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Conformational Equilibrium of an Enzyme Catalytic Site in the Allosteric Transition†

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ABSTRACT: The dynamic equilibrium of a catalytic site between active and inactive conformations, the missing link between the structure and function of allosteric enzymes, was identified using protein engineering and NMR techniques. Kinetic analyses of the wild-type and three mutants of *Thermus* L-lactate dehydrogenase established that the allosteric property of the enzyme is associated with a concerted transition between the high-affinity (R) and low-affinity (T) states. By introducing mutations, we prepared an enzyme in which the R and T states were balanced. The conformation of the enzyme-bound coenzyme, NAD^+, which interacts directly with the substrate, was analyzed using NMR spectroscopy. NAD^+ bound to the mutant enzyme was in a conformational mixture of the active and inactive forms, while NAD^+ took on predominantly one of the two forms when it was bound to the other enzymes we had analyzed. We interpret this to mean that the catalytic site is in equilibrium between the two conformations. The ratio of the coformers of each enzyme agreed with the [T]/[R] ratio as determined by kinetic analyses. Therefore, it is the identified conformational equilibrium of the catalytic site that governs the allosteric regulation of the enzyme activity.

The allosteric regulation of enzyme activity has been attributed to the transition of the protein conformation between two states since the proposal of relevant models in the 60's (Monod et al., 1965; Koshland et al., 1966). Crystallographic studies on several enzymes have led to the successful identification of two different conformations, which explain the difference in activity between the two states (Kantrowitz & Lipscomb, 1988; Barford & Johnson, 1989; Gouaux & Lipscomb, 1990; Schirmer & Evans, 1990; Ke et al., 1991). On the other hand, we know little about the dynamic transition of allosteric enzymes between different conformations, though almost all the structural research on allostericity was based on the assumption of a two-state transition. Even for the global conformation, there have been few demonstrations of the allosteric transition (Hervé et al., 1985; Eisenstein et al., 1990). In particular, we know nothing about the conformational

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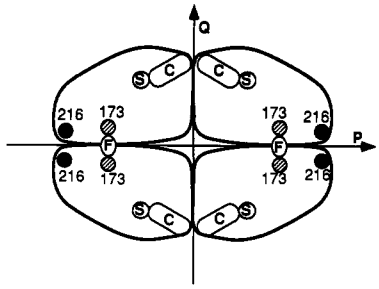

FIGURE 1: Schematic diagram of an LDH tetramer looking down the molecular 2-fold axis R. The other two molecular 2-fold axes are labeled P and Q. The model was drawn on the basis of the crystal structure of *Bacillus stearothermophilus* LDH (Piontek et al., 1990). The substrate binding sites are denoted by "S", the coenzyme sites by "C", and the FBP sites by "F". The positions of the residues to which mutations were introduced are denoted by circles with their residue numbers.

dynamics of the catalytic site, which actually governs enzyme activity, and its linkage to the global conformation transition. According to the concerted transition model (the MWC model) which satisfactorily describes kinetic properties for some allosteric enzymes (Monod et al., 1965; Blangy et al., 1968), allosteric enzymes are in the conformational equilibria between two states. Consequently, their catalytic sites should be in the equilibria between two conformations. However, there are two difficulties in demonstrating the conformational equilibria of the catalytic sites. First, in natural allosteric enzymes, the equilibria are usually one-sided so that one of two conformations exists dominantly, whether they are in the free forms or in the complexes with their effectors. Second, there had been no methodology of quantifying the conformational equilibrium of the catalytic sites among the relatively large framework of allosteric enzymes. Though the allosteric mechanism of hemoglobin has been investigated extensively by structural analyses and protein engineering techniques (Perutz, 1989; Imai et al., 1991), such a conformational equilibrium of the oxygen binding site has not been identified. In the present study, we adopted protein engineering techniques to balance the two states, and analyzed the conformational equilibrium of the catalytic site through analyses on the conformation of the enzyme-bound coenzyme by means of NMR spectroscopy.

L-Lactate dehydrogenase (LDH,[1] EC 1.1.1.27) catalyzes the oxidoreduction between pyruvate and lactate, utilizing NAD^+ as a coenzyme (Holbrook et al., 1975). LDHs from bacteria are usually allosteric enzymes, which are activated by fructose 1,6-bisphosphate (FBP) (Garvie, 1980). An allosteric LDH comprises four identical subunits, and the tetramer exhibits *222* symmetry (Piontek et al., 1990). The tetramer has four substrate binding sites, two effector binding sites lying at the interface of two subunits (Figure 1). Though the kinetic and structural properties of the allosteric LDH–FBP complex as well as the nonallosterc counterparts from vertebrates have been extensively investigated (Holbrook et al., 1975; Grau et al., 1981; Abad-Zapatero et al., 1987; Clarke et al., 1989; Piontek et al., 1990), the mechanism of allosteric transition of bacterial LDHs had not been delineated. In the absence of an effector, allosteric LDHs show sigmoidal saturation curves relative to the concentration of the substrates, while they show no cooperativity for coenzyme binding (Clarke et al., 1985; Taguchi et al., 1985). Kinetic characterization of the allosteric properties of LDHs has not been sufficient to establish a quantitative model, though it was reported that changes in subunit assembly were involved in the allosteric transition in some LDHs (Götz & Schleifer, 1978; Mayr et al., 1980; Clarke et al., 1989).

LDH from *Thermus caldophilus* (tcLDH) is one of the extensively studied allosteric LDHs (Taguchi et al., 1982, 1985; Kunai et al., 1986). We had already demonstrated that the allosteric property of the enzyme can be manipulated with chemical and genetical modifications (Taguchi et al., 1984; Matsuzawa et al., 1988). We had also identified the conformational change of the enzyme-bound NAD^+ upon the binding of FBP (Machida et al., 1985) and upon chemical modification (Koide et al., 1989) by means of NMR spectroscopy. Therefore, tcLDH is advantageous for demonstrating the conformational equilibrium of the catalytic site.

In the present research, we prepared mutant enzymes and established a quantitative model describing the kinetic property of tcLDH. Then we analyzed the conformation of the coenzyme bound to the mutant enzymes to probe the conformation of their catalytic sites.

Experimental Procedures

All chemicals used were of the highest grade available, unless otherwise noted. NAD^+ (lithium salt), FBP, and an *Xho*I linker were purchased from Boehringer Mannheim. [$^2H_{11}$]Tris was from MSD Isotopes.

Enzyme Preparation. The *Xho*I–*Sac*I fragments of the WT and R173Q genes (Kunai et al., 1986; Matsuzawa et al., 1988) were inserted at the multiple cloning sites in an M13 mp10 (Messing & Viera, 1982) derivative which contained an *Xho*I linker at the *Sma*I site. They were used as the templates for mutagenesis. Site-directed mutagenesis was performed following the method of Kunkel et al. (1987) with a synthetic oligonucleotide containing a mismatch which changed the Arg216 codon (CGG) to Leu (CTG). The *Xho*I–*Stu*I fragments of positive clones were sequenced to confirm the design and then inserted into an LDH expression vector (Koide et al., 1991).

All enzymes were purified from *Escherichia coli* containing the LDH expression vectors and quantified spectrophotometrically as described previously (Koide et al., 1991). R173Q (1 mg/mL) was irreversibly modified with 20 mM 2,3-butanedione for 24 h as described (Taguchi et al., 1984) and then passed through a monoQ column (Pharmacia) to remove the reagent and ligands (Koide et al., 1991).

Kinetic Analyses. The assay mixture for pyruvate reduction was comprised of 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS)–NaOH buffer (pH 6.75), 0.2 mM NADH, and pyruvate and FBP at appropriate concentrations. The enzyme activity was measured at 30 °C as described (Taguchi et al., 1984). The $S_{0.5}$ values, the concentrations for half-maximal activity, were determined by manual examination, because there was no model describing the dependence of activity upon the pyruvate concentration (Taguchi et al., 1985).

The assay mixture for lactate oxidation was comprised of 50 mM Tris-HCl buffer (pH 7.1 at 25 °C), 5 mM LiNAD, and lithium lactate and FBP at appropriate concentrations. KCl was added to the mixture so that the sum of the concentrations of lithium lactate and KCl was kept at 1 M. Lactate oxidation activity of LDH was measured at 40 °C by monitoring the increase of NADH with a Milton Roy Spec-

[1] Abbreviations: LDH, L-lactate dehydrogenase; tcLDH, *Thermus caldophiluc* LDH; bsLDH, *Bacillus stearothermophilus* LDH; WT, wild-type tcLDH; R173Q, R216L, and R173Q:R216L, mutant tcLDHs which contain the Arg173→Gln, the Arg216→Leu, and both mutations, respectively; FBP, fructose 1,6-bisphosphate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; TRNOE, transferred nuclear Overhauser effect.

tronic 3000 spectrophotometer. The enzyme concentration for the reaction was 0.6 μg/mL. One unit was defined as the amount of enzyme which produced 1 μmol of NADH per minute.

The data for lactate oxidation were interpreted with the equation for the concerted transition between the high-affinity (R) and low-affinity (T) states (Monod et al., 1965):

$$v = \frac{V([\text{lactate}]/K_{R(\text{lactate})})(1 + [\text{lactate}]/K_{R(\text{lactate})})^3}{L/(1 + [\text{FBP}]/K_{R(\text{FBP})})^2 + (1 + [\text{lactate}]/K_{R(\text{lactate})})^4}$$

where $K_{R(\text{lactate})}$ and $K_{R(\text{FBP})}$ are the microscopic dissociation constants of lactate and FBP in the R state, respectively, L is the [T]/[R] ratio in the absence of ligands, and V is the maximum velocity. We inferred that lactate was bound to the R state exclusively, because $K_{T(\text{lactate})}$ was estimated to be at least 1000 times larger than the K_R value for WT, R173Q, and R216L, and we also assumed that FBP was bound to the R state exclusively (Monod et al., 1965). All parameters were determined by nonlinear least-squares fitting with Igor (WaveMetrics, OR) to the date for lactate concentrations below 100 mM, in order to exclude effects of the slight substrate inhibition observed at higher lactate concentrations.

Equilibrium Binding Study. The binding of NADH to LDH was monitored by essentially following the procedures of Clarke et al. (1985). The change in the polarization of NADH fluorescence was recorded by a Jasco FP770 spectrofluorometer at 30 °C, as LDH was added to a cuvette containing 2 μM NADH and 50 mM MOPS–NaOH buffer (pH 6.75). The dissociation constant was determined by nonlinear least-squares fitting based on a simple binding of LDH and NADH.

Molecular Mass Determination. WT tcLDH was eluted from a gel permeation column (GFA50; Asahi Chemical Industry) equilibrated with 50 mM MOPS–NaOH buffer (pH 7.0) containing 100 mM NaCl at 30 °C. The retention time was determined by monitoring the absorbance at 280 nm and the protein fluorescence. Molecular mass calibration was performed with standard proteins obtained from Boehringer Mannheim. The protein concentration was varied from 0.5 μg/mL to 10 mg/mL in the absence and presence of 5 mM FBP.

NMR Spectroscopy. A protein solution in buffered ^2H_2O containing 50 mM [$^2H_{11}$]Tris (MSD Isotopes)-^{2}HCl (pH* 7.1 at 25 °C, direct meter reading) and 1 M KCl was prepared by repeated ultrafiltration, as described previously (Koide et al., 1989). The final concentrations of the enzymes were between 0.14 and 0.30 mM expressed as the concentration of subunit. NAD^+ was added to the solution so that the molar ratio of NAD^+/LDH was kept at 10.

NMR measurements were performed with a Bruker AM600 spectrometer with an Aspect3000 computer, essentially following the previous procedures (Koide et al., 1989). NMR spectra were recorded at 40 °C, with 16K data points for a 9.6-kHz spectral width. The pulse sequence for time-dependent TRNOE was $(t_1\text{–}\pi/2\text{–AQ–}D_1)_n$, where t_1 represents selective saturating irradiation (0.005–0.6 s, 15 dB below 0.2 W) and D_1 is a delay for magnetization recovery. The duration of one cycle was set at 5 s. A total of 96–256 transients were accumulated, and a line broadening of 2 Hz was applied.

Initial slopes of time-dependent TRNOE were determined from the relative resonance intensities $I(t)/I_c(t)$, where $I(t)$ and $I_c(t)$ are the resonance intensities on irradiation at a proton resonance and at a control frequency for the duration of t, respectively. In order to compensate for differences among individual experiments, the initial slopes were expressed as the ratios to the slope for the H_N5–H_N4 pair, which was in the range of 1.1–1.7 in all the experiments. The accuracy of the initial slopes was within ±20%. Interproton distances were determined as described previously (Clore & Gronenborn, 1983; Koide et al., 1989).

Results and Discussion

Construction of Mutant Enzymes. We demonstrated that tcLDH is fixed in a high-activity state on the modification of Arg residues with 2,3-butanedione (Taguchi et al., 1984). Arg173, a target of the reaction, was identified as the residue constituting the FBP binding site (Matsuzawa et al., 1988) [the numbering of residues in this paper follows the N-system (Eventoff et al., 1977)]. R173Q, a mutant enzyme containing an Arg173→Gln mutation, is the first one used in this study.

To find other residues involved in the allosteric mechanism, we subjected R173Q to further modification with 2,3-butanedione. With this modification, the $S_{0.5}$ value for pyruvate, the concentration for half-maximal activity, of this enzyme was decreased from 3.2 mM to 5.1 μM in the absence of FBP, which is comparable to that of the wild-type (WT) enzyme with FBP (14 μM). On comparison of the elution profiles of the trypsin-digested fragments of the unmodified and modified R173Q enzymes on reverse-phase liquid chromatography (Matsuzawa et al., 1988), we found that Arg216 is partly modified after 3-h modification (data not shown). Other smaller differences in the profile were also observed, suggesting the modification of other residues.

To examine the involvement of Arg216 in the allosteric regulation of tcLDH, we replaced Arg216 of R173Q with Leu. As for the 216th residue, hydrophilic amino acids were found in the allosteric LDHs, while large hydrophobic amino acids (Leu and Met) were found in the nonallosteric ones. However, the region surrounding it shows low identity in the amino acid sequence even among the allosteric LDHs, and tcLDH is unique in having Arg at the position (Kunai et al., 1986). Therefore, the structure and function of the region may differ among allosteric LDHs. The $S_{0.5}$ value for pyruvate of the mutant R173Q:R216L in the absence of FBP was 57 μM, indicating that the R216 → L mutation modulated the kinetic property of tcLDH (for results in detail, see below). The equilibrium dissociation constant of NADH was also determined for WT, R173Q, modified R173Q, and R173Q:R216L in the presence and absence of FBP. In all cases, the dissociation constant was 0.9–1.2 μM, and the binding was uncooperative. This demonstrates that neither the binding of FBP nor the mutations influences the binding of the coenzyme. In addition, a single mutant, R216L, was prepared.

The location of the 216th residue is illustrated in Figure 1, according to the crystal structure of *Bacillus stearothermophilus* LDH (bsLDH) (Piontek et al., 1990). The residue is located on the protein surface near the subunit interface across the P-axis, a molecular 2-fold axis, and distant from both the substrate binding site and the FBP binding site.

Model for the Kinetic Property. We analyzed the kinetic properties of the mutant enzymes as well as WT. The use of the mutant enzymes was of great help in determining the essential mechanism underlying allosteric regulation, because a relevant model must describe the kinetic properties of all the enzymes satisfactorily. We found that the lactate oxidation assay enabled quantitative analysis of the allosteric property of tcLDH. In our previous studies, the kinetic property of tcLDH was analyzed for pyruvate reducing activity, but it was impossible to characterize the allosteric property in detail through such analysis, because of severe substrate inhibition, that is, a decrease in velocity at high substrate concentration

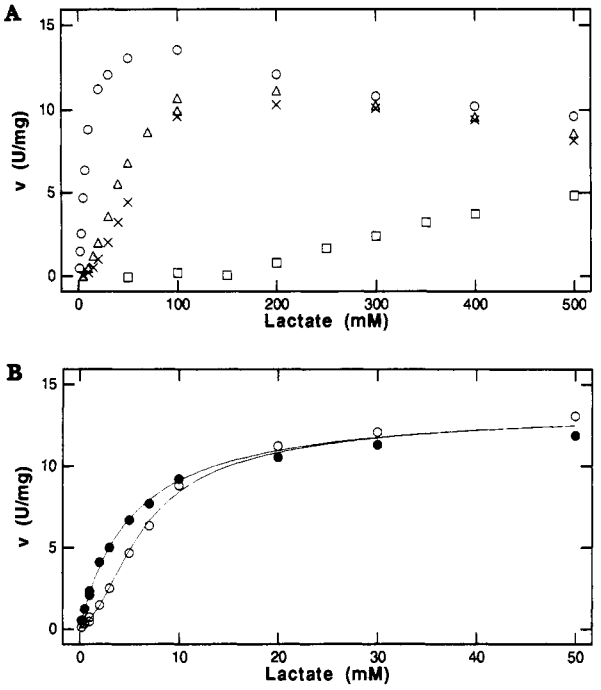

FIGURE 2: Lactate saturation curves for the WT (□), R173Q (△), R216L (×), and R173Q:R216L (○) tcLDHs in the absence of FBP (A), and results of fitting of the model to the data for R173Q:R216L (B) in the presence (●) and absence (○) of 10 mM FBP. The lines in (B) show the saturation curves obtained from the model.

(Taguchi et al., 1984, 1985; Matsuzawa et al., 1988).

By assaying the kinetics of the lactate oxidation reaction of WT, R173Q, R216L, R173Q:R216L, and modified R173Q, we could characterize the allosteric properties of the enzymes in detail. Since tcLDH shows no cooperativity for the binding of NAD^+ and NADH, and the affinity for the coenzymes is independent of FBP (Taguchi et al., 1985), we performed all the kinetic experiments in the presence of a saturated concentration of NAD^+. All but the modified R173Q were activated by FBP and showed sigmoidal saturation curves for lactate in the absence of FBP (Figure 2A). Then we interpreted the results with the concerted transition model between the high-affinity (R) and low-affinity (T) states for substrate (Monod et al., 1965). Examining several possible models which differ in the numbers of interacting sites, we found that the kinetic properties of all the enzymes were described well by the model in which the numbers of substrate sites and effector sites were 4 and 2, respectively, as expected from the crystal structure of bsLDH (Piontek et al., 1990). The allosteric and kinetic parameters are summarized in Table I. The differences among the properties of the enzymes were explained by the differences in the parameters. The modified R173Q was determined to be locked in the R state, and the reduction in V might be due to the inactivation resulting from nonspecific modification.

Both the R173 → Q and R216 → L mutations reduced the apparent $S_{0.5}$ values (Figure 2A), but their mechanisms for the activation were different. The R173 → Q mutation significantly decreased the L value, the [T]/[R] ratio in the absence of ligands. In contrast, the activation by the R216 → L mutation was due to the decrease in $K_{R(lactate)}$ and the slight decrease in L. The contributions of the mutations were additive (Table I). The $K_{R(lactate)}$ values differed by as much as a factor of 8 among the enzymes, but the difference was much smaller than that for substrate affinity between the R and T states (at least 1000-fold in the cases of WT, R173Q, and R216L). In addition, the properties of R173Q:R216L

Table I: Allosteric and Kinetic Parameters of the WT and Mutant tcLDHs[a]

enzyme	L	$K_{R(lactate)}$ (mM)	V (units/mg)	$K_{R(FBP)}$ (mM)
WT	$1.7\ (1.8) \times 10^5$	21 (6.6)	12.8 (1.6)	$5.3\ (1.5) \times 10^{-2}$
R216L	$1.8\ (1.2) \times 10^5$	2.8 (0.5)	13.3 (0.6)	$1.0\ (0.1) \times 10^{-2}$
R173Q	78 (36)	16.8 (2.7)	12.3 (0.6)	4.1 (0.4)
R173Q:R216L	6.8 (1.3)	5.1 (0.3)	13.7 (0.2)	2.0 (0.5)
modified R173Q	[b]	4.2 (0.3)	3.31 (0.08)	[b]

[a] Parameters were determined as described under Experimental Procedures, and standard deviations are shown in parentheses. [b] The Michaelis-Menten model was applied.

were described quite well by the model (Figure 2B). These suggest that the properties of the R and T states were essentially conserved in all the enzymes analyzed here.

On the other hand, the R173 → Q mutation drastically increased the dissociation constant for FBP from the R-state enzyme, while the R216 → L mutation only slightly altered it (Table I). These results are reasonable because Arg173 interacts directly with the effector but Arg216 is separate from the FBP site (Figure 1).

The L value of the R173Q:R216L enzyme was 6.8 (Table I), suggesting that both the T and R conformations existed in detectable proportions in the free enzyme. Therefore, we successfully obtained material for structural analysis.

The regulation mechanism of tcLDH in lactate oxidation was described essentially by the concerted model. Because the reaction catalyzed by LDH is reversible and homotropic and heterotropic cooperativities were observed in both directions, the regulation mechanism of tcLDH in the pyruvate reduction, the physiological direction, may be achieved essentially by the concerted transition between two states with different substrate affinity. However, the severe substrate inhibition in pyruvate reduction makes it difficult to obtain accurate kinetic data, and also complicates the interpretation of kinetic results (Taguchi et al., 1985). Equilibrium binding study using oxamate, a nonreactive analogue of pyruvate, would give detailed information on the substrate affinity.

Conformational Equilibrium of the Catalytic Site. First, we examined whether the tetramer form is preserved in the allosteric transition. Under the range of protein concentrations which covered those used in both the kinetic and NMR experiments, the elution volumes from the gel filtration column all equated to a molecular mass of 120 kDa (data not shown). This corresponds to the mass of a tetramer from the 33-kDa subunits (Kunai et al., 1986). This indicates that a change in the subunit assembly is not involved in the allosteric mechanism of tcLDH, in contrast to some LDHs (Götz & Schleifer, 1978; Mayr et al., 1980; Clarke et al., 1989). Therefore, the allosteric transition of tcLDH was essentially independent of the protein concentration, enabling direct comparison of results obtained at different protein concentrations.

Then we analyze the conformation of the catalytic site through analyses on the conformation of the enzyme-bound coenzyme. We have investigated the conformation of the enzyme-bound NAD^+ by analyzing the transferred nuclear Overhauser effect (TRNOE) of NMR spectroscopy. TRNOE utilizes magnetization transfer in a chemically exchanging system to determine the conformation of a small ligand bound to a macromolecule (Albrand et al., 1979; Clore & Gronenborn, 1983; Rosevear & Mildvan, 1989). Therefore, TRNOE is suitable for "site-directed" conformational analysis of a large protein. In fact, we had already demonstrated that the con-

Table II: Initial Slopes of the Time-Dependent TRNOE of NAD^+ in the Presence of Modified and Unmodified R173Q Enzymes and R173Q:R216L, and Derived Distances of Proton Pairs

proton pair		LDH				
		modified R173Q		R173Q		R173Q:R216L
irr[a]	obsd[b]	slope[c] (s^{-1})	dist[d] (Å)	slope[c] (s^{-1})	dist[d] (Å)	slope[d] (s^{-1})
		Nicotinamide Riboside Moiety				
H_N1'	H_N2	1.06	2.2	lag[e]	>4	0.11
H_N2'	H_N2	lag	>4	0.45	2.6	0.43
H_N3'	H_N2	lag	>4	0.35	2.7	0.44
H_N5'	H_N2	lag	>4	0.36	2.7	0.47
H_N5''	H_N2	lag	>4	0.25	2.8	0.29
H_N1'	H_N6	0.37	2.6	0.35	2.7	0.39
H_N2'	H_N6	0.83	2.3	0.43	2.6	0.37
H_N3'	H_N6	0.78	2.3	0.16	3.0	0.33
H_N5'	H_N6	0.29	2.7	0.05	3.6	0.24
H_N5''	H_N6	0.09	3.2	lag	>4	0.14
H_N2'	H_N1'	0.31	2.7	0.27	2.8	0.30
H_N5	H_N4	1	2.2	1	2.2	1
H_N5	H_N6	0.23	2.8	0.31	2.7	0.32
		Adenosine Moiety[f]				
H_A1'	H_A8	lag	>4	0.14	3.1	0.09
H_A2'	H_A8	0.86	2.2	0.67	2.4	0.91
H_A3'	H_A8	0.12	3.1	0.22	2.9	0.38

[a] Irradiated protons. [b] Observed protons. The numbering of the protons is shown in Figure 3A, and subscripts N and A denote the nicotinamide riboside moiety and the adenosine moiety, respectively. [c] Initial slope of time-dependent TRNOE. [d] Derived interproton distance. [e] A lag phase (Clore & Gronenborn, 1983) was observed prior to the time-dependent decrease in the relative intensities. [f] In all experiments, no direct TRNOEs were observed on the H_A2 proton on irradiation of the ribose protons of the adenosine moiety.

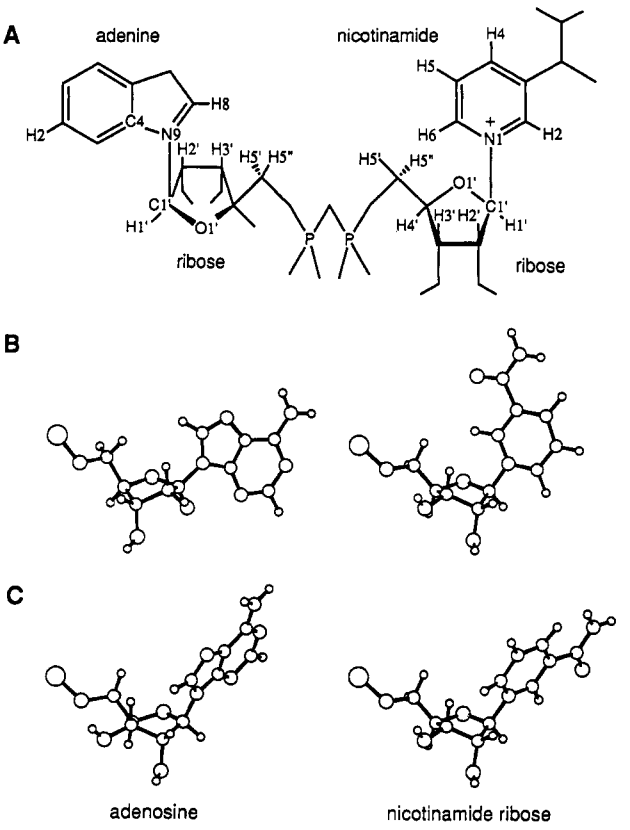

FIGURE 3: Numbering of atoms (A) and the conformations of NAD^+ bound to the unmodified (B) and modified (C) R173Q enzymes. The interproton distances listed in Table II were used.

formation of NAD^+ bound to tcLDH changes significantly upon binding of FBP (Machida et al., 1985) and chemical modification (Koide et al., 1989). The two conformations of bound NAD^+ should naturally represent the two different conformational states of the enzyme. LDHs catalyze the reaction through an ordered mechanism, in which the substrate is bound only to the enzyme–coenzyme complex (Holbrook et al., 1975; Clarke et al., 1985). In the reaction, stereospecific hydride transfer occurs between the substrate and the 4-*pro-R* proton (A-side proton) of the nicotinamide moiety of the coenzyme (Holbrook et al., 1975; Arnold et al., 1976). In the dehydrogenase of A-type specificity including LDH, the nicotinamide riboside moiety takes on an anti conformer, while it takes on a syn conformer in B-specific dehydrogenases (Rossmann et al., 1975; Levy et al., 1983; Eklund et al., 1984). In the case of tcLDH (Machida et al., 1984; Koide et al., 1989), the nicotinamide riboside moiety takes on an anti conformer when NAD^+ is bound to the enzyme–FBP complex and the chemically activated enzyme. The conformer is similar to those found in the A-specific dehydrogenases. On the other hand, when NAD^+ is bound to the free enzyme, the nicotinamide riboside moiety is in a syn conformer which is different from syn conformers found in other dehydrogenases. Therefore, the conformational change of the enzyme-bound coenzyme seems to be involved in the regulation mechanism of tcLDH, and the change can also be a structural probe for the catalytic site.

In this study, we analyzed the conformations of NAD^+ bound to the unmodified and modified R173Q enzymes, and R173Q:R216L, in the absence of FBP. Table II shows the initial slopes of the time-dependent TRNOE between the protons of NAD^+ in the presence of these enzymes. The initial slopes of the time-dependent TRNOE for proton pairs are inversely proportional to the sixth power of their interproton distances (Clore & Gronenborn, 1983). Accordingly, the conformations of NAD^+ bound to the unmodified and modified R173Q enzymes were determined from their interproton distances (Table II). In NAD^+ bound to the unmodified R173Q, the nicotinamide moiety took on the syn-C3′-endo form, the adenosine moiety being in the anti-C2′-endo form (Figure 3B; termed the S form). The dihedral angles, χ_N (C2–N1–C1′–O1′) and χ_A (C4–N9–C1′–O1′), were estimated to be about −10° and −100°, respectively. This conformation was almost identical to that of NAD^+ bound to the free WT (Machida et al., 1985). On the other hand, when NAD^+ was bound to the modified R173Q, both the moieties took on the anti-C3′-endo form (Figure 3C; termed the A form), being identical to that of NAD^+ bound to the WT–FBP complex and the modified WT (Machida et al., 1985; Koide et al., 1989). χ_N and χ_A were estimated to be about −75° and −70°, respectively.

The initial slopes of time-dependent TRNOE of NAD^+ in the presence of R173Q:R216L indicate that the H_N6 proton is close to the H_N2', H_N3', and H_N3' protons of the ribose. At the same time, the initial slopes indicate that the H_N2 proton is in proximity to the same ribose protons (Table II). Since the H_N2 and H_N6 protons are located symmetrically in terms of the glycoside bond (C1′–N1, Figure 3), these interproton distances cannot be satisfied in a single conformer. Therefore, the results obtained with R173Q:R216L were not interpretable in terms of a single conformation. We found that the slopes could be expressed as a mixture of those of the A state and those of the S state, that is:

$$\text{slope}_{ij}^{\text{obs}} = (a)\text{slope}_{ij}^{\text{A}} + (1 - a)\text{slope}_{ij}^{\text{S}}$$

where $\text{slope}_{ij}^{\text{obs}}$ is the normalized slope for the proton pair ij in the presence of R173Q:R216L, $\text{slope}_{ij}^{\text{A}}$ and $\text{slope}_{ij}^{\text{S}}$ are those

of the A form (here, modified R173Q) and the S form (R173Q), respectively, and a is the proportion of the A form. From the data in Table II, the proportion was determined to be 0.093 with a standard deviation of 0.076, by the least-squares method. This proportion corresponds to an [S]/[A] ratio = 9.7 ± 7.4.

These results and our previous studies (Machida et al., 1985; Koide et al., 1989) indicate that the conformation of NAD^+ bound to tcLDH is in equilibrium between the S and A forms. Only R173Q:R216L had an [S]/[A] ratio within the detectable range, while in the other enzymes, the equilibria were one-sided under these conditions so that only one conformation was detected. The results thus indicate the two-state conformational equilibrium of the catalytic site in contact with the bound coenzyme.

Allosteric Mechanism. We have established that the kinetic property of tcLDH is described by the concerted transition between the T and R states and demonstrated the dynamic equilibrium of the enzyme-bound NAD^+ between two conformers. According to the mechanism, FBP activates the enzyme by decreasing the [T]/[R] ratio. Actually, the conformation of NAD^+ bound to WT changes from the S form to the A form upon binding of FBP (Machida et al., 1985). The chemically modified enzymes were supposed to be locked in the R state, and the conformations of NAD^+ bound to them were the A form, which was identical to that of NAD^+ bound to the WT–FBP complex (Koide et al., 1989). NAD^+ dominantly took on the S form, when bound to R173Q with a large [T]/[R] ratio. As both mutations were introduced, the dynamic equilibrium of the NAD^+ conformation was shifted, and the mixture of the two conformers was observed. The [S]/[A] ratio of R173Q:R216L (ca. 10) agreed with its [T]/[R] ratio (=6.8), as determined by kinetic analyses. The agreement indicates that the conformation of the enzyme-bound NAD^+ is really a good probe of the conformation of the catalytic site, the most important part of the enzyme. The chemical and genetic modifications did not essentially alter the two conformations of the catalytic site, but changed the equilibrium between them. Therefore, we succeeded in identifying the dynamic equilibrium of the catalytic site, which has long been assumed to be the basis for allosteric regulation.

We have shown that the regulation of the activity of tcLDH is achieved through a significant difference in the substrate affinity between the R and T states. LDH catalyzes its reaction by an ordered mechanism, indicating that the correct configuration of the bound coenzyme is essential for the binding of the substrate. In the crystal structures, the nicotinamide moiety lies just beside the substrate and provides a hydrophobic environment for the substrate pocket (Abad-Zapatero et al., 1987; Clarke et al., 1989; Piontek et al., 1990). Therefore, the interconversion of the conformation of the nicotinamide riboside moiety must be involved in the change in the affinity for the substrate. However, the significant difference in affinity between the states suggested that another conformational rearrangement of the site might be also involved in the transition. Clarke et al. (1989) revealed that Arg171 plays a major role in substrate binding. On the other hand, the nicotinamide moiety of the coenzyme interacts with a hydrophobic pocket comprised of Val32, Val137, Leu167, and Ile250 in the ternary complex of dogfish LDH; Leu167 is a direct neighbor of Arg171 (Abad-Zapatero et al., 1987). These residues are completely conserved in tcLDH and bsLDH (Kunai et al., 1986). The connection of the conformational change between Arg171 and the hydrophobic pocket was suggested by the mutation of Ile250 of bsLDH which significantly decreased the affinity for the substrate (Clarke et al., 1989). Accordingly, the dynamic equilibrium of the region containing Arg171 and the nicotinamide binding pocket might possibly be the basis for the significant alteration of the affinity and the conversion of the coenzyme conformation.

The allosteric mechanism requires some machinery that combines the conformational equilibria of the individual catalytic sites in a concerted manner. It is suggested that the machinery involves the conformational equilibrium of the subunit interfaces, since each site lies in the individual subunit. Actually, differences in subunit interactions were revealed by comparisons of the crystal structures of the R and T states of other proteins (Kantrowitz & Lipscomb, 1988; Barford & Johnson, 1989; Perutz, 1989; Gouaux & Lipscomb, 1990; Schirmer & Evans, 1990; Ke et al., 1991). In tcLDH, the involvement of Arg216 in the allosteric transition suggested such differences in the subunit interaction between the R and T conformations, since Arg216 is situated near the P-axis interface (Figure 1). In bsLDH, the binding of FBP and the R173 → Q mutation shifted the tetramer–dimer equilibrium to the tetramer (Clarke et al., 1989), and the region near the 216th residue was recently demonstrated to switch the sensitivity of bsLDH to FBP (Zülli et al., 1990). In addition, the conformational difference in the adenosine moiety of the tcLDH-bound NAD^+ between the two states implies differences in the subunit interaction across the Q-axis, since the adenosine binding site is located near the Q-axis interface (Figure 1). All these suggest that the dynamic equilibria of the catalytic sites are linked to each other through the equilibrium of the global conformation between two states which differs in subunit interactions. Further studies should reveal the mechanism underlying the linkage between the local equilibria in the catalytic sites and the global one.

This research has provided fundamental evidence of the structure–function relationship of the allosteric enyzme, which links kinetic properties with the conformational equilibrium of the catalytic site. The conformational equilibrium between structurally defined states of the enzyme would be characterized directly by further studies, for example, specific incorporation of stable isotope-labeled amino acids followed by NMR spectroscopy. A protein conformation resides in a dynamic equilibrium between states in nature. The allosteric regulation is a distinct reflection of such an equilibrium.

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Analysis of Ground-State and Transition-State Effects in Enzyme Catalysis†

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ABSTRACT: "The entire and sole source of catalytic power is the stabilization of the transition state; reactant-state interactions are by nature inhibitory and only waste catalytic power". So reads a literature quote expressing the current view on enzyme catalysis proposed by Pauling over 40 years ago. Its validity is now examined by means of a "split-site" model in which an active site is subdivided into a region of binding and a region of reaction. Analysis of the resulting free energy levels clarifies several points of confusion regarding the nature of enzyme catalysis, including why enzyme/substrate complexes form if, indeed, they only "waste catalytic power". Circumstances are defined in which an evolving enzyme can both lower K_m (i.e., enhance substrate binding) and improve the forward catalytic rate while never meddling with the transition structure at the reactive site. It is argued that this process is most advantageously viewed as a substrate destabilization embodying "conserved" interactions at the binding region. Classical transition-state stabilization and an "anti-Pauling" effect are both capable of inducing rate accelerations. In certain circumstances, the latter can predominate as it does with many enzyme-like intramolecular reactions. Behavioral modes discussed herein are applicable to the chemistry of catalytic host/guest and abzyme systems.

M. I. Page listed no fewer than 21 published theories of enzyme catalysis including some with colorful names such as orbital steering, propinquity, vibrational activation, stereopopulation control, and group transfer hydration (Page, 1987). The recent trend, however, has been to adopt a simple and elegant idea originated over 40 years ago by Pauling (1946, 1948). In his view, an enzyme accelerates a reaction by binding strongly to a transition structure. This mode of catalysis, now known as "transition-state stabilization", will be deliberated in the present paper. For the moment, note simply

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