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On the Effect on Specificity of Thr246→Gly Mutation in L-Lactate Dehydrogenase of Bacillus stearothermophilus

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The function of the amino acid Thr246 in L-lactate dehydrogenase from Bacillus stearothermophilus has been investigated by site-directed replacement with glycine. Kinetic experiments with a number of 2-oxo acids showed strongly reduced activity for the mutated enzyme. However, the mutant enzyme shows a relative preference for the large hydropohobic sidechains of α-keto acids and an even higher specific activity than the wild-type lactate dehydrogenase for the polar oxaloacetate substrate. Graphics analyses indicate that the loss of one hydrogen bond, or intrusion of water into the active site, might be responsible for the reduced activity. The kinetic results suggest that the binding modes of bulky hydrophobic or polar substrates compensate to some degree for the partially disrupted active site.

The increasing application of enzymes as highly stereoselective catalysts in organic synthesis(1) is sometimes restricted by a narrow substrate specificity of some enyzmes. However, the techniques of protein engineering open up possibilities of expanding substrate specificity, and of evaluating and altering the factors controlling substrate accomodation.

Lactate dehydrogenase (LDH) is an ubiquitous oxidoreductase (L-LDH, E.C. 1.1.1.27) that in vivo catalyses the NADH-linked interconversion of pyruvate (1a) and lactate (2a) (eq.1).

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Abbreviations: LDH, lactate dehydrogenase; DMLDH, dogfish muscle LDH; BSLDH, Bacillus stearothermophilus LDH; NADH, nicotine adenosine dinucleotide (reduced form); FDP, fructose-1,6-diphospohate; PHLDH, pig heart LDH.

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$$H^+ + \underset{1}{RCOCOOH} + NADH \xrightarrow{BSLDH} \underset{2}{RCH(OH)COOH} + NADH \quad \text{(Equation 1)}$$

a: R = CH_3 **e**: R = $(CH_2)_4CH_3$
b: R = CH_2CH_3 **f**: R = $(CH_2)_5CH_3$
c: R = $(CH_2)_2CH_3$ **g**: R = $CH_2C_6H_5$
d: R = $(CH_2)_3CH_3$ **h**: R = CH_2COO^-

In addition to the natural substrates **1a**,**2a**, LDH's from different sources can catalyse the sterospecific conversion of a variety of achiral 2-oxo acids, such as **1b-h**, to the corresponding L-hydroxy acids, which are interesting synthons in organic synthesis(2). The L-LDH from Bacillus stearothermophilus (BSLDH) had been shown to be a very effective catalyst in such preparative scale reactions(3). It is a very stable(4), readily available(5,6) enzyme that exhibits a strict L-stereospecificity(3).

A number of mutants of BSLDH have already been made in order to explore aspects of substrate binding(7,8), to study the binding site of the allosteric activator fructose-1,6-diphosphate (FDP)(9) and to investigate the influence of various amino acids on the catalysis steps(10) and on the heat stability(11). In an attempt to expand further the substrate specificity of BSLDH, we have replaced the active site residue Thr246 by the smaller amino acid glycine by site-directed mutagenesis. The present paper reports the effect of this Thr246→Gly mutation.

Materials and Methods

The site-directed Thr246→Gly mutation was introduced into the LDH gene from Bacillus stearothermophilus using methods described previously (10a). The mutant gene was expressed in the pKK223-3 vector(5) and the mutant protein purified by two different methods: heat denaturation of E. coli proteins, followed by hydrophobic interaction chromatography on phenyl Sepharose CL-4B and purification on blue Sepharose(8b), or, chromatography on blue Sepharose and DEAE CL-6B Sepharose(7a). Reaction rates were determined under steady state conditions by monitoring the change in A_{340} due to the consumption of NADH (for details see Table 1).

Results

The two different purification procedures gave mutant enyzmes with effectively equivalent catalytic properties. The heat treatment step ensured that there was no contaminating host-LDH from E. coli. The Thr246 →Gly enzyme itself remained stable up to 65°C. Disappointingly, the Thr246→Gly mutation lowered the catalytic performance of BSLDH, as shown in Table 1, with the specificity constant, k_{cat}/K_M, being reduced by 1000-fold relative to that for the wild-type enzyme. Both the turnover number, k_{cat}, and the Michaelis constant, K_M, were equally affected. For the unbranched

Table I. Kinetic Parameters for Thr246→Gly Catalyzed Reductions of **1a** - **1h** [a,b]

Substrate	k_{cat} [s^{-1}]		K_M [mM]		k_{cat}/K_M [$M^{-1}xs^{-1}$]	
	T246	G246	T246	G246	T246	G246
1a	188	6.6	0.04	1.9	4.7E6	3.5E3
1b	155	0.5	0.34	5.7	4.6E5	8.9E1
1c	44	0.4	2.4	18	1.8E4	2.2E1
1d	25	0.8	1.5	30	1.7E4	2.7E1
1e	23	2.2	0.53	41	4.3E4	5.3E1
1f	32	4.1	0.35	19	9.1E4	2.1E2
1g	81	0.7	0.67	16	1.2E5	4.3E1
1h	6	0.9	1.5	0.2	4.0E3	4.7E3

[a] values determined under steady state conditons by monitoring the decrease in NADH adsorbance at 340 nm and 25°C, in 20mM triethanolamine-HCl buffer pH = 6.0, $[NADH]_b$= 0.2 mM, [FBP] = 5.0 mM, [E] = 0.7 - 5.0 μM; error values were ≤10%. [b] values for the wild-type enzyme are from ref. (9a). [c] values from ref.(7c)

aliphatic substrates **1a-f**, the k_{cat}-values were lowered by factors of 8-310 fold, with the smaller substrates (**1a** and **1b**) exhibiting the greatest k_{cat}-response. The K_M's were increased in all cases, with those of **1c-f** the most affected. A similar catalytic behaviour was observed for the aromatic substrate **1g**. On the other hand, the most polar substrate, oxaloacetate (**1h**), showed kinetic constants that were comparable for both the wild-type and mutant enzyme. In fact, the specificity constant (k_{cat}/K_M) for **1h** with the Thr246→Gly mutant was slightly higher than for the natural substrate pyruvate (**1a**).

Discussion

Residue 246 is close to a tight turn (aa 235-238) between two helices that, together with the mobile coenzyme loop (aa 99-110), shields the active site, with it's bound substrate and the nicotinamide moiety of NADH, from the solvent. From graphics analyses of the X-ray structures of PHLDH(12) and DMLDH(13) it appeared likely that the restriction of active site volume imposed by the Thr246 residue might be responsible for the specificity preference for α-keto acid substrates with small sidechains. Thr246 was therefore replaced by glycine in order to enlarge the space available for substrate sidechain accommodation.

Disappointingly, the Thr246→Gly mutation did not achieve the desired expansion of specificity, and the activity of the enzyme was significantly lowered relative to wild-type, with both k_{cat} and K_M being adversely affected for each of the substrates **1a-g** (Table 1). In our planning with Thr246→Gly mutation could be used to acommodate larger hydrophobic groups of α-keto acids **1e-g**. However, the kinetic data do not indicate that this occurs to a significant extent. On the other hand, when the side-chain of

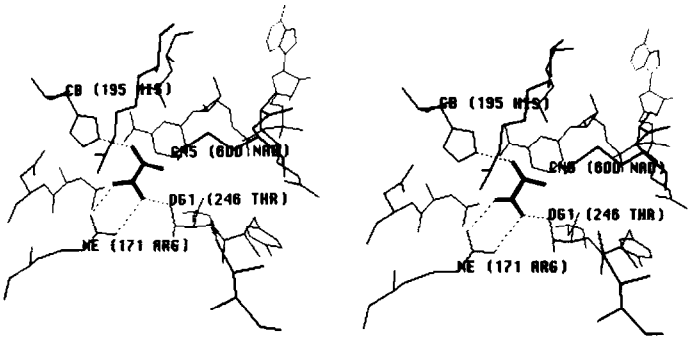

Figure 1. Graphics analysis of the mutant 246->Gly BSLDH structure with pyruvate (**1a**), drawn in heavy lines, at the active site. The BSLDH active site structures were modelled on that of the 2.1Å (R=17.3) data of the oxamate-NADH-DMLDH ternary complex(13) using the MIDAS program (14). All of the amino acid residues displayed are identical for both DMLDH and BSLDH. The Arg171 guanidinium-to-COO^- and His195-to-CO binding interactions are shown in dotted lines. Additional interaction of **1a** with the OH of Thr246 to the COO^- of pyruvate (d=2.52 Å) are also shown. Replacing Thr246 by Gly could also be responsible for creating room for intrusion of solvent water into the active site, thereby interfering with the substrate orientation as reflected by the reduced specificity constants.

the substrate is charged, as in the case of oxaloacetate (**1h**), the deleterious effect of the 246→Gly mutation is reversed. Now the 246→Gly mutant is more active towards **1h** than as the wild-type protein, mainly due to better binding, as reflected by the K_M values.

These data are explained by the graphics analyses observation that, in the native enzyme, the hydroxyl group of Thr246, which is part of the active site wall, is favourably located to form a hydrogen bond of length 2.54 Å to the carboxylic group of the substrate (Figure 1). Replacement of Thr246 by Gly eliminates this H-bonding possibility, and substrate orientation and binding suffer correspondingly. In addition, the graphics picture shows that the active site in the Gly-mutant is more accessible the external solution, such that a water molecule may now gain access to the site formerly occupied by the Thr $CH_3CH(OH)$ sidechain. This explains the strongly reduced specificity constants with the substrates **1a-g** and the improved performance with oxaloacetate **1h**. The latter is the only substrate capable of preventing an extra external water from intruding into this critical active site region.

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