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The Substrate Specificity of the Muscle L-Lactate Dehydrogenase of Spiny Dogfish (*Squalus acanthis*)

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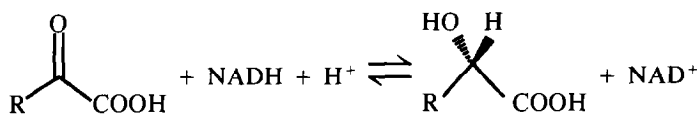
The substrate specificity of the L-lactate dehydrogenase (L-LDH) from the muscle of the spiny dogfish (DMLDH) was evaluated for 13 structurally varied α-ketoacid substrates and the results were compared with the specificity data for the L-LDH from *Bacillus stearothermophilus* (BSLDH), the latter being the most valuable L-LDH for preparative-scale production of chiral α-hydroxy acid synthons of asymmetric synthetic value. DMLDH has a significantly narrower specificity than BSLDH, and also than heart LDHs. Reasons for its narrower specificity, based on X-ray data analyses that provide new insights into the factors determining the specificities of L-LDHs are advanced. © 1992 Academic Press, Inc.

INTRODUCTION

Lactate dehydrogenases (LDHs) catalyze C=O to CH(OH) transformations of the type shown below. Both L- and D-LDHs are available, with the L-enzymes being the best documented.



1 **2**

a, R = CH_3-
b, R = $HOCH_2$-
c, R = CH_3CH_2-
d, R = $CH_3(CH_2)_2$-
e, R = $CH_3(CH_2)_3$-
f, R = $CH_3(CH_2)_5$-
g, R = $(CH_3)_2CH$-
h, R = $(CH_3)_2CH_2CH$-
i, R = C_6H_5-
j, R = $C_6H_5CH_2$-
k, R = HOOC-
l, R = $HOOCCH_2$-
m, R = $HOOC(CH_2)_2$-

The natural ketoacid substrate for all L-enzymes is pyruvate (**1a**), but reductions of other structurally varied 2-ketoacids to the corresponding 2-hydroxyacids have

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been reported for the L-LDHs from *Bacillus stearothermophilus* (BSLDH (*1, 2*)), rabbit muscle (*3–5*), porcine heart (*1, 3, 6*), bovine heart (*3, 7–9*), chicken liver (*3*), lobster tail (*3*), and mouse testes (*10, 11*). All such L-LDH-catalyzed reductions are stereospecific and several have been exploited to produce α-hydroxy acid synthons of asymmetric synthetic value (*1, 3*).

The first purification of an L-LDH (*12*) that resulted in crystals suitable for high resolution X-ray crystallography was that from the muscle of spiny dogfish, *Squalus acanthis* (DMLDH (*13, 14*)). Although X-ray structures have subsequently been determined for L-LDHs from pig heart (*15*), mouse testes (*16*) and *B. stearothermophilus* (*17*), the most accurate LDH-structural data currently available remains that for the dogfish enzyme. The definitive structure is that of the ternary DMLDH–NAD–oxamate complex (*18*). This has been widely employed as a reference structure for computer graphics analyses upon which mechanistic and site-directed mutagenesis studies have been based. Despite this keystone position in the field it is surprising that, in the nearly 30 years since its isolation, the substrate specificity of DMLDH has not been examined. To our knowledge, it has been assayed only with pyruvate. We have therefore purified DMLDH and evaluated its specificity toward the representative structural range of α-keto acids **1a–1m** and compared its specificity with those of L-LDHs that are of asymmetric synthetic value.

MATERIALS AND METHODS

Whole frozen fresh dogfish was obtained from Carolina Biological Supply Co. BSLDH was prepared as described previously (*2*). The α-keto acids (**1a–1m**) and L- and D-lactate substrates were purchased from Sigma. Fructose -1,6-diphosphate, NAD+, and NADH were from Boerhinger Mannheim. Oxamate–Sepharose was prepared by the method of Schär *et al.* (*19*). Purified DMLDH was analyzed by SDS–polyacrylamide gel electrophoresis and silver staining, and by amino acid analysis by the analytical facility of the Department of Biochemistry, University of Toronto. DMLDH concentration was determined using the Bradford method (*20*) using a standard curve constructed from the concentration determined by amino acid analysis.

Purification of DMLDH

The purification procedure of Pesce *et al.* (*12*) was modified and combined with affinity chromatography. Approximately 500 g of frozen dogfish muscle was ground up in a meat grinder and diluted to a 1-liter volume with distilled water. To achieve more complete homogenization, the tissue was further disrupted with a Polytron (Brinkman Instruments). Most of the fat still present in the preparation was removed by straining twice through two layers of cheesecloth. Subsequently, solid ammonium sulfate was added to the suspension to a saturation of 45% and the resulting precipitate was removed by centrifugation at 10,000 rpm, for 1 h. The supernatant was then taken to 70% saturation with solid ammonium sulfate. After

centrifugation of the mixture at 10,000 rpm for 1 h, the resulting precipitate was dissolved in 90 ml of buffer 1 (50 mM piperazine–HCl pH 6.0, containing 0.05% sodium azide) and dialyzed against the same buffer for 1.5 h. To 35 ml of this dialyzed solution was added NADH to a final concentration of 750 μM (buffer 2), and the resulting solution was loaded on an oxamate-Sepharose column (1.6 × 11 cm). The column was washed with 60 ml of buffer 2 and the DMLDH was eluted with triethanolamine–HCl (20 mM, pH 8.5, buffer 3). DMLDH eluted as a single peak immediately after buffer 3 had passed through the entire column. This purification yielded 96% recovery based on specific activity determinations of crude and purified preparations.

Kinetic Assays

The kinetic assays for the DMLDH-catalyzed reductions of the α-keto acids **1a–1m** were performed in piperazine–HCl buffer (20 mM, pH 6.0, buffer 4) that was 0.2 mM in NADH. The DMLDH-catalyzed oxidations of L- and D-lactate (**2a**) were performed in triethanolamine–HCl buffer (100 mM, pH 8.5) that was 10 mM in NAD^+. The comparison kinetic assays for BSLDH were done as for DMLDH except that the assay solutions contained the allosteric activator fructose 1,6,-diphosphate (5 mM). In each case, the assays were initiated by the addition of the substrate. The initial rates were obtained by recording the changes in absorbance at 340 nm during the first minute using a Pharmacia 4054 Ultraspec Plus UV/visible spectrophotometer equipped with a 1-cm-path-length cell. An NADH absorption coefficient of 6.3×10^3 liters $\cdot$ $mol^{-1} \cdot cm^{-1}$ was used to calculate the change in NADH concentration. Kinetic parameters were calculated using nonlinear regression with the computer program "GraFit" from Erithacus Software (Staines, U. K.). Assays were performed in duplicate, with errors of $\pm$ 10% for K_M and $\pm$ 25% for k_{cat} based on calibrations of the measuring equipment used, the method of protein determination, and the standard errors supplied by the nonlinear regression calculations. The results are recorded in Table 1.

Graphics Analyses

The specificity data of Table 1 were analyzed by examining the possible ES-complexes of the substrates with DMLDH–NADH, using the ternary DMLDH–NAD–oxamate structure (Brookhaven PDB file 1LDM) as the basis structure. Comparisons with BSLDH were based on BSLDH–NAD–oxamate X-ray structure coordinates received from D.B. Wigley. (*21*) The modeling was performed on a Silicon Graphics Personal Iris work station using Insight II software (BioSym Technologies, San Diego, CA).

RESULTS AND DISCUSSION

The purification procedure yielded approximately 50 mg of DMLDH that was homogeneous according to silver-staining of samples analyzed by SDS–polyacrylamide gel electrophoresis. The gels showed a band of the appropriate molecular

TABLE 1

Kinetic Parameters for DMLDH-Catalyzed Reductions of α-Keto-Acids (**1a–1m**) and Oxidation of L-Lactic Acid (**2a**)[a]

	RCOCOOH, R =	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (M^{-1} s^{-1})
1a	CH_3-	760 (250)[b]	0.085 (0.04)	8.9×10^6 (6.3×10^6)
1b	$HOCH_2$-	660 (125)	0.14 (0.16)	4.7×10^6 (7.8×10^5)
1c	CH_3CH_2-	59 (155)	1.5 (0.34)	3.9×10^4 (4.6×10^5)
1d	$CH_3(CH_2)_2$-	4.4 (44)	2.5 (2.4)	1.8×10^3 (1.9×10^4)
1e	$CH_3(CH_2)_3$-	5.4 (25)	1.5 (1.5)	3.6×10^3 (1.7×10^4)
1f	$CH_3(CH_2)_5$-	0.092 (32)	0.83 (0.35)	1.1×10^2 (9.1×10^4)
1g	$(CH_3)_2CH$-	0.22 (0.49)	5.8 (17)	3.8×10^1 (2.8×10^1)
1h	$(CH_3)_2CH_2CH$-	~0[c] (0.51)	— (4.9)	— (1.0×10^2)
1i	C_6H_5-	~0[c] (0.96)	— (14)	— (6.3×10^1)
1j	$C_6H_5CH_2$-	0.27 (81)	1.5 (0.67)	1.8×10^2 (1.2×10^5)
1k	HOOC-	4.8 (21)	8.5 (0.99)	5.6×10^2 (2.1×10^4)
1l	$HOOCCH_2$-[d]	1.5 (6.0)	1.6 (1.5)	5.2×10^3 (4.0×10^3)
1m	$HOOC(CH_2)_2$-	0.15 (4.1)	2.3 (3.9)	6.5×10^1 (1.1×10^3)
2a	L-Lactate (R = CH_3)	99 (9)	7.4 (40)	1.4×10^4 (2.3×10^2)
	D-Lactate (R = CH_3)	~0[c] (~0[c])	— (—)	— (—)

[a] Reductions determined at 25°C, in 20 mM piperazine–HCl, pH 6.0, that was 0.2 mM in NADH. Oxidations determined at 25°C, in 100 mM triethanolamine–HCl, pH 8.5, that was 10 mM in NAD^+.

[b] For comparison purposes, the values (Refs. (*1, 2*)) for BSLDH-catalyzed reductions (**1a–1m**) and oxidation (**2a**) are included in parentheses.

[c] Undetectable activity. Lower limit for detection of activity is 0.5–1 μmol of α-ketoacid reduced (or α-hydroxyacid oxidized) per minute.

[d] The effect of pyruvate contamination due to decarboxylation of oxalacetate was eliminated by the method of Parker *et al.* (*21*).

weight (36,000 Da) without any contaminating bands visible. The determination of amino acid composition of the purified enzyme matched closely that predicted from the primary sequence of the enzyme (*23*). The activity of the preparation decreased significantly after storage for several days at 4°C in piperazine–HCl buffer of pH 6.0. In contrast, no significant loss of activity was detected for saturated ammonium sulfate suspensions that were stored for up to 2 months.

The kinetic constants for the DMLDH-catalyzed reductions of **1a–1m** are shown in Table 1. Kinetic parameters for DMLDH–pyruvate determined by Pesce *et al.* (*12*) are: K_M = 0.33 mM, with a maximal activity of 454 s^{-1} at 0.2 mM pyruvate. These deviations from the Table 1 values are considered to be due to the differences in assay conditions, primarily the lower pH used in our experiments (pH 6.0 vs 7.5). Also, Pesce *et al.* (*12*) used a definition of turnover number as maximal activity which, as a result of substrate inhibition, is significantly lower than the k_{cat} determined in this study by nonlinear regression analysis.

The substrate specificity of DMLDH is seen to be much narrower than that of BSLDH (Fig. 1). DMLDH's relative activity decreases dramatically as the size of the substituent on the α-keto acid is increased. For example, DMLDH-catalyzed

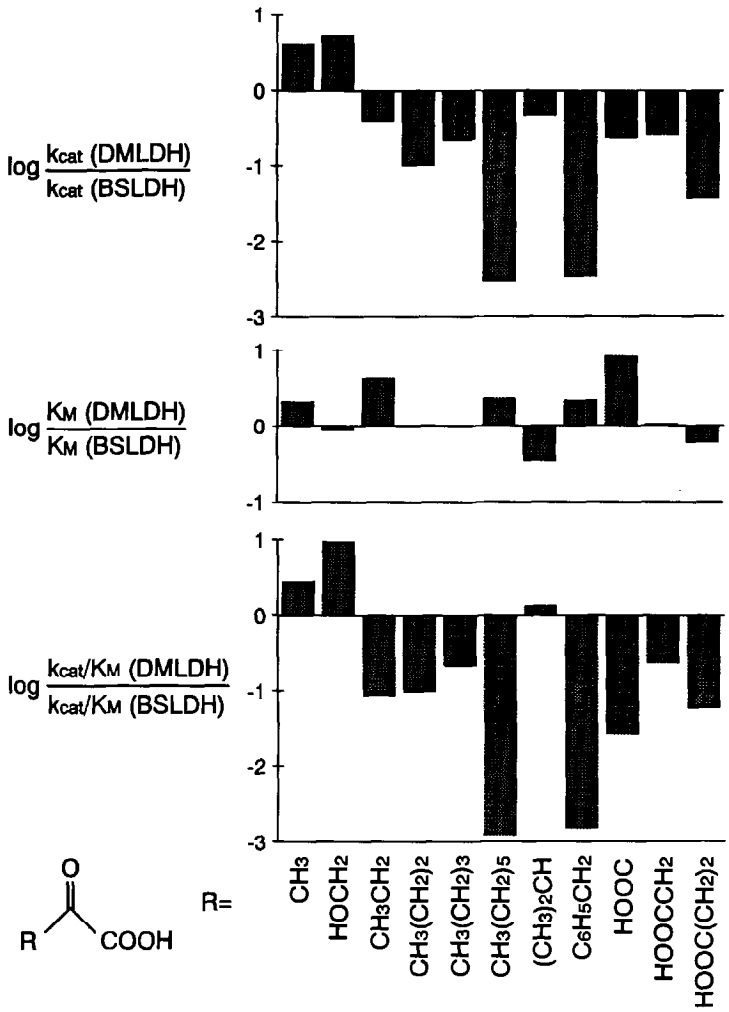

FIG. 1. Comparative ratios of the kinetic parameters for DMLDH and BSLDH with substrates **1a–1m**.

reductions of ketooctanoate (**1f**) and phenylpyruvate (**1j**) exhibit approximately 100,000-fold reductions in k_{cat}/K_M compared to pyruvate, but only 50-fold reductions for the corresponding BSLDH-catalyzed reductions. The K_M values for the various substrates are similar for DMLDH and BSLDH so that the differences in overall activity manifest for the larger substrates are primarily due to decreased k_{cat} values with DMLDH as the catalyst. However, for small substrates the turnover numbers with DMLDH are significantly greater than with BSLDH (Table 1). DMLDH was also tested for its ability to catalyze the oxidation direction, using L-lactate as the substrate. The results (Table 1) show that, as expected, L-lactate is a good substrate for the enzyme but that the D-enantiomer is not, thus confirming

the strict L-stereospecificity of the enzyme. The higher activity observed for reduction of small ketoacid substrates with DMLDH is also apparent for the oxidation of L-lactate, with k_{cat} being 10-fold greater than with BSLDH (Table 1).

Hydroxypyruvate (**1b**) exhibits a very high DMLDH activity, almost equivalent to that of pyruvate. Again this activity is much higher than that observed with BSLDH. Substrates such as **1c–1e** with moderately sized side chains are reasonably well tolerated by DMLDH, with only a 10-fold decrease in k_{cat}/K_M levels relative to BSLDH. Surprisingly, ketoisovalerate (**1g**) is as good a substrate for DMLDH as for BSLDH. However, the structurally similar compound ketoisocaproate (**1h**), which differs from **1g** only by an extra methylene group, is such a poor DMLDH-substrate that no reduction could be detected. A similar result is apparent from comparisons of the oxalacetate (**1l**) and ketoglutarate (**1m**) data. An interesting exception to this trend is the very poor DMLDH-activity of ketomalonate (**1k**), the smallest of the dicarboxylic acids (**1k–1m**) assayed. This may indicate that the presence in a substrate of a planar, sp^2, C-3 carbon atom is deleterious since benzoylformate (**1i**), in which C-3 is similarly planar, is virtually a nonsubstrate. These effects, and the degree to which the specificity of DMLDH is narrower than that of BSLDH is clearly evident in the Fig. 1 comparison of the kinetic constants for the two enzymes.

Reasons for the differences in substrate activities of DMLDH for the series **1a–1m** were sought using computer graphics analyses of the possible active site binding modes for each substrate.The analyses, which provide new insights into the factors determining the structural specificities of L-LDHs, show that DMLDH has a generally more constrained active site than BSLDH. In particular, arginine 101, and to some extent tyrosine 245, residues in DMLDH block an active site crevice that could prevent the accommodation of longer side chains (Fig. 2). The corresponding residues in BSLDH are glutamine (101) and alanine (245), which are smaller and therefore do not block the crevice to the same extent. This analysis is also consistent with the higher activity of BSLDH with longer substrates. Pig heart LDH, whose sequence is identical to DMLDH at positions 101 and 245, also has very poor activity with longer substrates such as **1f** and **1j.** Interestingly, Arg 101 also appears to interact with the pyrophosphate moiety of NADH (not shown). This contact could be partly responsible for the higher pyruvate turnover number for DMLDH compared to BSLDH since such an additional electrostatic interaction could accelerate the rate-limiting loop closure step.

However, the importance of Arg 101 as a specificity determinant is called into question by the broad specificity of LDH-X, which also has arginine at position 101. While this uncertainty cannot be settled from X-ray data since no ternary complex structure is available for LDH-X, it is likely that the numerous amino acid changes in the active site loop region of LDH-X drastically alter the loop conformation, thereby changing the position of Arg. 101. Thus it is possible that Arg 101 in LDH-X neither blocks the active site crevice nor interacts with NADH, as it appears to in DMLDH. Indeed, the apoenzyme crystal structure of LDH-X shows the loop closed over the active site, whereas all other L-LDH apoenzyme structures show the loop extended into solution. This different loop conformation has also been suggested as a reason for the lower k_{cat} observed with LDH-Xs (*24*).

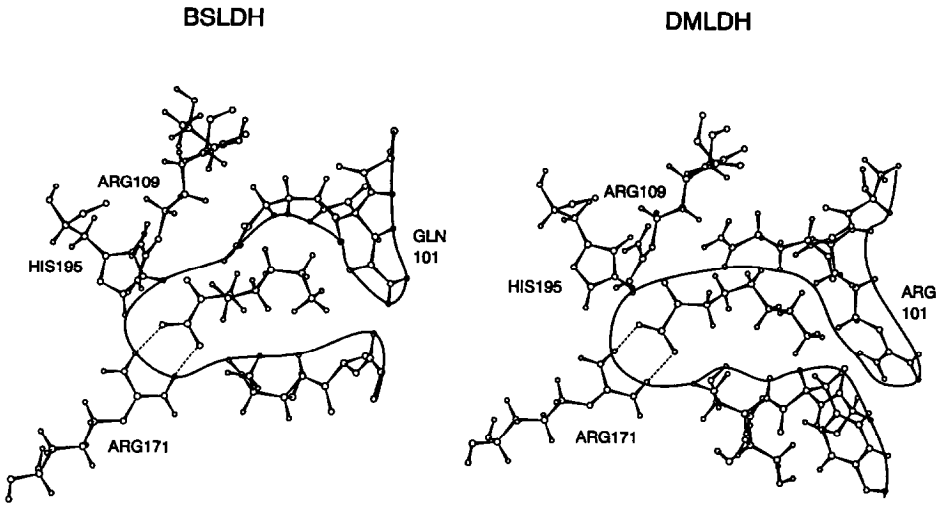

FIG. 2. Comparison of BSLDH and DMLDH active sites. Ketooctanoate (**1f**) is shown modeled into the active sites of each enzyme with the C-1 carboxylate hydrogen bonded to arginine 171. NADH, which would be located directly behind the ketooctanoate in this orientation, has been omitted for clarity.

Structural reasons for the poor tolerance of **1i** and **1k** are not clear. The active sites of BSLDH and DMLDH are very similar in the C-3 region of pyruvate (inferred from the position of NH_2 of oxamate), with equivalent residues in the two enzymes being within 1 Å of each other. The anomalously good relative activity for ketoisovalerate (**1g**) could possibly be due to its greater conformational flexibility at C-3 compared to benzoylformate (**1i**) or ketovalerate (**1k**).

An examination of the LDH literature reveals that, relative to the corresponding heart LDHs, the muscle isozymes of vertebrate L-LDHs typically have higher turnover numbers and weaker affinities for the NAD/H cofactor, are subject to less severe substrate inhibition, and show less tolerance for larger ketoacid substrates (*25*). The results presented here agree with this general trend, with DMLDH showing similarities in activity profiles to those of other muscle LDH isozymes (*7, 17, 18*). However, the dogfish L-LDH is significantly more active toward pyruvate than some of the other muscle enzymes. This may be a result of metabolic adaptation since the specific pyruvate activity of the L-LDH from the related cartilaginous fish, *Raja erinacea* (infraclass elasmobranchii), is also considerably higher than for other L-LDHs (*26*).

The present data provide an interesting additional perspective into the specificities of the L-hydroxyacid dehydrogenases in general, which are a family of enzymes that have very similar amino acid sequences (*27*) and three-dimensional structures (*28*). They include L-malate (L-MDH), L-lactate (L-LDH), and L-hydroxyisocaproate (L-HicDH) dehydrogenases. MDH is the most specific, reducing only **1k** and **1l** (*29*). DMLDH, as reported above, is also highly specific, having generally low tolerance for larger substrates. L-HicDH is specific for large 2-ketoacid sub-

strates, with relatively lower activity with pyruvate (*30*). PHLDH and BSLDH have significantly better activities with larger ketoacid substrates than DMLDH (*2*). LDH-X is the only truly "broad" specificity L-hydroxyacid dehydrogenase as it seems to show good activities with all substrates tested (*31*). A strong correlation is evident between maximal activity toward the best substrate and tolerance of varied substrate structure. The narrow specificity enzymes, MDH and DMLDH, have the highest activities with their best substrates. The more substrate-structure tolerant PHLDH, BSLDH, and L-HicDH are of intermediate best-substrate activity, and the broadest specificity enzyme, LDH-X, has the lowest activity of any of the L-hydroxyacid dehydrogenases. In addition to clarifying the nature of the specificity determinants in this series of enzymes, the present data also demonstrate that in the development of the truly "broad" specificity LDHs, of most value in asymmetric synthesis, a price may have to be paid in terms of the maximal activity achievable.

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