (15 min, 7000*g*). The supernatant was 25% saturated with solid ammonium sulfate and loaded onto a phenyl-Sepharose column (6 mL/100 mL overnight culture) previously equilibrated in buffer A, 25% saturated with ammonium sulfate. A step gradient down to 0% ammonium sulfate saturation in buffer A removed bulk proteins, and the mutant LDH was eluted with a linear gradient of ethylene glycol (0%–50%) in buffer A. Fractions were assayed by standard methods for BSLDH activity at 25 °C[8d] and dialyzed against buffer A containing EDTA (1 mM) and mercaptoethanol (1 mM). The BSLDH protein was then further purified by chromatography on blue Sepharose.[10b] The dialyzed sample was loaded and the column (1 mL of gel/5 mg of protein) washed extensively with buffer A. BSLDH was then eluted with NADH (1 mM) in buffer A, and fractions containing BSLDH were dialyzed against buffer A containing EDTA (1 mM) and mercaptoethanol (1 mM).

Preparation of BSLDH. Native BSLDH was prepared and purified as described earlier[11] (basically as described above for the Gln102 → Asn mutant), except that phosphate buffer was employed instead of buffer A for the hydrophobic chromatography and oxamate–Sepharose instead of blue Sepharose for the affinity purification.

Kinetic Studies. For the kinetic measurements on **1e–g** the pig heart H_4 (PHLDH) enzyme suspension in ammonium sulfate was centrifuged and redissolved in phosphate buffer (100 mM, pH 7.0). Concentrations were determined spectrophotometrically with $A_{280}^{0.1\%,1cm}$ = 1.37 for PHLDH[24] and $A_{280}^{0.1\%,1cm}$ = 0.91 for wild-type and mutant BSLDH.[9d] All kinetic measurements were done at 25 °C and under steady-state conditions. For both native and mutant BSLDH, the measurements were made on solutions in TEA buffer (20 mM, pH 6.0) containing saturating concentrations of FDP (5 mM) and NADH (0.2 mM). PHLDH kinetics were carried out in phosphate buffer (100 mM, pH 7.0) with saturating levels of NADH (0.2 mM). Enzyme concentrations between 5 × 10^{-6} and 5 × 10^{-9} M were used. The reactions were initiated by the addition of substrate solution (3–1000 mM) to give a final concentration between $0.1K_M$ and $3.0K_M$ in a 1-mL cuvette of 1-cm path length. Reactions were monitored by recording the decrease in NADH absorbance at 340 nm. The extinction coefficient for NADH was taken to be 6220 L^{-1} M^{-1} cm^{-1}.[25] Individual data points, and also each kinetic run, were determined in duplicate. A minimum of six substrate concentrations between $0.1K_M$ and $3.0K_M$ were measured. Substrate inhibition was observed for **1b** at concentrations of $>2K_M$. Michaelis constants (K_M) and turnover numbers (k_{cat}) were determined from initial rate measurements at a fixed enzyme concentration by the Lineweaver–Burk[26] method, the correlation coefficients being >0.995 in each case.

Graphics Analyses. Graphics analyses were performed with new, highly refined, X-ray coordinates for the ternary complex of DMLDH–oxamate–NADH[16] with the MIDAS molecular-modeling program,[27] run on a Silicon Graphics Iris 2400 system.

Acknowledgment. This work was supported by a Natural Sciences and Engineering Research Council of Canada (NSERC) Strategic Grant (to J.B.J., J.D.F., and M.G.). The award of a Stiftung für Stipendien auf dem Gebiete der Chemie, Switzerland (to M.A.L.) is gratefully acknowledged. We thank Dr. J. J. Holbrook for the BSLDH gene, Professor M. G. Rossmann for giving us his most recent DMLDH X-ray data prior to publication, Drs. A. R. Clarke and K. Piontek for helpful discussions, and I. S. Graham for his patient assistance in solving some difficult programming problems. The graphics analyses were performed with MIDAS software developed by Professor R. Langridge and licensed from the Regents of the University of California, San Francisco.

(21) Igarashi, M.; Midorikawa, H. *J. Org. Chem.* **1963**, *28*, 3088.
(22) Wermuth, C. G. *Bull. Soc. Chim. Fr.* **1966**, *4*, 1435.
(23) Russel, M.; Kidd, S.; Kelly, M. R. *Gene* **1986**, *45*, 333.
(24) Jeckel, D.; Pfleiderer, G. *Hoppe Seyler's Z. Physiol. Chem.* **1969**, *350*, 903.
(25) Horecker, B. L.; Kornberg, A. *J. Biol. Chem.* **1948**, *175*, 385.
(26) Lineweaver, H.; Burk, D. *J. Am. Chem. Soc.* **1934**, *56*, 658.
(27) Ferrin, T. E.; Huang, C. C.; Jarvis, L. E.; Langridge, R. *J. Mol. Graphics* **1988**, *6(2)*, 13.
(28) Eventhoff, W.; Rossmann, M. G.; Taylor, S. S.; Torff, H. J.; Meyer, H.; Keil, W.; Kiltz, H. H. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 2677.