Improved Specificity toward Substrates with Positively Charged Side Chains by Site-Directed Mutagenesis of the L-Lactate Dehydrogenase of *Bacillus stearothermophilus*†

James K. Hogan,‡ Carlos A. Pittol,§ J. Bryan Jones,*,§ and Marvin Gold*,‡

Department of Molecular and Medical Genetics and Department of Chemistry, University of Toronto, Toronto, Ontario, Canada M5S 1A1

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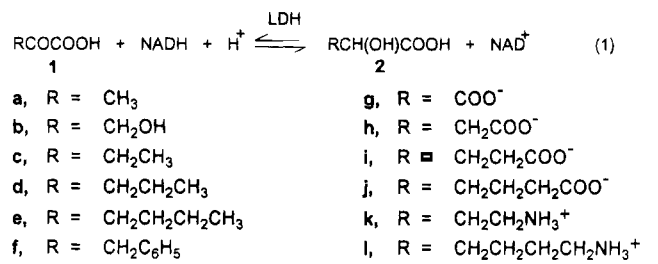
ABSTRACT: The substrate specificities of L-α-hydroxy acid dehydrogenases, including L-lactate dehydrogenases (L-LDH's), can often be quite broad. However, an LDH with high catalytic activity toward α-keto acids with positively charged side chains, such as those containing ammonium groups, has not been described, even though there is evidence from metabolic studies that a natural dehydrogenase with such activity might exist in Nature. L-ω-Amino-α-hydroxy acids are important intermediates in the synthesis of pharmacologically active compounds, and enzymatic reduction of ω-amino-α-keto acids represents an attractive route to these compounds. Graphics analysis indicated that introduction of acidic amino acids at position 102 of the L-LDH of *Bacillus stearothermophilus* (BSLDH) would favor binding of such side chain ammonium groups. Accordingly, Q102E and Q102D mutant BSLDH's were constructed and the steady state kinetic parameters determined for these mutants for a broad range of α-keto acids, including ω-amino-α-keto acids. The results obtained show that, compared to WT-BSLDH, these mutants show up to 25-fold improvements in k_{cat}/K_m values for ω-amino-α-keto acid substrates.

α-Hydroxy acid dehydrogenases, particularly lactate dehydrogenases (LDH's,[1] EC 1.1.1.27), are a class of enzymes that have been widely studied, and whose structures and specificities continue to be of current interest (Wigley *et al.*, 1992; Piontek *et al.*, 1990; LaReau & Anderson, 1989; Goldberg *et al.*, 1994; Lamzin *et al.*, 1994, Sakowicz *et al.*, 1993; Hall *et al.*, 1992; Iwata *et al.*, 1994; Deng *et al.*, 1994; Nobbs *et al.*, 1994). LDH's have also been widely applied as as catalysts for organic synthesis (Kim & Whitesides, 1988). Synthetic interest in LDH's as catalysts is due to the fact that their α-hydroxy acid products are valuable chiral synthetic intermediates (Hanessian, 1983; Mori, 1981; Seebach *et al.*, 1980), and because they are highly stereospecific and accept a relatively broad range of 2-keto acid substrate structures (Kim & Whitesides, 1988). However, there remains a need to extend still further the structural range of substrates that an LDH's active site can accept, and some progress in expanding the structural specificity of the L-LDH from *Bacillus stearothermophilus* (BSLDH) in a controlled manner has been achieved by site-directed mutagenesis (Luyten *et al.*, 1989; Wilks *et al.*, 1990).

L-LDH's are NAD(H)-dependent oxidoreductases that catalyze stereospecific C=O ⇌ CH(OH) transformations of the type shown in eq 1 (Holbrook *et al.*, 1975). The natural, and best, α-keto acid substrate for L-LDH's is pyruvate (**1a**), but reductions of other structurally varied α-keto acids (*e.g.*, **1b**−**i**) have been demonstrated, usually at significantly reduced rates, for a number of L-LDH's, including BSLDH (Bur *et al.*, 1989; Luyten *et al.*, 1989; Kim & Whitesides, 1988; Lane & Dekker, 1969; Hirschbein & Whitesides, 1982; Holbrook & Stinsen, 1973; Meister, 1950, 1952; Eisman *et al.*, 1965; Hawtrey & Goldberg, 1970; Schatz & Segal, 1969).



In addition to the several L-LDH's which generally favor α-keto acid substrates with small substituents (*e.g.*, **1a,b**), the related enzymes L-malate dehydrogenase and L-hydroxyisocaproate dehydrogenase are specific for α-keto acids with negatively charged (*e.g.*, **1h**) or large (*e.g.*, **1e,f**) substituents, respectively (Birktoft *et al.* 1982; Wilks *et al.*, 1988; Schuette *et al.*, 1984). However, L-LDH's accepting ω-amino-α-keto acids such as **1k,l** bearing positively charged substituents at physiological pH's, whose reduction would afford ω-amino-L-α-hydroxy acid products, have not yet been reported in the literature although the existence of a **1l** → **2l** conversion step in one pathway of L-lysine degradation has been predicted from studies using ^{14}C-labeled intermediates (Fowden, 1960). ω-Amino-L-α-hydroxy acids are important intermediates in the synthesis of a number of biologically active compounds, such as the L-$H_2NCH(R)CH(OH)COOH$ of isoserine and the peptide antibiotics ediene and tabutine

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* Correspondence should be addressed to either author.
‡ Department of Molecular and Medical Genetics.
§ Department of Chemistry.

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[1] Abbreviations: LDH, lactate dehydrogenase; BSLDH, *Bacillus stearothermohilus* LDH; WT, wild-type; FBP, fructose 1,6-bisphosphate; MDH, malate dehydrogenase.

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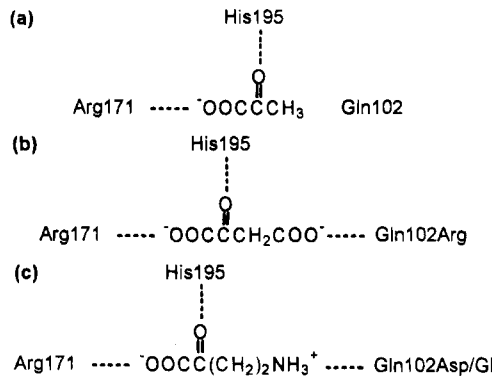


FIGURE 1: Schematic representation (derived from graphics analysis as described by Kallwass *et al.* (1992) using the X-ray data of Wigley *et al.* (1992)) of the relative orientations of (a) the Arg171 and His195 residues of WT-BSLDH responsible for pyruvate COO^- group binding and C=O polarization, respectively, and the Gln102 residue adjacent to the pyruvate methyl side chain in the active complex. In (b) is depicted the orientation for oxaloacetate (**1h**) in the active complex with Q102R-BSLDH. The orientation shown is favored by the electrostatic interaction between the side chain CH_2COO^- and the guanidinium residue of the introduced 102R (Wilks *et al.*, 1988). The situation depicted in (c) is complementary to that of (b), except that the Q102D/E mutation is now biased in favor of binding positively charged ammonium side chains of substrates such as **1k**.

(R = H), of the antitumor agent bestatin (R = benzyl), of the peptide antibiotic amastatin (R = isopropyl), and of the L-γ-amino-α-hydroxybutyric acid inhibitor of GABA uptake that is also a component of the peptide antibiotic butyrosin. Addition of such new specificity properties to the BSLDH arsenal by site-directed mutagenesis would therefore be of considerable synthetic value and would also contribute to an eventual understanding of the factors controlling enzyme–substrate interactions.

BSLDH is an attractive choice for such site-directed mutagenesis and structure–function studies because the BSLDH gene has been cloned and overexpressed in *Escherichia coli* (Barstow *et al.*, 1986) and high resolution X-ray crystal structures are available (Wigley *et al.*, 1992; Piontek *et al.*, 1990). Purification is straightforward because of high levels of expression of active BSLDH in *E. coli* and because the thermostability of BSLDH allows removal of most contaminating *E. coli* proteins by heat denaturation (Luyten *et al.*, 1989).

For WT-BSLDH, orientation of pyruvate as substrate is dominated by the interaction of its COO^- group with the guanidinium residue of R171. In the active complex, pyruvate's methyl side chain is then postulated to sit under the 98–110 loop, whose closure is rate-determining (Dunn *et al.*, 1991), adjacent to the Q102 side chain (Wigley *et al.*, 1992; Piontek *et al.*, 1990), as indicated schematically in Figure 1a. In their elegant studies in 1988, Wilks *et al.* showed that a single, Q102R, amino acid substitution in BSLDH created a mutant enzyme that favored oxaloacetate (**1h**), and also other dicarboxylic acid substrates (Kallwass *et al.*, 1992), over monocarboxylic acids such as pyruvate (**1a**) as a result of the beneficial opportunity provided for the COO^- of the oxaloacetate side chain to interact with the guanidinium group of the introduced 102R residue (Figure 1b). This successful engineering of the active site to accommodate a negatively charged function in a location designed by Nature to best receive a methyl group suggested that the converse effect, that of inducing a favorable environment for positively charged groups, such as the ammonium groups of **1k,l**, could be achieved in a complementary manner, for example, by Q102D/E replacements. Graphics analysis analogous to that carried out on the Q102R mutant (Kallwass *et al.*, 1992) supported the view that for ω-amino-α-keto acid substrates, favorable ammonium-to-side chain COO^- interactions could be expected for Q102D and E mutant BSLDH's (Figure 1c). This paper reports the preparation of both of these mutant enzymes and their catalytic behavior toward the representative γ-keto acid substrates **1a–l**.

EXPERIMENTAL PROCEDURES

Mutagenesis of BSLDH. Site-directed mutagenesis was performed using the Amersham protocol (Sayers *et al.*, 1988) as previously described (Kallwass *et al.*, 1992). The mutagenic oligonucleotide primer used was 5′-pGGCGC-CAACGA(G/C)AAACCAGGCGAGACG-3′ , the underlined bases showing the codon for amino acid position 102 (50% GAG and 50% GAC, giving E and D, respectively). Mutants were identified by dideoxy DNA sequencing (Sanger *et al.*, 1977) of the 102 region, and then the entire BSLDH gene was sequenced to check for inadvertent mutations.

Purification of WT and Mutant BSLDH. Purification of protein was performed as previously described (Kallwass *et al.*, 1992). Briefly, overnight cultures of *E. coli* expressing mutant BSLDH's were centrifuged and pelleted cells resuspended in 50 mM triethanolamine, pH 7.4 (buffer 1), and then disrupted by ultrasonic radiation. Cell debris was removed by centrifugation, and the supernatants were heated to 60 °C for 1 h. After heat treatment and centrifugation, supernatants were dialyzed against buffer 1 at 4 °C overnight. The dialyzed preparation was then loaded onto a Pharmacia MonoQ HR 10/10 anion exchange column on a Pharmacia FPLC system. The column was washed with 25 mL of buffer 1, and then 20 mL of 150 mM NaCl in buffer 1. BSLDH was eluted with a linear gradient of 150–350 mM NaCl in buffer 1 (100 mL), and the column was washed with 1 M NaCl in buffer 1. Fractions containing BSLDH were identified by enzyme assays and confirmed by SDS–PAGE on a Pharmacia Phast system. BSLDH-containing fractions were pooled, and solid ammonium sulfate was added to 20% saturation (at room temperature). This suspension was then filtered and loaded onto a Pharmacia phenyl-Superose HR 5/5 hydrophobic-interaction column. The column was washed with 5 mL of 20% saturated ammonium sulfate in buffer 1, and then BSLDH was eluted with a decreasing linear gradient of 20–0% ammonium sulfate in buffer 1 (100 mL). Fractions containing BSLDH were identified as previously described for the MonoQ chromatography.

Substrates: Starting Materials and Reagents. α-Keto acid substrates **1a–j**, and CBZ-L-lysine, were purchased from Sigma. L-CBZ-α,γ-diaminobutyric acid was prepared from L-α,γ-diaminobutyric acid (Aldrich), with selective reaction at the γ-position being induced via the copper complex (Horiuchi *et al.*, 1976). Purification of L-CBZ-α,γ-diaminobutyric acid was achieved by hydrophobic interaction chromatography (XAD-2 from Aldrich), as follows: L-CBZ-α,γ-diaminobutyric acid (1 g) was dissolved in 100 mL of H_2O and loaded onto 120 g of XAD-2 in a glass column (4.5 × 50 cm). The column was then washed with 500 mL

Table 1: Steady State Kinetic Parameters for WT-, Q102E-, and Q102D-BSLDH-Catalyzed Reductions of **1a−g,i−l**[a]

	k_{cat} (s^{-1})			K_M (mM)			k_{cat}/K_M (M^{-1} s^{-1})		
	WT	Q102E	Q102D	WT	Q102E	Q102D	WT	Q102E	Q102D
1a	188[b]	150 ± 3	50 ± 1	0.04[b]	0.65 ± 0.03	3.5 ± 0.2	4.7×10^6	2.3×10^5	1.4×10^4
1b	125[b]	52.5 ± 1.2	27.2 ± 0.6	0.16[b]	3.2 ± 0.2	11 ± 0.6	7.8×10^5	1.6×10^4	2.5×10^3
1c	155[b]	53.0 ± 1.8	42.0 ± 0.9	0.34[b]	3.2 ± 0.2	5.1 ± 0.3	4.6×10^5	1.7×10^4	8.2×10^3
1d	44[b]	13.6 ± 0.4	16.2 ± 0.6	2.4[b]	6.6 ± 0.6	7.3 ± 0.7	1.8×10^4	2.1×10^3	2.2×10^3
1e	25[b]	23 ± 1	16 ± 1	1.5[b]	4.7 ± 0.1	3.0 ± 0.3	1.7×10^4	4.9×10^3	5.3×10^3
1f	81[b]	46 ± 2	78 ± 3	0.67[b]	1.5 ± 0.2	2.1 ± 0.2	1.2×10^5	3.1×10^4	3.7×10^4
1g	21[c]	28 ± 1	9.5 ± 0.3	1.0[c]	10.2 ± 0.9	11.4 ± 0.6	2.1×10^4	2.8×10^3	8.3×10^2
1i	4.1[c]	3.0 ± 0.1	2.3 ± 0.1	3.9[c]	12.1 ± 0.8	10.9 ± 0.4	1.1×10^3	2.5×10^2	2.1×10^2
1j	5.3 ± 0.2	3.2 ± 0.3	3.6 ± 0.1	1.6 ± 0.1	4.7 ± 0.9	3.0 ± 0.2	3.3×10^3	6.8×10^2	1.2×10^3
1k	0.3 ± 0.03	1.5 ± 0.03	0.6 ± 0.02	38 ± 5	10 ± 0.4	19 ± 1.2	7.9	1.5×10^2	32
1l	0.008 ± 0.0004	0.1 ± 0.008	0.03 ± 0.002	22[d] ± 1.6	11[d] ± 1.8	14[d] ± 1.9	0.36[d]	9.0[d]	2.1[d]

[a] Determined in 20 mM piperazine hydrochloride buffer (pH 6.0), 5 mM FBP, and 0.2 mM NADH, at 25 °C with [S] 0.2−5K_M. [b] From Luyten *et al.*, 1989. [c] From Kallwass *et al.*, 1992. [d] True values of K_m are lower, and values of k_{cat}/K_M correspondingly higher, for all enzymes because of the **1l** ⇌ **3** equilibrium.

of H_2O, the L-CBZ-α-γ-diaminobutyric acid component was eluted with 50% ethanol in H_2O, and the fractions were monitored by ninhydrin staining. The ninhydrin-positive fractions were pooled, and the solvent was removed by rotary evaporation to give pure L-CBZ-α,γ-diaminobutyric acid (800 mg, CBZ-blocked only at the γ-amino position): mp 210−212 °C; $[\alpha]^{21}_D$ 13.4° (*c* 1, 1 M HCl); ^{1}H NMR (200 MHz, D_2O) δ 2.12−2.27 (CH_2CHNH_2COOH), 3.48 (t, 2H, *J* = 7.5 Hz, $CH_2CH_2CH(NH_2)COOH$), 4.13 (t, 1H, *J* = 8 Hz, $CH(NH_2)COOH$), 5.15 (s, 2H, $C_6H_5CH_2$), 7.45 (s, 5H, $C_6H_5CH_2$). HRMS: Calcd for $C_{12}H_{16}N_2O_4$: 253.1188. Found: 253.1191.

Preparation of ω-Amino-α-keto Acids ***1k,l.*** Unpurified venom from *Crotalus adamanteus* was purchased from Sigma (catalog no. A9253). Crude venom (100 mg) was suspended in 20 mM tris(hydroxymethyl)aminomethane (Tris) buffer and dialyzed overnight against this buffer at 4 °C. The suspension was then centrifuged at 5000*g* for 10 min at 4 °C. The supernatant containing active L-amino acid oxidase was then decanted.

*ω-Amino-α-ketobutyric acid (**1k**).* L-CBZ-α,γ-diaminobutyric acid (800 mg, 3.17 mmol) was incubated overnight in 500 mL of 20 mM Tris, pH 7.5, containing 100 mg of L-amino acid oxidase (prepared as described above) at 37 °C with gentle shaking (100 rpm). The mixture was then acidified to pH 2 with 6 M HCl and extracted with ethyl acetate (4 × 100 mL). The ethyl acetate extracts were dried ($MgSO_4$) and then rotary evaporated to give CBZ-**1k** (750 mg). This material was then resuspended in 200 mL of H_2O, the pH adjusted to 7 with 1 M NaOH, and then chromatographed on XAD-2 resin (as described above for L-CBZ-α,γ-diaminobutyric acid). α-Keto acid-containing fractions, identified using BSLDH assays, were combined and lyophilized to give CBZ-**1k** as a free-flowing powder: mp 178−180 °C; IR (KBr) 3334, 1708, 1691, 1647, 1633, 1583, 1543, 1456, 1415, 1384 cm^{-1}; ^{1}H NMR (200 MHz, D_2O) δ 2.95 (t, 2H, *J* = 6 Hz, $CH_2COCOOH$), 3.37 (t, 2H, *J* = 12 Hz, $CH_2CH_2COCOOH$), 5.08 (s, 2H, $C_6H_5CH_2$), 7.39 (s, 5H, $C_6H_5CH_2$). HRMS: Calcd for $C_{12}H_{13}O_5NNa$ (M + H + Na): 274.0691. Found: 274.0682.

The above CBZ-**1k** (250 mg, 0.99 mmol) was suspended in 1 mL of 30% HBr in AcOH. After 1 h, 10 mL of diethyl ether was added and the precipitate obtained filtered off, then washed with diethyl ether (3 × 1 mL) and dried under vacuum to give ω-amino-α-ketobutyric acid (**1k**, 82 mg): mp 144−148 °C (lit. mp 130−140 °C (Chen & Koeppe, 1970)); IR (KBr) 3226, 3087, 2995, 1723, 1594, 1570, 1496, 1463, 1386, 1363, 1309 cm^{-1}; ^{1}H NMR (200 MHz, D_2O) δ 2.13 (t, 2H, *J* = 6 Hz, $CH_2COCOOH$), 3.10 (t, 2H, *J* = 6 Hz, $CH_2CH_2COCOOH$).

*ω-Amino-α-ketocaproate (**1l**).* This was prepared by the procedure described above for **1k**, with CBZ-L-lysine (800 mg, 2.85 mmol) being converted to CBZ-**1l**: mp 99−101 °C; IR (KBr) 3535−2334, 3253, 1950, 1760, 1698, 1674, 1423, 1349 cm^{-1}; ^{1}H NMR (200 MHz, D_2O) δ 1.40−1.66 (m, 4H, $CH_2CH_2CH_2COCOOH$), 2.76 (t, 2H, *J* = 5 Hz, $CH_2CH_2CH_2CH_2COCOOH$), 3.14 (t, 2H, *J* = 5 Hz, $CH_2CH_2CH_2CH_2COCOOH$), 5.11 (s, 2H, $C_6H_5CH_2$), 7.43 (s, 5H, $C_6H_5CH_2$). HRMS: Calcd for $C_{14}H_{16}NO_4$ (M + H − H_2O): 262.1079. Found: 262.1088. Elemental analysis: Calcd for $C_{14}H_{17}NO_5$: C, 60.21; H, 6.14. Found: C, 60.18; H, 6.37.

Treatment of the CBZ-**1l** (250 mg, 0.89 mmol) with HBr in AcOH gave, after Dowex 50 × 8 ion exchange column purification, ω-amino-α-ketocaproate (**1l**, 78 mg, in equilibrium with Δ^1-piperidine-2-carboxylic acid (**3**) (Macholan & Svatek, 1960): mp 176−181 °C; IR (KBr) 3484, 3394, 3294−2072, 1935, 1878, 1754, 1703, 1645, 1437, 1390, 1360, 1335 cm^{-1}; **1l**: ^{1}H NMR (200 MHz, D_2O) δ 1.68−1.82 (m, 4H, $CH_2CH_2CH_2COCOOH$), 2.12−2.24 (m, 2H, $CH_2COCOOH$), 3.00 (t, 2H, *J* = 16 Hz, $CH_2(CH_2)_3COCOOH$). HRMS: Calcd for $C_6H_9NO_2$: 127.0633. Found: 127.0632. **3**: ^{1}H NMR (200 MHz, D_2O) δ 0.60−1.50 (m, 4H, C3- and C4-CH_2), 2.75−2.95 (m, 2H, C2-CH_2), 3.65−4.00 (m, 2H, C5-CH_2).

Kinetic Studies. Steady state kinetic parameters for each of **1a−l** were determined, at least in duplicate, as described previously (Kalwass *et al.*, 1992; Sakowicz *et al.*, 1993) at 25 °C in 20 mM piperazine hydrochloride buffer (pH 6.0) containing 5 mM FBP, and with an NADH concentration (0.2 mM) that was saturating for WT and mutant enzymes. The substrate concentrations were varied within the range 0.2−5K_m, and the kinetic parameters obtained by nonlinear regression analysis using the Grafit program (Erithacus Software Ltd., Staines, U.K.). The kinetic parameters obtained are recorded in Table 1.

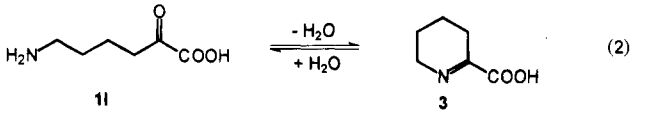
RESULTS

Purifications of BSLDH Q102E and Q102D were achieved using methods previously established for WT-BSLDH. Levels of protein expression were comparable to that for the

WT enzyme, being approximately 30% of soluble cytoplasmic protein. Thermostability was not significantly altered by the mutations, thereby allowing the use of a heat-denaturation step to remove most of the contaminating *E. coli* proteins. The unchanged resistance to thermal denaturation was as anticipated since amino acid residue 102 is in a flexible, active site loop, region and is thus not expected to influence thermostability. In this regard, the previously constructed Q102R and Q102N mutants (Kallwass *et al.*, 1992; Luyten *et al.*, 1989) exhibited WT-like thermostability also.

MonoQ anion exchange chromatography was successful in removing most of the *E. coli* proteins remaining after the heat-denaturation step. Interestingly, both Q102D and Q102E bound to the MonoQ column slightly more strongly than WT, thereby requiring an approximately 5% higher sodium chloride concentration for elution. This is reasonable considering that an additional negative charge has been added at the surface of the mutant proteins. Hydrophobic chromatography on phenylSuperose removed the remaining minor protein and nucleic acid contaminants.

In the preparations of the ω-amino-α-keto acid substrates **1k,l**, the ω-CBZ derivatives of the ω-amino-α-keto acid targets were obtained from the corresponding ω-CBZ-ω,α-diamino acids using the L-amino acid oxidase from *Crotalus adamanteus* venom (Meister, 1952). The ω-amino-α-ketobutyric acid (**1k**) contained a minor impurity (^{1}H NMR δ 3.20) not removable by Dowex chromatography and whose concentration increased on storage in aqueous solution. Accordingly, **1k** was used as soon as feasible after preparation. In aqueous solution, the ω-amino-α-ketocaproate (**1l**) was always in partial equilibrium with the cyclic structure **3** (eq 2), with relative concentrations of **1l** and **3** being pH dependent. ^{1}H NMR analysis, with proton decoupling, showed that at pH 1, at a concentration of 20 mg/mL in D_2O, the ratio of open chain to cyclized forms was approximately 1:1. After the Dowex 50 × 8 purification steps involving acetic acid, the **1k** preparations also contained up to 20% sodium acetate. However, this level of sodium acetate does not detectably affect the BSLDH kinetic parameters, with rates of reaction changed insignificantly (<2%) for WT-BSLDH, Q102E, and Q102D using concentrations of sodium acetate up to 100 mM. This parallels the situations observed for the L-LDH from pig muscle (Saburova *et al.*, 1983).



1l **3** (2)

The kinetic parameters for WT-, Q102E-, and Q102D-BSLDH-catalyzed reductions of the α-ketoacid substrates **1a−g** and **1i−l** are recorded in Table 1. The determination of kinetic parameters for **1h** was not possible due to our inability to separate the "burst phase" reduction of the ever-present, due to facile decarboxylation of **1h**, contaminant pyruvate (**1a**) from that of oxalacetate (**1h**) itself for the mutant-catalyzed reactions (Parker & Holbrook, 1981). This measurement shortcoming could also reflect an inadvertent similarity in k_{cat} and K_m values for pyruvate and oxalacetate with the mutant BSLDH's. Determinations of K_m and k_{cat}/K_m for ϵ-amino-α-ketocaproate (**1l**) were not corrected for the reduction in concentration of **1k** due to partial or full equilibration with **3** (eq 2). Thus the actual K_m for **1l** will be lower, and the true k_{cat}/K_m correspondingly higher, than recorded in Table 1. The determinations of the **1l** parameters were done under identical conditions for WT and mutant BSLDH's, so that comparisons between all the values are valid.

DISCUSSION

As shown in Table 1, the specificity profiles of Q102E and Q102D are quite different from that of WT-BSLDH. As anticipated, kinetic parameters with pyruvate (**1a**) are less favorable for the mutants, with Q102E exhibiting a small (40%) decrease in k_{cat}, and a 16-fold increase in K_m, for pyruvate (**1a**) relative to WT. This significantly reduced binding affinity for pyruvate (**1a**), without a large change in maximal rate of turnover, is consistent with loop closure being relatively unaffected by the Q102E mutation and remaining the rate-determining step. While for Q102D the 5-fold decrease relative to WT in pyruvate's k_{cat} is somewhat larger, the same basic conclusion remains valid since the unfavorable (88-fold) increase in K_m is again proportionally much greater than the effect on k_{cat}. Similar inferences can be drawn for the other small side chain substrates hydroxypyruvate (**1b**) and 2-ketobutyrate (**1c**). Interestingly, as the side chain length increases, as in **1d−f**, the kinetic parameters of the mutant BSLDH's approximate more closely those of the WT enzyme. Kinetic differences between Q102E and Q102D themselves also diminish as the substrate side chain length increases. These trends presumably reflect the fact that the larger α-keto acids are already such relatively poor substrates for WT-BSLDH, an enzyme with an active site optimized for small substrates such as pyruvate (**1a**), that changes to the active site, as in Q102D/E, have little further effect.

A somewhat surprising result was that the dicarboxylic α-keto acids **1g, i−j** were relatively good substrates for the mutant enzymes, with k_{cat}/K_m values ranging from 0.21 to 2.8×10^3 M^{-1} s^{-1}. It might have been expected that, under the pH 6 assay conditions, the new, negatively charged, side chains that 102D/E mutations introduce into the active site would adversely affect the binding of an α-keto acid with a negatively charged COO^- side chain of its own. However, the kinetic parameters are no more affected than those of substrates with uncharged side chains of similar size, such as **1d,e**, whose k_{cat}/K_m's are in the range of 2.1 to 5.3×10^3 M^{-1} s^{-1}. This apparent contradiction is rationalizable in terms of the effects on binding of the ionization state of the side chain carboxylate ($COOH \leftrightarrow COO^-$) of oxalacetate (Parker & Holbrook, 1981), which indicated that it is the uncharged form of the side chain carboxylate that is the preferred structure. It thus appears that the dicarboxylic keto acids **1g,i,j** are also most actively reduced by both WT-BSLDH and Q102E/D mutants when in their uncharged, COOH-state, forms.

With the major goal of this study being the development of an enzyme catalyst favoring reduction of ω-amino-α-keto acids to useful chiral ω-amino-α-hydroxy acid intermediates, the improved specificity of Q102D/E toward **1k,l** is very encouraging. Enhancements of the mutant enzymes's acceptance of ω-amino-α-keto acid substrates are evident in both the increased k_{cat} and decreased K_m values for **1k,l** relative to WT. The magnitudes of the k_{cat} increases (4−

13-fold) are larger than the K_m decreases (2-fold) for the larger of the two substrates, ϵ-amino-α-ketocaproate (**1l**). For the smaller substrate, γ-amino-α-ketobutyrate (**1k**), the k_{cat} and K_m values are increased and decreased, respectively, to the same ~2−5 fold extent and thus contribute equivalently to the amplified activities. The Q102E mutant is a significantly better catalyst than Q102D for both ω-amino-α-keto acid substrates, a result that is in agreement with its generally better activity with most of the Table 1 substrates. Interestingly, the increase in k_{cat}/K_m for Q102E compared to WT is 20−25-fold for both γ-amino-α-ketobutyrate (**1k**) and ϵ-amino-α-ketocaproate (**1l**) although the absolute values of k_{cat}/K_m for **1l** are substantially lower, by 15−17-fold, than for both Q102D/E with **1k** as substrate. However, the reduced concentration of **1l** due to its interconversion with Δ^1-piperidine-2-carboxylic acid (**3**) undoubtedly reduces the actual k_{cat}/K_m values for **1k** dramatically, and the true kinetic parameters for **1k,l** are undoubtedly much closer in magnitude than Table 1 indicates.

The Q102E substitution is the more conservative in terms of matching sizes of the respective amino acid side chains and seems to be better tolerated by the enzyme than the Q102D mutation. The smaller (**1k**) of the two ω-amino-α-keto acids **1k,l** is the better substrate, as one would anticipate for an active site optimized for reduction of small α-keto acids. The dominance of the k_{cat} increase (18-fold) relative to the K_m decrease (2-fold) on k_{cat}/K_m, relative to WT, for Q102E-catalyzed reduction of ϵ-amino-α-ketocaproate (**1l**) points to the rate-determining active site loop closure being facilitated for this mutant−substrate combination.

Although the Q102E substitution introduces a significant improvement in activity toward ω-amino-α-keto acid substrates relative to WT-BSLDH, the absolute value of k_{cat}/K_m (1.5×10^2 M^{-1} s^{-1}) for γ-amino-α-ketobutyrate (**1k**) is still very low compared to the WT value of 4.7×10^6 M^{-1} s^{-1} for pyruvate (**1a**). In contrast, for the Q102R mutant, the k_{cat}/K_m of 1.1×10^7 M^{-1} s^{-1} for oxalacetate (**1h**) is very similar to that of WT-BSLDH with pyruvate (**1a**) (Kallwass *et al.*, 1992). The new substrate COO^--to-Arg interaction possible with the Q102R mutant is the simplest interpretation of the dramatic alteration of specificity brought about by the single Q102R amino acid substitution. However, this approach will usually be too simplistic to be applied generally when new charge-interactions are involved in ES complexes. In the Q102R-**1h** situation, the straightforward ion-pairing design basis applied (Wilks *et al.*, 1988) for improving substrate side chain−COO^- binding was reasonable because of the close evolutionary relationship between L-MDH and L-LDH (Birktoft *et al.*, 1982). Consequently, introduction of 102R created an environment in BSLDH matching that already known to be favorable in L-MDH (Wilks *et al.*, 1988; Birktoft *et* al., 1982). On the other hand, the opposite type of polarity pairing envisaged for a Q102E−ω-amino-α-keto acid complex need not necessarily be as favorable. In fact, it has been suggested to be problematical on the basis of molecular modeling (Clarke *et al.*, 1991). However, the present and previous experimental results demonstrate that the ion-pairing strategy is fundamentally sound in both +/− and −/+ directions. A further consideration in the present cases may also be that the R109-to-substrate carbonyl interaction (Wigley *et al.*, 1992), that facilitates the hydride-transfer step in WT-catalyzed reductions, may be disturbed by formation of a new salt bridge between R109 and the additional active site carboxylate functions of Q102D/E. Such an interaction would be expected to reduce k_{cat} values for normal, uncharged α-keto acid substrates, as is observed. On the other hand, with the amino keto acid substrates, the interaction between the substrate ammonium group and the Q102D/E carboxylates could permit the normal R109 substrate C=O polarization to be reestablished, thereby raising k_{cat}'s relative to those of the corresponding WT reductions of **1k,l**, in accord with the experimental results (Table 1).

Further support of the validity of the ion-pairing approach to tailoring specificity is the analogous approach taken with aspartate aminotransferase, for which an R292D mutation achieved k_{cat}/K_m increases of 6−9-fold, relative to WT, for diamino acid substrates such as L-arginine, L-lysine, and L-ornithine (Cronin & Kirsch, 1988). These levels of improvement match closely the 6−25-fold improvements in k_{cat}/K_m for α-amino-α-keto acids achieved with the BSLDH Q102D and Q102E mutations. Moreover, the two enzymes parallel each other in that molecular modeling has suggested that the 292D−diamino acid ion pair in aspartate aminotransferase is also not stabilized by a prepolarized active site environment (Hwang & Warshel, 1988). Furthermore, it is interesting to note that there are several known aminotransferases that have activity toward diamino acids (EC 2.6.1.*n*; Webb, 1984), indicating that there may be a more favorable location for a negative charge in the active site of these enzymes. In this regard, for the current mutant BSLDH's, an additional site-directed introduction of a basic amino acid residue capable of neutralizing (Wilks *et al.*, 1988; Gelpi *et al.*, 1993) the overall extra negative charge of the Q102D/E could also be beneficial. Preparative applications of the Q102D/E mutant enzymes for the general synthesis of ω-amino-L-α-hydroxy acids are now being explored.

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