Threonine 246 at the Active Site of the L-Lactate Dehydrogenase of *Bacillus stearothermophilus* Is Important for Catalysis but Not for Substrate Binding†

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ABSTRACT: Threonine 246 is an active site residue that is conserved in all known L-lactate dehydrogenase (LDH; EC 1.1.1.27) sequences. In order to investigate the role of Thr246 in *Bacillus stearothermophilus* LDH, this residue was altered by site-directed mutagenesis to valine, alanine, leucine, and serine, respectively. The effects of these mutations, as observed in both steady-state and single-turnover kinetic measurements with different substrates, demonstrated the importance for catalysis of a hydroxyl group in the 246 amino acid residue. In contrast, no significant contribution of the OH group of Thr246 to productive pyruvate binding was observed. Instead, it is proposed that the role of Thr246 may be to facilitate hydride transfer from the nicotinamide ring of the NADH cofactor to the pyruvate carbonyl group.

L-Lactate dehydrogenases (L-LDHs,[1] EC 1.1.1.27) are well-characterized enzymes that catalyze C=O ⇌ CH(OH) conversions of the type shown in eq 1, of which the conversions of **1a–f** to the corresponding L-hydroxy acids **2a–f** are representative.

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

a: R = CH_3
b: R = CH_3CH_2
c: R = $CH_3CH_2CH_2$
d: R = $(CH_3)_2CH_2$
e: R = $C_6H_5CH_2$
f: R = $HOCH_2$

Enantiomerically pure L-2-hydroxy acids, such as **2a–f**, are valuable chiral synthons in asymmetric synthesis (Bernardi et al., 1990; Pearson & Hines, 1989). Since LDHs exhibit an extremely high degree of stereospecificity, they have been used to catalyze the formation of preparative-scale amounts of these hydroxy acids (Bur et al., 1989b; Bradshaw et al., 1991). The importance of the specificity of LDHs to their synthetic potential had previously prompted us to probe the factors controlling both structure and stereospecificity, using the L-LDH from *Bacillus stearothermophilus* (BSLDH) as the target enzyme.

BSLDH is an attractive vehicle for this purpose, since it is a stable, mechanistically well studied enzyme (Schaer & Zuber, 1979; Clarke et al., 1989) whose three-dimensional structure has been deduced by X-ray crystallography (Wigley et al., 1992; Piontek et al., 1990) and whose gene has been cloned and expressed in *Escherichia coli* (Zuelli et al., 1987; Barstow et al., 1986). Crystal structure data (Wigley et al., 1992) indicate the involvement of several active site amino acid residues in enzyme–substrate (ES) interactions. These are summarized in Figure 1.

From the X-ray structure, Thr246 was identified as an active site residue that could play a role in control of catalysis and specificity. Thr246 is an important active site residue (Bur et al., 1989a) that is conserved in all known LDH sequences. A recent X-ray structure of a BSLDH–NADH–oxamate complex (Wigley et al., 1992) demonstrates that the hydroxyl group of Thr246 can form a 2.5-Å hydrogen bond to the carboxyl group of the substrate analog oxamate. This interaction has been proposed as one of the "fail-safe" interactions that contribute to retaining the L-stereospecific orientation even in the absence of the key substrate binding residue Arg171 (Luyten et al., 1989b). Furthermore, the side chain of Thr246 restricts the volume of part of the active site that could otherwise by filled by the R side chain of a bulky 2-keto acid substrate. In an attempt to enlarge the active site volume. Thr246 was mutated to glycine (Bur et al., 1989a), but the resulting enzyme was 3 orders of magnitude less active than the WT toward pyruvate. The low activity was attributed both to the loss of the Thr246-OH to substrate-COO^- hydrogen bond and to permitting access of external water to the active site. Yet another possibility, not considered at the time, was a distortion of the active site structure due to enhanced main chain flexibility caused by the introduction of glycine (Fersht & Serrano, 1993). To better evaluate the role of Thr246, it was changed (a) to valine, which is isosteric with threonine but lacks hydrogen-bonding capacity, (b) to serine, the lower homolog with the hydroxyl group retained, (c) to alanine, which lacks both the methyl and hydroxyl groups of the threonine side chain, and (d) to leucine, with its hydrophobic side chain that is one methylene group larger than that of valine. In addition to learning more about the catalytic role of Thr246, it was hoped that the enlarged active site volume of the T246S and T246A mutants would facilitate the reductions of keto acids with larger aliphatic R groups, especially those, such as **1d**, with β-branched chains. It has already been found that the hydroxyl group of Thr246 plays a crucial role in promoting substrate inhibition (Sakowicz et

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[1] Abbreviations: LDH, L-lactate dehydrogenase; BSLDH, *Bacillus stearothermophilus* LDH; WT, wild type; FBP, fructose 1,6-bisphosphate; NADD, [4*R*-^{2}H]nicotinamide adenine dinucleotide, reduced form; CD, circular dichroism.

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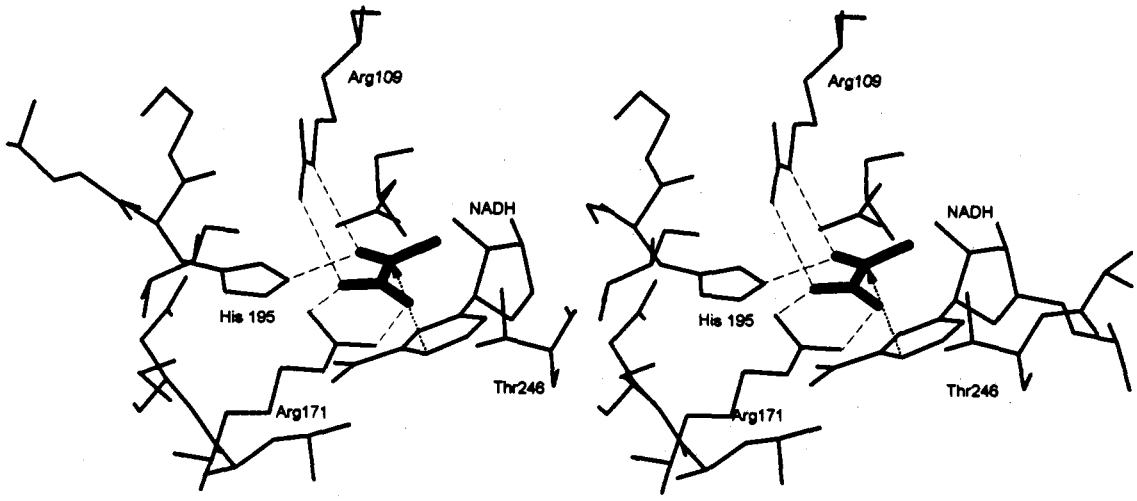

FIGURE 1: Normal stereo representation of pyruvate (in bold-face, modeled into the oxamate position of the X-ray structure of the BSLDH–oxamate–NADH complex (Wigley et al., 1992)) at the active site of BSLDH, and of some of the key amino acid residues involved in catalysis and binding. The dashed lines show the interactions of pyruvate-carboxylate with Arg171 and Arg109, and of His195 and Arg109 with the pyruvate-carbonyl group. The direction of hydride delivery to the re-face of the pyruvate-carbonyl that leads to L-lactate formation is shown by the arrow (dotted line).

al., 1992). The current work was carried out to seek a further understanding of the part played by Thr246 in specificity control and in the catalytic mechanism of BSLDH.

EXPERIMENTAL PROCEDURES

Materials and Strains. Horse liver alcohol dehydrogenase and yeast aldehyde dehydrogenase were obtained from Sigma. Perdeuterated (C_2D_5OH) ethanol was from MSD Isotopes (Montreal). *E. coli* strain TG2 was used exclusively for all procedures involving a bacterial host. All kinetic measurements were done in 20 mM piperazine buffer, pH 6.0.

Mutagenesis. The BSLDH gene cloned into plasmid pTZ-R18 served as a source of single-stranded DNA for mutagenesis and sequencing as described previously (Luyten et al., 1989a). An oligonucleotide doped in two positions (AAA AAG GAG CG(G/T) (C/T)GT ACT ACG GGA) was used to create four different Thr246 mutant BSLDHs: T246A, T246V, T246S, and T246L. The site-directed mutagenesis method employing thio analogs of nucleotides (Sayers et al., 1988) was used, in the version supplied by Amersham. Mutants were identified by sequencing. For each of the mutants, the entire protein coding region was sequenced to confirm that no inadvertant mutations had been introduced during *in vitro* mutagenesis procedures.

Protein Purification. Both mutant and WT proteins were expressed from the same pTZ plasmid construct used in mutagenesis. Initial purification steps consisting of sonication of bacterial cells and heat denaturation of host proteins were carried out as described previously (Luyten et al., 1989a) except that 50 mM piperazine, pH 6.0, was used in all steps. Subsequently, the WT, T246A, T246S, and T246V BSLDHs were purified by affinity chromatography on oxamate Sepharose (Sakowicz et al., 1992). Because of the lower affinity for pyruvate, T246L was purified on an anion-exchange MonoQ column (Pharmacia). Finally, all proteins were chromatographed on hydrophobic-interaction phenyl-Superose (Pharmacia) columns to remove tightly bound nucleotides obstructing spectroscopic measurements. The purified proteins gave a single band on SDS PAGE gels and a clear 280-nm maximum peak in UV spectra.

Steady-State Kinetics. Progress of the BSLDH-catalyzed reaction was followed by monitoring the decrease in NADH absorption at 340 nm. All measurements were done at 25 °C in 20 mM piperazine, pH 6.0, buffer in the presence of 5 mM FBP (unless otherwise stated). Kinetic parameters k_{cat} and K_M for pyruvate were determined at saturating NADH concentrations (0.2 mM). The apparent K_M for NADH was determined at a saturating pyruvate concentration (1 mM). Numerical values of all kinetic parameters were obtained by fitting experimental data points with the nonlinear regression program Grafit (Erithacus Software Ltd., Staines, U.K.). Enzyme concentrations for all mutant proteins and for the WT enzyme were determined spectrophotometrically at 280 nm using an extinction coefficient of 0.91 g^{-1} L^{-1} (Clarke et al., 1985a).

Single-Turnover Experiments. Rapid-mixing single-turnover experiments were performed according to Clarke et al. (1985b). A stopped-flow apapratus (Hi-Tech, Wilts, U.K.; dead time 2 ms), set to record changes in absorbance at 340 nm, was used in all measurements. Typical experiments involved rapid mixing of equal volumes of a solution containing 30 μM enzyme, 30 μM NADH, and 5 mM FBP with a solution containing saturating concentrations of pyruvate (20 mM) and 5 mM FBP. Each reported rate constant represents the mean of five experiments.

Primary Kinetic Isotope Effects. NADD, specifically deuterated in the 4*R*-position of the nicotinamide ring, was prepared by enzymatic reduction of NAD^+ coupled with oxidation of perdeuterated ethanol (Viola et al., 1979). It was used for single-turnover experiments in place of NADH within 5 h without further purification. Rate constants with NADD were compared with those obtained with NADH in an identical experimental setup.

CD Spectroscopy. Circular dichroism (CD) spectra of WT and T246A, V BSLDHs were obtained as previously described (Kallwass et al., 1992). Sodium phosphate buffer (30 mM, pH 7.0) was used as the solvent.

RESULTS

Steady-State Kinetic Parameters. Initial velocity studies under steady-state conditions were performed as described previously (Kallwass et al., 1992), using an NADH concentration (0.2 mM) that was saturating for all the mutant enzymes. The kinetic parameters are recorded in Table I.

Introduction of a bulky leucine side chain at position 246 resulted in a significant decrease in turnover number and a

Table I: Kinetic Parameters of BSLDH and Its Mutants[a]

substrate	constant	enzyme				
		WT	T246S	T246A	T246V	T246L
pyruvate (**1a**)	k_{cat} (s^{-1})	166	132	5.1	10	10
	K_M (mM)	0.04	0.07	0.045	0.09	40
	k_{cat}/K_M (M^{-1} s^{-1})	4.2×10^6	1.9×10^6	1.1×10^5	1.1×10^5	250
pyruvate (without FBP)	k_{cat} (s^{-1})	247	133	5.2	11.8	nd[b]
	K_M (mM)	5	10	0.44	0.97	
	k_{cat}/K_M (M^{-1} s^{-1})	4.9×10^4	1.3×10^4	1.2×10^4	1.2×10^4	
2-ketobutyrate (**1b**)	k_{cat} (s^{-1})	124	40	1.7	6.9	1
	K_M (mM)	0.4	1.1	0.44	4.8	47
	k_{cat}/K_M (M^{-1} s^{-1})	3.1×10^5	3.6×10^4	3.9×10^3	1.4×10^3	21
2-ketovalerate (**1c**)	k_{cat} (s^{-1})	17	9.4	1.2	0.6	0.09
	K_M (mM)	2.6	3.7	4.2	6.9	50
	k_{cat}/K_M (M^{-1} s^{-1})	6.5×10^3	2.5×10^3	290	87	1.8
2-ketoisovalerate (**1d**)	k_{cat} (s^{-1})	0.26	0.23	0.20	*c*	*c*
	K_M (mM)	22	25	14	*c*	*c*
	k_{cat}/K_M (M^{-1} s^{-1})	12	9.2	14	*c*	*c*
phenylpyruvate (**1e**)	k_{cat} (s^{-1})	75	25	4.8	2.7	0.18
	K_M (mM)	0.74	1.8	3.1	2.7	4.6
	k_{cat}/K_M (M^{-1} s^{-1})	1.0×10^5	1.4×10^4	1.5×10^3	1.0×10^3	39
hydroxypyruvate (**1f**)	k_{cat} (s^{-1})	162	74	20	78	nd[b]
	K_M (mM)	0.43	0.54	1	9	
	k_{cat}/K_M (M^{-1} s^{-1})	3.8×10^5	1.4×10^5	2.0×10^4	8.6×10^3	
NADH	K_M (μM)	13	6.6	0.8	0.7	nd[b]

[a] All steady-state kinetic parameters were determined at least in duplicate (<±15% error limits) as described previously (Kallwass et al., 1992) in 20 mM piperazine, pH 6.0, 5 mM FBP, 25 °C. [b] nd = not determined. [c] No activity detected with enzyme concentrations ≤10 μM and substrate concentrations ≤30 mM.

substantial increase of K_M for all substrates, leading to a very low activity enzyme. However, the other substitutions at the 246 position were significantly less disruptive. An interesting pattern can be observed for reactions with the natural substrate, pyruvate (**1a**). Mutants devoid of a hydroxyl group in the 246-residue side chain (T246A,V) were over 40-fold less active, in terms of k_{cat}/K_M values, than WT, while only a 3-fold deactivation was observed for T246S-BSLDH, in which the side-chain hydroxyl was retained. Surprisingly, none of the three T246A,V,S mutations significantly affected the apparent K_M for pyruvate. The kinetic differences were manifest mostly in the k_{cat} values.

When the activator FBP was omitted from the reaction mixture, the maximum turnover rate was not altered for the T246S,A,V mutant enzymes, a situation paralleling that reported for the WT protein (Clarke et al., 1985a,b). However, the increase in pyruvate K_M resulting from the omission of FBP was 5–10 times less for T246A,V than for WT and T246S.

Activities toward substrates with longer aliphatic side chains, such as 2-ketobutyrate (**1b**) and 2-ketovalerate (**1c**), were generally lower for the mutant BSLDHs than for WT. Nevertheless, the β-branched compound 2-ketoisovalerate (**1d**) was accommodated similarly by WT and T246A. WT BSLDH catalyzes the reduction of hydroxypyruvate (**1f**) at a rate comparable to, but slightly slower than, that of pyruvate. A similar pattern was observed for the T246S mutant. On the other hand, T246A and T246V mutants showed significantly elevated turnover rates with hydroxypyruvate, exhibiting k_{cat} values 5–8 times higher for this substrate than for pyruvate.

Apparent K_M's for NADH were determined for WT and T246A,V,S BSLDHs (Table I). Relative to WT, there is almost a 20-fold decrease in the coenzyme K_M(app) for the T246A,V mutants lacking the OH group at position 246. In contrast, for the T246S mutation, in which the side-chain hydroxyl is retained, K_M(app) for NADH decreases only 2-fold over the WT situation.

Single-Turnover Kinetics. Catalytic constants obtained under steady-state conditions are subject to some experimental uncertainties, including difficulties in determining precisely the concentration of the active sites, and product or substrate inhibition. Most of these problems can be avoided by performing an experiment under single-turnover conditions. In fact, the rate constants obtained in this way correlated well with those from the steady-state experiments, with the deviations being less than ±20%. The single-turnover kinetic data, with pyruvate as substrate, are presented in Table II. The mutant with the most reduced rate constant was T246A, which displayed a deuterium kinetic isotope effect of 2.4, in contrast to the WT enzyme, for which no such kinetic isotope effect was detected.

Table II: Summary of Single-Turnover Kinetic Data for WT and Mutant BSLDHs[a]

k (s^{-1})	enzyme			
	WT	T246A	T246S	T246V
with NADH (k_H)	235 ± 4	5.7 ± 0.2	119 ± 12	11.9 ± 0.4
with NADD (k_D)	210 ● 5	2.3 ± 0.04	nd[b]	nd[b]
(k_H/k_D)	1.12 ± 0.04	2.4 ± 0.1	nd[b]	nd[b]

[a] All single-turnover kinetics were determined in 20 mM piperazine, pH 6.0, 5 mM FBP, 25 °C. [b] nd = not determined.

Circular Dichroism Spectra. The T246A and T246V mutants display no detectable differences in CD spectra in comparison to the case of WT BSLDH. The spectra are virtually superimposable in the peptide-bond region (178–225 nm) and also in the aromatic region (250–320 nm).

DISCUSSION

One of the objectives of our mutagenesis studies at the 246 position was to determine whether enlargement of the active site volume by introducing a small residue would result in better accommodation of β-branched substrates. Unfortunately, interpretation of our data is not straightforward, since in addition to the steric effects on binding caused by mutation, mutants at the 246 position with or without a side-chain hydroxyl function might affect the hydride-transfer step of the reaction in a different and difficult-to-predict manner. Consequently, meaningful comparisons can only be made

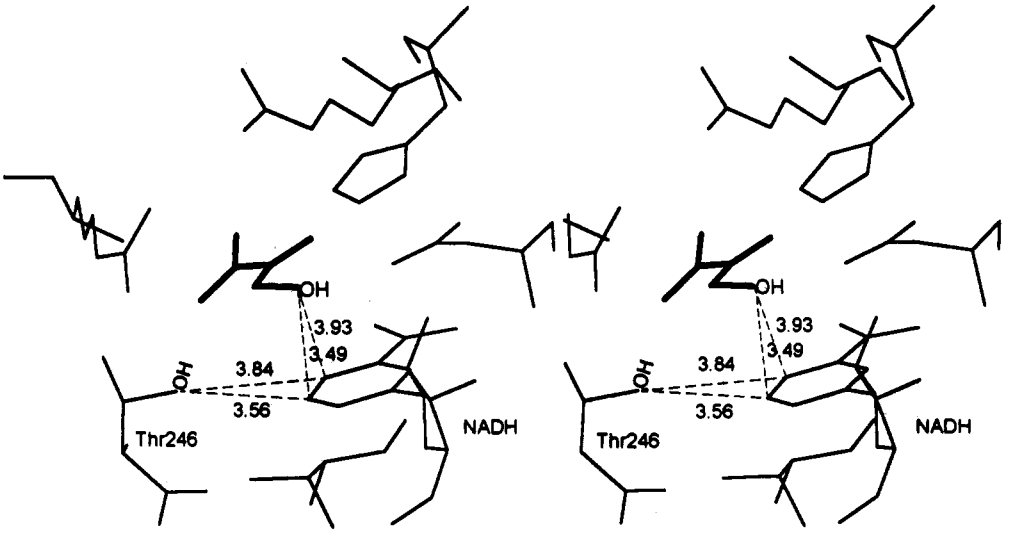

FIGURE 2: Normal stereo representation showing hydroxypyruvate (in bold-face, modeled into the oxamate position of the X-ray structure of Wigley et al. (1992)) at the active site of BSLDH. The OH group of hydroxypyruvate can be favorably located adjacent to the C-4 and C-5 atoms of the nicotinamide ring of NADH such that its oxygen-to-C-4 and -C-5 distances (3.93 and 3.49 Å, respectively) match those (3.84 and 3.56 Å, respectively) of the hydroxyl group of Thr246. In this way, the OH group of hydroxypyruvate can, to a degree, mimic the Thr246-hydroxyl function in mutants lacking a side-chain OH at the 246 position, such as T246A and T246V.

between BSLDHs retaining or lacking a 246 hydroxyl. The kinetic data observed with ketoisovalerate (**1d**) illustrate this dilemma well (Table I). Ketoisovalerate is a very poor substrate for the wild-type enzyme (Luyten et al., 1989a). It is thus predictable that the removal of the hydroxyl group from the 246 resiue, which produced a sharp decrease in the activity toward pyruvate, would result in even lower ketoisovalerate reduction rates. This prediction is fulfilled for T246L and T246V, both of which are virtually inactive with this substrate. However, in the case of T246A, the loss in activity due to the missing hydroxyl group appears to be compensated by the increased active site volume, giving an enzyme that has a specificity constant comparable to that of WT BSLDH. This suggests that it is the hydroxyl group itself that represents the major steric constraint with respect to β-branched substrates. This conclusion is supported by the observation that removing the methyl group of the Thr246 side chain, as in T246S, affords no improvement in k_{cat}/K_M value for ketoisovalerate reduction over that of the WT enzyme. The benefits of an enlarged substrate-binding pocket are also evident for substrates with larger, more hydrophobic, side chains than that of pyruvate, such as ketobutyrate (**1b**), ketovalerate (**1c**), and phenylpyruvate (**1e**), each of which is better accommodated by T246A than T246V.

As noted above, Thr246 is conserved in all known lactate dehydrogenase sequences. Also, in soluble pig heart malate dehydrogenase, which is very structurally similar to the LDHs, the corresponding position is occupied by Ser241 (Birktoft et al., 1989). This strongly indicates that for these families of enzymes, a side-chain hydroxyl group at this position is important for proper catalytic function. The need to maintain a hydrogen bond to the COO^- of the substrate, as deduced from X-ray structures (Luyten et al., 1988b; Wigley et al., 1992), could account for this remarkable evolutionary conservation. However, if this were truly the case, removal of the hydroxyl interaction should have resulted in a weaker substrate-binding, thereby leading to an increased K_M. The results in Table I clearly rule this out as a unique explanation of the Thr246 role. The mutant proteins T246A and T246V, for which side-chain hydrogen bonding to the substrate is impossible, have virtually the same pyruvate K_M's as those capable of forming OH–COO^- hydrogen bonds, *i.e.* WT and T246S. Even more striking is the observation that, in the absence of the FBP activator, the apparent pyruvate K_M's for T246A and T246V are lower than those for WT and T246S. Removal of the FBP causes a sharp decrease in the ability of WT BSLDH to bind pyruvate (Clarke et al., 1985c). Both the K_M for pyruvate and the K_D for the substrate analog oxamate are increased >100-fold, but no effect on the turnover number or the coenzyme binding is observed (Clarke et al., 1985c). This weakened pyruvate affinity has been attributed to a change in the orientation of two most important binding residues Arg171 and His195 (Clarke et al., 1985b). This is relevant to the present results, since a decreased contribution to binding from the Arg171 and His195 side chains should in turn intensify the effect of any auxiliary binding interactions, such as the putative Thr246-hydroxyl to pyruvate-carboxylate hydrogen bond. However, our current experimental data argue against such interactions being catalytically significant. In this regard it must be borne in mind that for two-substrate reactions of the BSLDH-type, an apparent K_M can be a complex kinetic constant that may not reflect only the substrate binding step. However, in the case of the BSLDH, the extensive mutagenesis and binding studies of the Holbrook group have demonstrated that changes in the apparent K_M for pyruvate, whether induced by altered enzyme conformation (Clarke et al., 1985c) or by active site mutations (Clarke et al., 1986; Hart et al., 1987; Clarke et al., 1988), do in fact relate closely to the corresponding changes in the K_D for oxamate.

The most dramatic effect of hydroxyl group removal from the Thr246 side chain is manifest in the single-turnover-number experiments (Table II), for which a >40-fold decrease in the rate constant between WT and T246A is observed. The steady-state kinetic data also show this effect. The single-turnover results eliminated the possibility that the decreased mutant k_{cat} values might be due to phenomena unrelated to the actual catalytic process, such as variations in product-dissociation contributions, errors in active site concentration determinations, and substrate or product inhibition. Furthermore, the primary kinetic isotope effect of 2.4 displayed by T246A demonstrated that hydride transfer was not at least partially rate-limiting for this mutant. For WT BSLDH, the conformational change associated with the rate of closure of the

flexible 98–110 loop over the active site is rate-determining at 250 s^{-1} (Dunn et al., 1991) while the hydride-transfer step has a rate constant of >750 s^{-1} (Clarke et al., 1986). Thus for the T246A enzyme, this latter step is at least 150-fold slower.

That the hydroxyl group of Thr246 effectively facilitates catalysis is reflected by the fact that its presence affects only k_{cat}, and not K_M. One possible explanation of its beneficial influence might have been that the Thr246-OH-to-substrate-COO^- hydrogen bond can only form in the transition state of the reaction, thereby contributing to lowering the activation energy barrier (Fersht, 1985). This interpretation was excluded by the kinetic data for hydroxypyruvate (**1f**) reduction (Table I). With a hydroxyl group in the substrate, but not at the 246 position as in T246A,V, formation of a stabilizing OH–COO^- hydrogen bond is precluded, yet the T246S and T246V mutants have the same k_{cat} values for hydroxypyruvate. Thus, the data for pyruvate and hydroxypyruvate show that loss of the side-chain hydroxyl of Thr246 can be partially compensated by its incorporation into the substrate, a situation reminiscent of the "substrate-assisted" catalysis observed with subtilisin BPN′ (Carter & Wells, 1987). Another potential role for the Thr246 hydroxyl group could be to ensure proper orientation of pyruvate with respect to hydride attack from the coenzyme's dihydronicotinamide ring. This could arise from restriction of the mobility of the pyruvate-carboxyl movement by steric interactions between the oxygen atoms of the substrate-carboxyl and Thr246-hydroxyl groups. Such interactions could account for the pyruvate K_M values being substantially higher for the WT and T246S BSLDHs that retain the hydroxyl at the 246 position than for the T246A,V mutant proteins that lack this function, particularly in the absence of FBP when overall pyruvate binding is weakened. On the other hand, T246A,V enzymes the compensatory effect of a hydroxyl group supplied on the substrate molecule, as for hydroxypyruvate, would be hard to account for in these terms. Furthermore, if steric control of the above type were important, the isosteric T246V replacement should have induced a substantially less disruptive effect.

Another possible explanation of the catalytic benefit conferred by the Thr246-hydroxyl, which agrees with all experimental observations, is that it activates the dihydronicotinamide ring of the NADH-cofactor. Activating effects of proximal hydroxyl groups on the reducing potential of dihydronicotinamides are well documented in nonenzymatic model reactions (Sigman et al., 1978). These effects have also been proposed to play a role in enzymatic catalysis of dihydrofolate reductase (Filman et al., 1982; Farnum et al., 1991). In such systems it is generally accepted that in the transition state preceding hydride transfer, a localized positive charge develops at C-4 of the nicotinamide ring (LaReau et al., 1989; Rotberg & Cleland, 1991), although the question remains as to whether this charge formation is associated with ring-tilting or boat-like puckering. By analogy, we propose that the hydroxyl group of Thr246 could participate in stabilizing a developing positive charge at C-4 of NAD/H in the transition state leading to hydride transfer.

The side-chain hydroxyl of Thr246 is suitably located in this regard. In the X-ray structure of the BSLDH ternary complex, the oxygen atom of the T246 side-chain hydroxyl group is adjacent to the C-4 and C-5 carbons of the dihydronicotinamide ring of NADH, at distances of 3.8 and 3.6 Å, respectively. These distances are comparable with those observed for the analogous oxygen atom of the Thr46 implicated in activation of the cofactor-nicotinamide ring in dihydrofolate reductase catalysis (Farnum et al., 1991). Moreover, for BSLDH, the out-of-plane location of the Thr246 hydroxyl relative to the pyruvate carboxyl precludes strong OH–COO^- hydrogen bonding. The ability of a substrate-hydroxyl group to ameliorate the catalytically detrimental absence of a side-chain OH in the T246A,V mutant-catalyzed reductions of hydroxypyruvate is also highly significant. When hydroxypyruvate is modeled into the oxamate location in the BSLDH–NADH–oxamate structure, its hydroxyl function can be favorably positioned close to the dihydronicotinamide ring of NADH such that the OH to C-4 and C-5 distances are comparable to those of the Thr246 hydroxyl group (Figure 2). Furthermore, although no particular amino acid residues have yet been implicated, Thr246 may also be involved in the activation of the nicotinamide ring upon cofactor binding to LDH (Burgner & Ray, 1984; Deng et al., 1992) as well as in the distortion of the nicotinamide ring of NADH on binding (Deng et al., 1989). Further studies addressing the role of Thr246 and adjacent residues in coenzyme binding and activation are planned. In this regard, it is expected that NADH, with its uncharged and hydrophobic dihydronicotinamide ring, should bind more strongly to the T246A,V mutant enzymes lacking the position-246 side-chain hydroxyl group than to WT or T246S.

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