Regulatory Mechanism of *L*-Alanine Dehydrogenase from *Bacillus subtilis*

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L-alanine dehydrogenase from *Bacillus subtilis* exhibits allosteric kinetic properties in the presence of Zn^{2+}. Zn^{2+} induces the binding of substrate (*L*-alanine) to be cooperative at pH 8.0. The effect of pH variation between pH 7.0 and pH 10.0 on the inhibition by Zn^{2+} correlates with the pH effect on the K_m values for *L*-alanine within these pH range indicating that Zn^{2+} and substrate compete for the same site. No such cooperativity is induced by Zn^{2+} when the reaction is carried out at pH 10. At this higher pH, Zn^{2+} binds with the enzyme with lower affinity and noncompetitive with respect to *L*-alanine. Inhibition of *L*-alanine dehydrogenase by Zn^{2+} depends on the ionic strength. Increase in KCl concentation reduced the inhibition, but allosteric property in Zn^{2+} binding is conserved. A model for the regulatory mechanism of *L*-alanine dehydrogenase as a noncooperative substrate-cooperative cofactor allosteric enzyme, which is compatible in both concerted and the sequential allosteric mechanism, is proposed.

Introduction

L-Alanine dehydrogenase (*L*-alanine: NAD^+ oxidoreductase, EC 1.4.1.1) is a NAD^+ dependent amino acid dehydrogenase which catalyzes the reversible oxidative deamination of *L*-alanine to pyruvate. The enzymological and kinetic properties of the enzyme have been investigated from various bacteria[1-6] and several bacterial *L*-alanine dehydrogenase genes have been cloned recently.[7-9] Although the equilibrium of the reaction favors the reductive aminating direction, *L*-alanine dehydrogenase is found to catalyze the deamination of *L*-alanine in many *Bacillus* species. Therefore, in these organisms, the enzyme apparently plays as a key enzyme in the catabolism of *L*-alanine to pyruvate and for further catabolism by tricarboxylic acid cycle in order to provide the energy necessary for the sporulation during differentiation of these cells.[3,8,10] *B. subtilis L*-alanine dehydrogenase is an oligomeric protein which is consisted of six identical subunits of molecular weight between 38,000 and 48,000 and has been known to be a nonallosteric enzyme which is stable over the pH range 5-11.[11]

In the previous paper, we reported that under certain conditions, *L*-alanine dehydrogenase from *B. subtilis* exhibits an unusual allosteric properties in the presence of the allosteric competitive inhibitor, Zn^{2+}, otherwise it behaves as a normal Michaelis-Menten type enzyme.[12] The present work was undertaken to study the nature of the Zn^{2+} binding site further and to discuss the regulatory mechanism for this enzyme.

Experimental Section

Kinetic studies of *L*-alanine dehydrogenase catalyzed reactions. Pretreatment of purchased *L*-alanine dehydrogenase was done as descrived elsewhere.[12] To study the pH effect, 0.025 M of various buffer of pH range 7.0 to 10.0, were used. Buffers generally used are HEPES buffer (pH 7.0-8.0), Tris buffer (pH 8.0-8.8), glycine buffer (pH 8.8-9.8) and Na-bicarbonate buffer (pH 9.5-10.0). For the kinetic run, standard assay mixture contained 0.025 M Tris-HCl buffer, pH 8.0, 1 mM NAD^+, 0.6 mM-12 mM *L*-alanine and enzyme in final volume of 0.5 mL. Free *L*-alanine concentrations are corrected for metal complex formation using the logarithm of stability constants, log K_1=5.1 M^{-1} for Zn-alanine.[13] Reactions were started by the addition of enzyme and the initial velocities were measured spectrophotometrically by monitoring the increase of NADH at 340 nm at 25 °C using Shimadzu UV 3101 PC Spectrophotometer equipped with thermoelectrical temperature controller, TCC-260 and UV 2101/3101 Optional Kinetic Software.

Data Analysis. The initial velocity data were analyzed on a computer with a nonlinear least square analysis program. Velocity data were plotted and fitted iteratively to Michaelis-Menten equation for hyperbolic saturation curves and to Hill equation when the cooperative properties are noticed.

Results and Discussion

Effect of pH variation on the inhibition of *L*-alanine dehydrogenase by Zn^{2+}. *L*-Alanine dehydrogenase catalyzed deamination reaction is inhibited by divalent metal ions. Inhibition by Zn^{2+} is of particular interest because of this metal ion is shown to induce the positive cooperativity in binding of substrate.[12] In order to study the nature of Zn^{2+} binding site, the effect of pH variation between pH 7.0 to 10.0 on the kinetic parameters of *L*-alanine dehydrogenase and on the degree of inhibition by Zn^{2+} are compared. As shown in Figure 1, the effects on the inhibition by Zn^{2+} correlates with the variation of K_m values for *L*-alanine within the chosen pH range. These results especially at the lower pH's affirm our interpretation that Zn^{2+} at low concentration competes for the same site with substrate. The Hill coefficients determined in the substrate binding curves in the pre-

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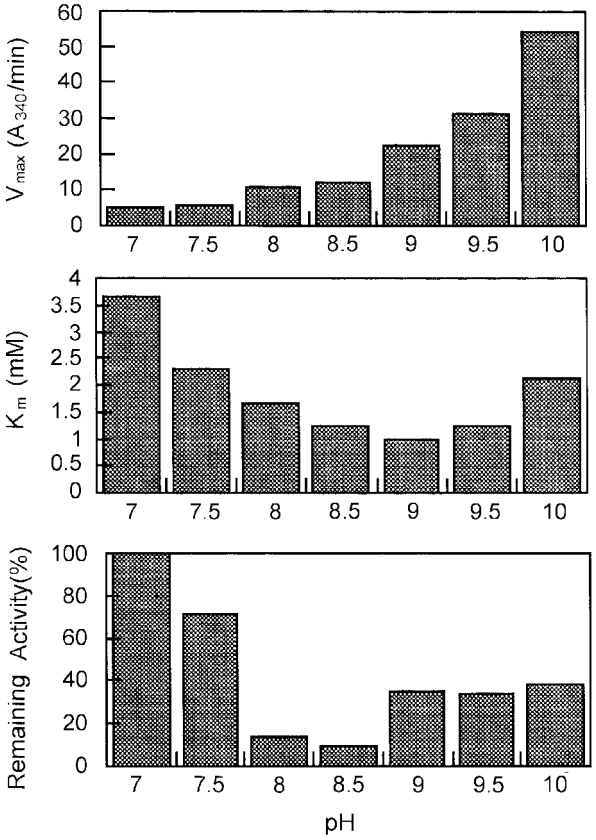

Figure 1. pH effect on the kinetic parameters and Zn^{2+} induced inhibition of *L*-alanine dehydrogenase activity. At each indicated pH, V_{max} and K_m were determined from the initial velocity data. Remaining activity was obtained from the velocity data measured in the presence of 20 μM Zn^{2+} in the assay mixture.

sence of Zn^{2+} are near unity at the higher pH's indicating that binding of substrate is not cooperative in the presence of metal ion at those pHs (Table 1). The mutual exclusion of noncooperative substrate and cooperative inhibitor at enzyme active site can cause transformation of substrate binding from noncooperative to cooperative.[14,15] On the basis of site directed mutagenesis, Kuo *et al.*[15,16] suggested that an active site Cys residue is the site for metal and substrate compete with each other in the *E. coli* ornithine transcarbamoylase. It was also proposed that Zn^{2+} binds to ornithine transcarbamoylase in two consecutive steps; at first, reversible binding with conformation change by subunit interaction, then irreversible inactivation by tight binding. For *L*-alanine dehydrogenase, although the results from pH studies might imply the involvement of the Cys sulfhydryl group in binding of Zn^{2+}, this is not confirmed. No time dependent inactivation of *L*-alanine dehydrogenase upon Zn^{2+} binding was observed (data not shown) and thus the allosteric property observed with this enzyme can be explained by the simple direct exclusion of cooperative cofactor by noncooperative substrate.

Table 1. pH effect on the inhibition of *L*-alanine dehydrogenase from *Bacillus subtilis* by Zn^{2+}

pH	% Inhibition	Hill coefficient (n_H)
7.00	3.1	2.16
7.56	28.4	1.92
8.00	85.7	1.60
9.17	59.8	0.97
10.0	64.8	1.02

Two Zn^{2+} binding sites of the *L*-alanine dehydrogenase from *B. subtilis*. Previously we have reported that at pH 8.0, Zn^{2+} binds to *L*-alanine dehydrogenase with homotropic coopeative characteristics. Kinetics of *L*-alanine binding with the enzyme at pH 8.0 showed typical Michaelis-Menten type hyperbolic curve. Zn^{2+} at μmole level of concentration, inhibits the enzyme competitive with respect to *L*-alanine as shown by Lineweaver-Burk plot, but the lines concaved upward revealing the positive cooperativity of *L*-alanine binding (Figure 2(A)). The alternate possible causes for the apparent cooperativity such as changes in enzyme polymerization and depolymerization[17] and the random

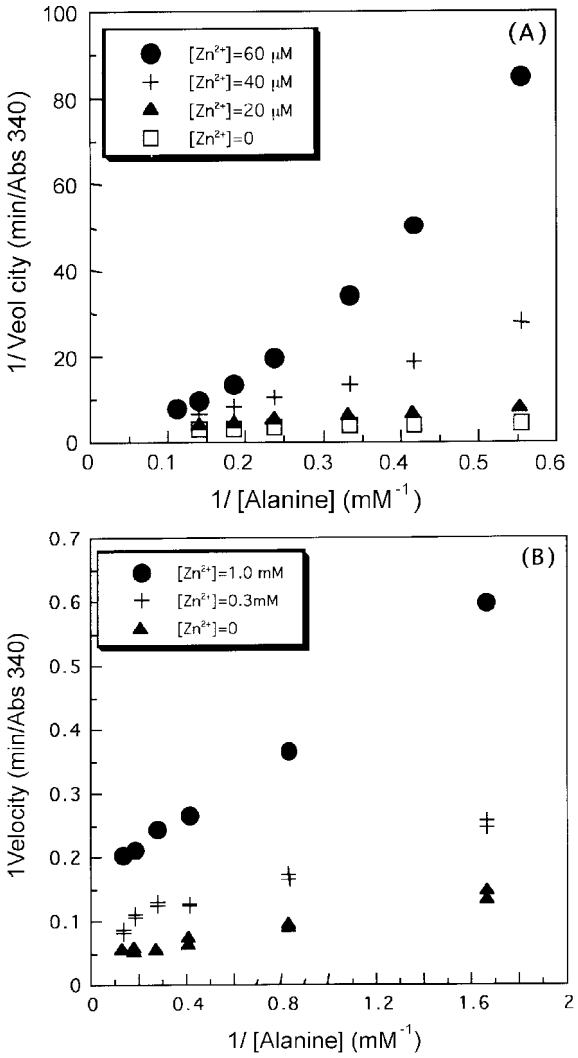

Figure 2. Effect of Zn^{2+} on *L*-alanine dehydrogenase activity at pH 8.0 and pH 10.0. Kinetic data are analyzed by double reciprocal plot at indicated concentration of Zn^{2+}. (A): pH 8.0, velocity lines concaved upward and (B): pH 10.0, velocity lines are linear even at 1 mM Zn^{2+}.

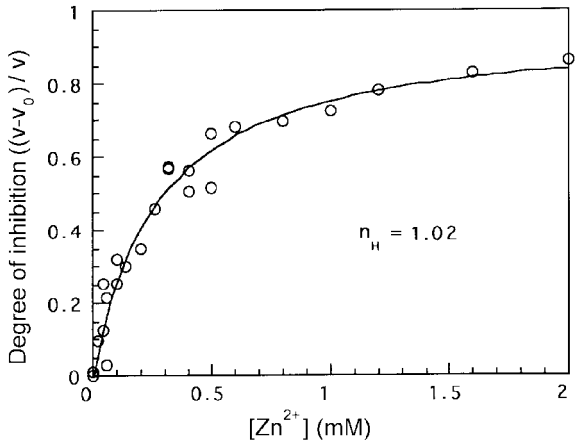

Figure 3. Zinc ion binding curve of *L*-alanine dehydrogenase at pH 10.0. Zn^{2+} binding curve is obtained by plotting the degree of inhibition, $(v-v_o)/v$, versus concentrartion of zinc ion where v is the velocity in the absence of inhibitor. Experimental data are fitted to Hill equation.

bindings of 2 substrates with enzyme[18] are ruled out. Binding of NAD^+, the other substrate, is not affected by Zn^{2+}.

These results leaded a conclusion that substrate and Zn^{2+} competes for a same site of the enzyme active site. A quite different situation is found when Zn^{2+} reacts with *L*-alanine dehydrogenase at pH 10.0 (Figure 2(B) and Figure 3). Double reciprocal plot of *L*-alanine binding in the presence of Zn^{2+} is linear and the inhibition appeared to be noncompetitive. This is in accordance with the early finding by Yoshida and Freese.[11] Zn^{2+} binding curve is hyperbolic (Figure 3). Such results may be suggestive of the existence of at least two distinct Zn^{2+} binding sites in *L*-alanine dehydrogenase, one is the same site as substrtate binding site and the other, different from substrate site. It is the substrate site that Zn^{2+}

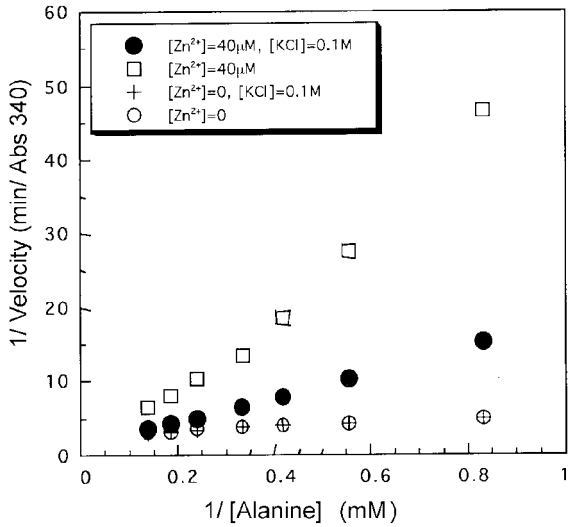

Figure 4. Effect of KCl on the inhibition of *L*-alanine dehydrogenase by Zn^{2+}. *L*-Alanine dehydrogenase catalyzed reaction velocities were measured with varied concentration of alanine and 0.1 M KCl was added to both Zn^{2+} inhibited and control assay mixture.

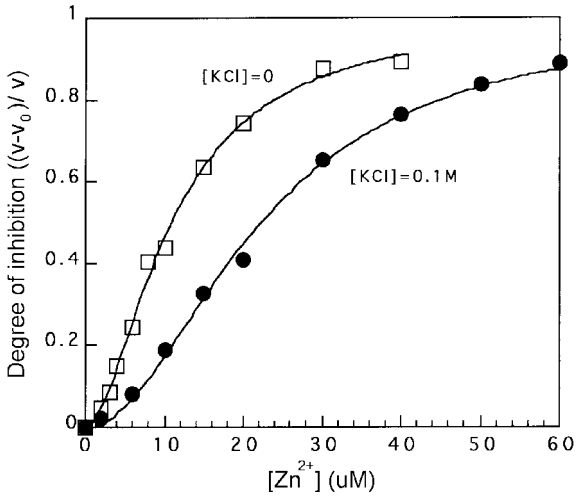

Figure 5. Effect of ionic strength on the *L*-alanine dehydrogenase inhibition by Zn^{2+}. Velocities are measured with Zn^{2+} concentration varied. Kinetic data are fitted to the Hill equation and the Hill coefficients are determined as 1.767 and 1.768 with and without KCl, respectively.

binds with relatively higer affinity and acts as an allosteric inhibitor at pH 8.0.

Effect of ionic strength on the inhibition of *L*-alanine dehydrogenase by Zn^{2+}. Zn^{2+} inhibits *L*-alanine dehydrogenase activity and exerts the apparent cooperativity in alanine binding with enzyme in the absence of KCl. The increase of KCl concentration reversed the inhibition and reduced the cooperativity of substrate binding as shown in Figure 4. The sigmoidity of Zn^{2+} binding is not affected by KCl, but I_{05} is increased (Fiure 5). It is reasonable to believe that KCl doesn't change the allosteric property of binding of Zn^{2+} but reduces the number of bound Zn^{2+} to be displaced by substrate. The reversal of Zn^{2+} induced inhibition by KCl is shown to be concentration dependent. Zn^{2+} induced inhibition is reduced upto the concentration of KCl is increased to 0.1 M and beyond this concentration, the inhibition was increased again. The fact that KCl doesn't affect the cooperative binding of Zn^{2+} but increases the concentration of Zn^{2+} to give same degree of inhibition may imply that the affinity between protein and ligand is influenced by electrostatic interaction but the changes in ionic strength does not modify the allosteric equilibrium, T/R.

Effect of competitive inhibitor on Zn^{2+} binding. In Figure 6, the effect of D-Phe, a substrate competitive inhibitor on the Zn^{2+} binding with *L*-alanine dehydrogenase is shown. Zn^{2+} binding curve in the presence of D-Phe is shifted to right without altering Hill coefficient. A comparable curve for the Zn^{2+} binding could be obtained with increased substrate concentration.[12]

Regulatory mechanism of *L*-alanine dehydrogenase from *Bacillus subtilis*. It has been known for a few cases in which a specific substrate binds with enzyme cooperatively only in the presence of competitive allosteric cofactor. For instance, *A. parasticus* mannitol-1-phosphate dehydrogenase catalyzed reactions, fructose-6-phosphate binds with enzyme with positive cooperativity in the peresence of cer-

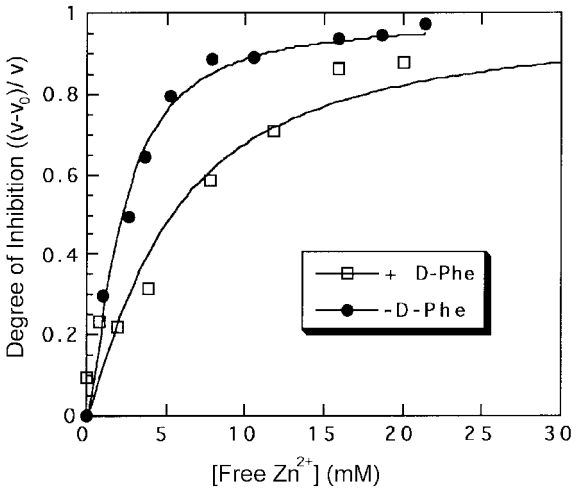

Figure 6. Effect of the substrate competitive inhibitor on the Zn^{2+} binding with *L*-alanine dehydrogenase. Enzyme reaction velocities were measured with varied concentration of Zn^{2+} with or without 30 mM of D-Phe.

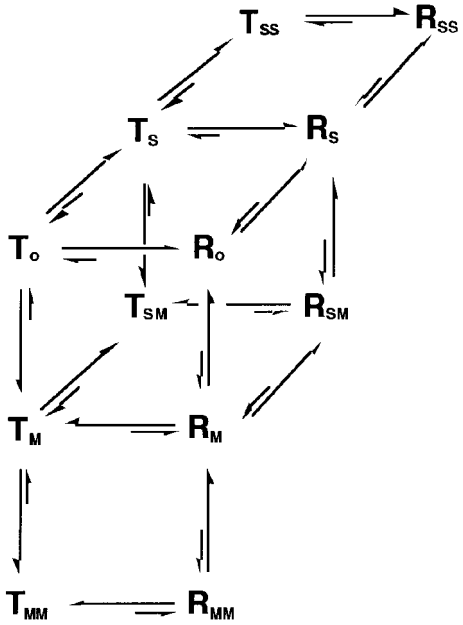

Figure 7. Proposed allosteric model for *L*-alanine dehydrogenase from *Bacillus subtilis*. Although the exact number of binding site of *L*-alanine dehydrogenase is not known, 2 binding sites are assumed in this model for the simplicity. M: metal inhibitor S: substrate.

tain divalent metal ions[19] and ATP, a competitive inhibitor for phosphorylase b is known to induces kinetic cooperativity in the binding of glucose-1-phosphate, the substrate.[20] In these enzymes, substrate and cofactor (inhibitor) bind to separate sites and exert mutual allosteric effect each other. Cofactor has much stronger preference for allosteric conformation (T or R) than substrate does so that only cofactor binding is seen as cooperative. On the other hand, in ornithine transcarbamoylase, substrate and cofactor compete for the same site. Substrate binding is not cooperative, *i.e.*, substrate binds to both R and T state with same affinity and only cofactor shows the preference for T/R state and exhibits cooperativity in binding. Kuo[14,21] concluded that the apparent cooperativity of substrate binding in ornithine transcarbamoylase is the result of competitive binding of Zn^{2+} and *L*-ornithine.

The results from this work and earlier studies indicate that *L*-alanine dehydrogenase from *B. subtilis* is an allosteric enzyme in which Zn^{2+} is an allosteric cofactor that binds with enzyme at the same site as substrate at pH < 9.0. Alanine binding is not cooperative but Zn^{2+} binding with the same site is. Thus *L*-alanine dehydrogenase is believed to be a "non cooperative substrate-cooperative cofactor allosteric system".

We propose here a plausible regulatory model for *B. subtilis L*-alanine dehydrogenase to explain our experimental results (Figure 7). In accordance with MWC allosteric model,[22] the enzyme exists in two conformations, T and R states. The substrate binds to both T and R state with similar affinity and a competitive inhibitor (M) binds to the same site only in T conformation. Although *B. subtilis L*-alanine dehydrogenase is known as a hexameric protein, a dimeric enzyme with one binding site in each subunit is assumed for simplicity. In the absence of M, R_o/T_o is large and substrate(S) binds to both R_o and T_o as well and thus no cooperativity in binding can be observed. M binds to one subunit site of T_o to form T_m so that the allosteric equilibrium is shifted toward T state. Binding of M to one site will results in the conformational change due to subunit interactions. Zn^{2+} binding with enzyme is thus positive cooperative and Zn^{2+} binding curve will be sigmoidal. When the enzyme is in primarily T_{MM} state, an S molecule is required to displace an M molecule from one subunit to bind the same site to form T_{MS}. The second M will be displaced more easily and substrate can bind faster than the first. This makes the substrate binding curve sigmoid in the presence of Zn^{2+}. This model is compatible with both MWC model and KNF model. The exclusive binding of inhibitor fit to the former concerted model but the competitive binding between substrate and inhibitor can be explained by the latter, sequential model.[23]

The physiological function of the Zn^{2+} induced cooperativity is not clear. Since *L*-alanine dehydrogenase is more active in deaminating catabolic pathway, this enzyme might be involved in the regulation of this catabolic pathway and provides the control mechanism to meet physiological requirement. At pH 8.0 or lower, the enzyme must be primarily in ibhibited state by cellular Zn^{2+} concentration (recall that K_m for alanine is lower mM and $I_{0.5}$ of Zn^{2+} is lower micromolar) and upon the physiological call for the catabolic flux, the enzyme activity can be regulated successfully by cotrolling the level of substrate. At higher pH, Zn^{2+} binds to enzyme site distinct from substate binding site without cooperativity. In this nonphysiological conformation, substrate binding site must be desensitized for the binding of Zn^{2+} thus exhibits noncompetitive and noncooperative bindings.

It has been reported that a nonallosteric enzyme could be transformed to an allosteric enzyme by point mutation without the aid of external cofactor.[24] Therefore, it can't be com-

pletely ruled out the possibility of *L*-alanine dehydrogenase to have isomeric form with intrinsic allosteric property in parallel with the evolutionary transformation to the cofactor aided allosterism.

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