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Unusual Allosteric Property of L-alanine Dehydrogenase from *Bacillus subtilis*

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Kinetic studies of L-Alanine dehydrogenase from *Bacillus subtilis*-catalyzed reactions in the presence of Zn^{2+} were carried out. The substrate (L-alanine) saturation curve is hyperbolic in the absence of the metal ion but it becomes sigmoidal when Zn^{2+} is added to the reaction mixture indicating the positive cooperative binding of the substrate in the presence of zinc ion. The cooperativity of substrate binding depends on the zinc ion concentration: the Hill coefficients (n_H) varied from 1.0 to 1.95 when the zinc ion concentration varied from 0 to 60 μM. The inhibition of AlaDH by Zn^{2+} is reversible and noncompetitive with respect to NAD^+ ($K_i = 5.28 \times 10^{-5}$ M). Zn^{2+} itself binds to AlaDH with positive cooperativity and the cooperativity is independent of substrate concentration. The Hill coefficients of substrate biding in the presence of Zn^{2+} are not affected by the enzyme concentration indicating that Zn^{2+} binding does not change the polymerization–depolymerization equilibria of the enzyme. Among other metal ions, Zn^{2+} appears to be a specific reversible inhibitor inducing conformational change through the intersubunit interaction. These results indicate that Zn^{2+} is an allosteric competitive inhibitor and substrate being a non-cooperative *per se*, excludes the Zn^{2+} from its binding site and thus exhibits positive cooperativity. The allosteric mechanism of AlaDH from *Bacillus subtilis* is consistent with both MWC and Koshland's allosteric model.

Keywords: Alanine dehydrogenase, Allosteric inhibitor, Enzyme regulation, Kinetics.

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Introduction

L-Alanine dehydrogenase (AlaDH, L-alanine: NAD^+ oxidoreductase, EC 1.4.1.1) catalyzes the reversible oxidative deamination of L-alanine to pyruvate.

$$\text{L-Alanine} + NAD^+ + H_2O \rightleftarrows \text{Pyruvate} + NH_3 + NADH + H^+$$

The enzyme was purified and some enzymatic properties were investigated from *B. subtilis* (Yoshida and Freese, 1964; Yoshida, 1965), *B. sphaericus* (Ohshima and Soda, 1979), *B. lichenformis* (McCowen and Phibbs, 1974), *S. clavulgerus* (Ahranowitz and Friedrich, 1980), *S. phaeochromogens* (Itoh and Morikawa, 1983), *P. lapideum* (Sawa *et al.*, 1994) and soybean bacteroids (Smith and Emerich, 1993). Recently several bacterial AlaDH genes have been cloned (Kuroda *et al.*, 1990; Andersen *et al.*, 1992; Siranosian *et al.*, 1993). Kinetic studies have shown that the *B. subtilis* enzymes (Grimshaw and Cleland, 1981; Grimshaw *et al.*, 1981) and those from *S. phaeochromogens* (Lee *et al.*, 1991) react by sequential ordered mechanism. The enzymes from some nitrogen-fixing bacterial systems are reported to follow the Theorell–Chance mechanism (Rowell and Stewart, 1976; 1982; Smith and Emerich, 1993). Although the equilibrium of the reaction favors the reductive aminating direction, AlaDH is found to catalyze the deamination of L-alanine in many *Bacillus* species. In these organisms, the enzyme apparently plays a key role 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 (McCowen and Phibbs, 1974; Frankel and Jones, 1980; Siranosian *et al.*, 1993).

B. subtilis AlaDH is a hexameric protein which consists of six identical subunits of molecular weight between 38,000 and 48,000 (Yoshida and Freese, 1964; Yoshida, 1965 and 1967). The enzyme is known as a nonallosteric

enzyme and is inhibited by SH-blocking reagents (Yoshida and Freese, 1970). Some divalent metal ions such as Hg^{2+}, Cu^{2+} or Zn^{2+} are known as potent inhibitors of the enzyme (Yoshida and Freese, 1970). Among others, inhibitions caused by Zn^{2+} are of particular interest in relation to the regulatory property of certain anabolic enzyme (Kuo *et al.*, 1982) and NAD^+-dependent dehydrogenase (Foreman and Niehaus, 1985).

In the present report, the kinetics of *B. subtilis* AlaDH-catalyzed reactions in the presence of Zn^{2+} were studied to show for the first time that only in the presence of the allosteric competitive inhibitor would the enzyme exhibit an unusual allosteric property; otherwise it behaves as a normal Michaelis–Menten type enzyme.

Materials and Methods

Materials *B. subtilis* AlaDH, β-NAD^+, L-alanine, Trizma base, dithiothreitol (DTT) and ethylenediaminetetraacetate (EDTA) were purchased from Sigma Chemical Co. (St. Louis, USA.). $ZnCl_2$, $HgCl_2$, $CuCl_2$, and $NiSO_4$ were obtained from Aldrich Chemical (Milwaukee, USA). NANOpure deionized water of conductivity no less than 17.5 Mohm-cm at 25°C was used throughout this study.

Assay of L-alanine dehydrogenase activity AlaDH from *B. subtilis* purchased from manufacturer as a suspension in 2.4 M ammonium sulfate was centrifuged and the precipitate was redissolved in 0.01 M potassium phosphate buffer, pH 7.2 containing 1 mg/ml bovine serum albumin and 0.1 mM DTT. The enzyme was desalted and concentrated using Amicon ultrafiltration system and YM-10 membrane. For the kinetic run, standard assay mixture contained 0.025 M Tris-HCl buffer, pH 8.0, 1 mM NAD^+, 0.6–12 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}, 8.5 M^{-1}, and 6.0 M^{-1} for Zn-alanine, Cu-alanine and Ni-alanine, respectively (O'sullivan, 1969). Reactions were started by 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 (1) for hyperbolic saturation curves and Hill equation (2) when the cooperative properties were noticed.

$$v = \frac{V_{max} \times [S]}{K_m + [S]} \quad (1)$$

$$\frac{v}{V_{max}} = \frac{L^{nH}}{K + L^{nH}} \quad (2)$$

Where S is the substrate, L is a ligand, K is a constant, and nH is the Hill coefficient.

Results and Discussions

Effect of Zn^{2+} on the AlaDH activity The initial velocities (v_0) of the AlaDH-catalyzed reactions as a function of L-alanine concentration with or without Zn^{2+} are shown in Fig. 1. In the absence of Zn^{2+} AlaDH follows the Michaelis–Menten kinetics as shown by the hyperbolic saturation curve (Fig. 1a). However, when the inhibitor, Zn^{2+} is added to assay mixture, the saturation curves become significantly sigmoidal indicating the positive cooperative binding of substrate. The data were fitted into Hill equation into obtain the Hill coefficients, n_H = 1.0, 1.3, 1.7, and 1.95 at Zn^{2+} concentrations of 0, 0.02, 0.04, and 0.06 mM, respectively. These results may indicate that AlaDH has two or more interacting binding sites for L-alanine and the extent of cooperativity of substrate binding depends on Zn^{2+} concentration. Figure 1b represents the Lineweaver–Burk plot of the same data. Without the inhibitor, the plot is linear (K_m for L-alanine = 1.66 mM), but in the presence of Zn^{2+}, the curves concaved upward revealing the positive cooperativity of AlaDH in the presence of Zn^{2+}. Upon extrapolation, the plots converged at abscissa indicating that Zn^{2+} binds to enzyme competitively with L-alanine.

Effect of Zn^{2+} on the binding of NAD^+ In order to investigate the effect of Zn^{2+} on the binding of the other substrate for the deaminating reaction of AlaDH, the initial velocities were determined at varying concentrations of NAD^+ (0.4–2.0 mM) and saturating level of L-alanine. In Fig. 2, double reciprocal plots are shown with or without the added Zn^{2+}. As can be seen in this figure, Zn^{2+} inhibits AlaDH linear noncompetitively with respect to NAD^+ and no sigmoidity is observed. Inset is the secondary plot of the slopes of this plot versus $[Zn^{2+}]$, and $K_i = 5.28 \times 10^{-5}$ M was obtained.

Kinetic determination of Zn^{2+} binding to AlaDH At fixed concentrations of both L-alanine and NAD^+, the initial velocities were measured at varying concentrations of Zn^{2+} (Fig. 3). Velocity data are plotted in terms of inhibition degree $((v - v_0)/v)$, where v is the velocity in the absence of inhibitor, versus Zn^{2+} concentration at three different fixed concentrations of L-alanine to obtain saturation curve for Zn^{2+} binding (Kuo *et al.*, 1982; Kuo, 1983). The inhibition degree increases with increasing concentration of Zn^{2+} and binding curves constructed indirectly from kinetic data are sigmoidal indicating the cooperative binding of Zn^{2+} to the enzyme. When the L-alanine concentration is increased, the curve is shifted to the right and $I_{0.5}$ increases as expected for competitive binding of L-alanine and Zn^{2+}. The data are analyzed according to Hill equation and the Hill coefficients of 1.75–1.80 were obtained for these plots. From these results it can be said that Zn^{2+} binds to the AlaDH with homotropic cooperativity by the conformational change in

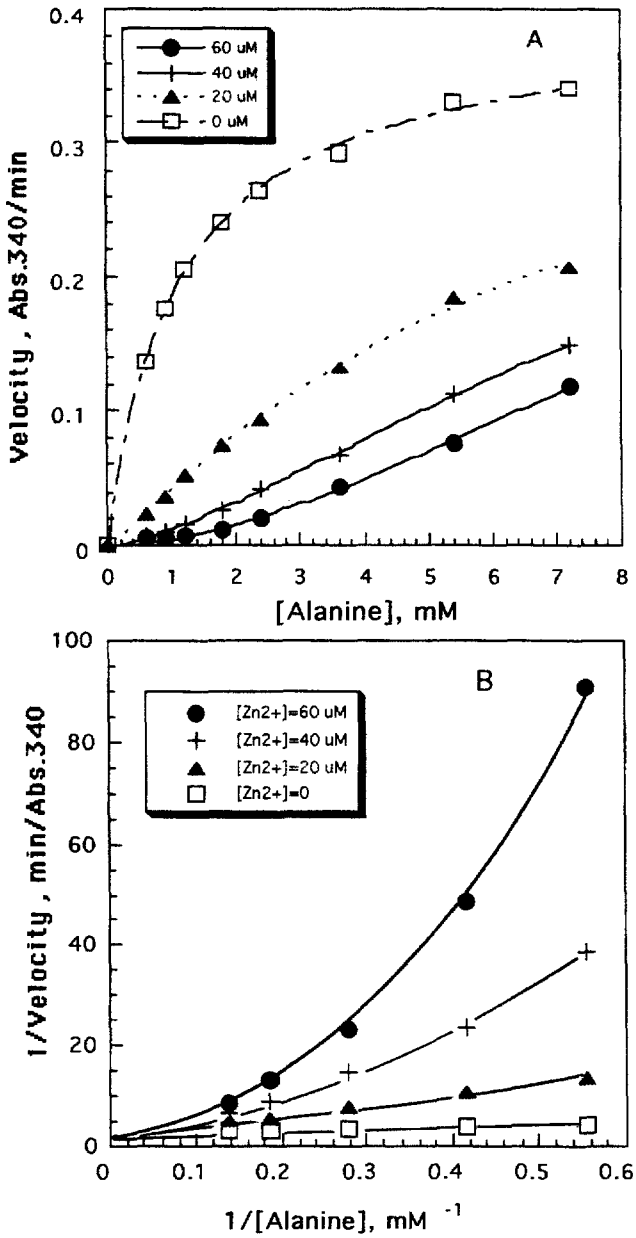

Fig. 1. Activity of AlaDH from *B. subtilis* as a function of L-alanine concentration in the absence of, or presence of 0.02 mM, 0.04 mM, or 0.06 mM Zn^{2+}. (A) Direct velocity versus [L-alanine] plot. Double kinetic measurements were carried out at 25°C. L-Alanine concentrations were corrected for the Zn–alanine complex formation. Standared assay mixture contained 0.025 M Tris-HCl buffer, pH 8.0, 1 mM NAD^+, fixed three different concentrations of Zn^{2+} and L-alanine concentrations as indicated in a final volume of 0.5 ml. Reactions were started by the addition of enzyme. Lines are the best fitted line to Hill equation. The Hill coefficients obtained are varied with varying concentration of Zn^{2+}. (B) Double reciprocal plot of data (A).

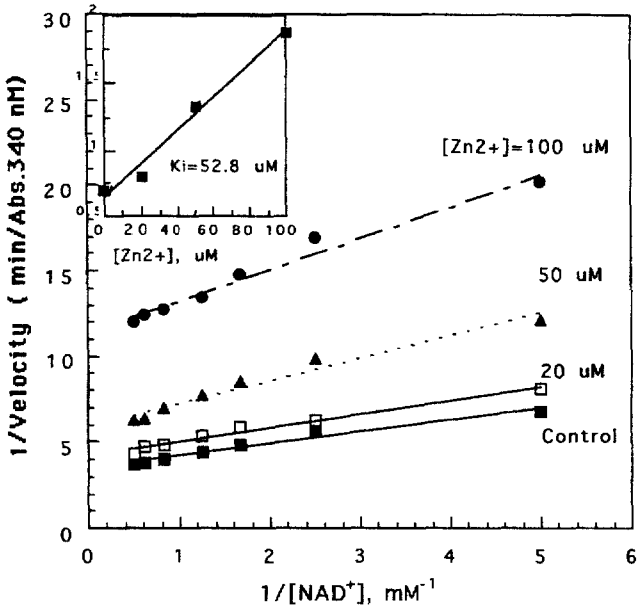

Fig. 2. Inhibition of AlaDH by Zn^{2+} as a function of NAD^+ concentration. Assay condition is the same as in Fig. 1. Alanine concentration is fixed to 12 mM, and four differnt Zn^{2+} concentrations as indicated. Inset is the replot of the slopes of the plots versus Zn^{2-}.

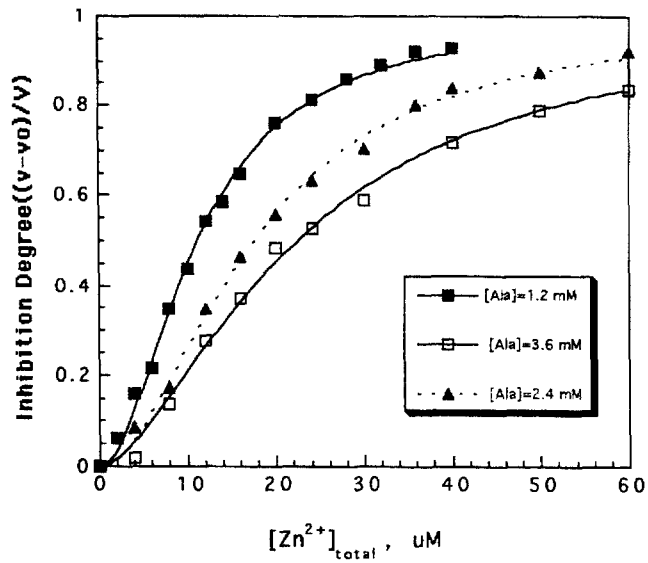

Fig. 3. Inhibition of AlaDH as a function of Zn^{2+} concentration. Assay conditions are the same as in Fig. 1. Velocities are measured with Zn^{2+} concentration varied as indicated and three different fixed L-alanine concentrations. Alanine concentrations are shown as of the total concentration. Experimental data were fitted to Hill equation and the lines are computor drawn using the best fit parameters.

protein through the subunit interaction and this cooperativity is not affected by the concentration of substrate. Substrate binds to enzyme at the same site as Zn^{2+}, but without cooperativity and the only effect is the displacement of the inhibitor from its binding site. The apparent cooperative binding of L-alanine in the presence of Zn^{2+}, is, therefore, attributed by the direct competitive binding of Zn^{2+}, and not the real heterotropic effect of the

allosteric inhibitor. Since NAD^+ does not compete with Zn^{2+} for the binding site, the binding of Zn^{2+} does not induce the cooperativity in the binding of NAD^+.

Effect of enzyme concentration on the Zn^{2+}-induced cooperativity The apparent cooperativity in the binding of a ligand can also be caused by the enzyme having a different number of binding sites in the enzyme monomer and the polymer existing in equilibrium (Hathaway and Criddle, 1966; Nichol *et al.*, 1967). Since AlaDH is a hexamer and the polymerization-depolymerization equilibrium depends on the amount of enzyme, the existence of a change in the polymerization-depolymerization in the presence of a competitive inhibitor would be reflected by the enzyme concentration dependence of the sigmoidal saturation curve (Hammes, 1982). To test this, the L-alanine binding studies in the presence of a fixed concentration of Zn^{2+} were carried out at three different concentrations of enzyme. Figure 4a shows that all the substrate satruration curves are sigmoidal and their V_{max} decrease with decreasing enzyme concentration. These results are replotted in terms of v_0/V_{max} vs L-alanine concentration (Fig. 4b). The Hill coefficients obtained by fitting to the Hill equation were 1.73, 1.77, and 1.76 with enzyme concentrations of 5.55 pM, 11.10 pM and 22.20 pM, respectively.

These results indicate that Zn^{2+} induced cooperativity of AlaDH is independent of enzyme concentration and therefore, is not the result of the changes in the polymerization-depolymerization equilibria induced by Zn^{2+}.

The effect of Zn^{2+} on the AlaDH is reversible. Both enzymes incubated in reaction buffer with or without 0.04 mM Zn^{2+} achieved the same activities after equimolar concentrations of EDTA were added. The apparent kinetic cooperativity in the bisubstrate oligomeric enzyme may also be observed under certain conditions, when the two substrates bind in random addition mechanism (Wells *et al.*, 1976; Neet, 1980). In such a case, the cooperativity in the saturation curve of one substrate decreases with increase in the concentration of the second substrate and eventually disappears at the saturating level of the second substrate. Zn^{2+}-induced cooperativity of AlaDH is not changed significantly when the concentration of NAD^+ is increased to saturating level. This observation can rule out the possible change of mechanism to random binding by the addition of Zn^{2+}. Zn^{2+}-induced cooperativity of AlaDH depends on the pH. Previously, Zn^{2+} was reported to inhibit the *B. subtilis* AlaDH with linear noncompetitive behavior at its optimum pH (10.5) for deamination reaction (Yoshida and Freese, 1970). Zn^{2+}-binding curve obtained indirectly by kinetic determination was hyperbolic at pH 10, and $I_{0.5}$ was higher (more than tenfold) than that at pH 8.0 (data not shown). The fact that Zn^{2+} induces cooperativity only at physiological condition may imply the significance of such a regulation mechanism in relation to the overall metabolism of this bacteria. Thorough investigation for the pH dependence of the Zn^{2+} induced cooperativity of AlaDH is currently ongoing.

Effect of other divalent metal ions on AlaDH activity Some divalent metal ion inhibitors of AlaDH

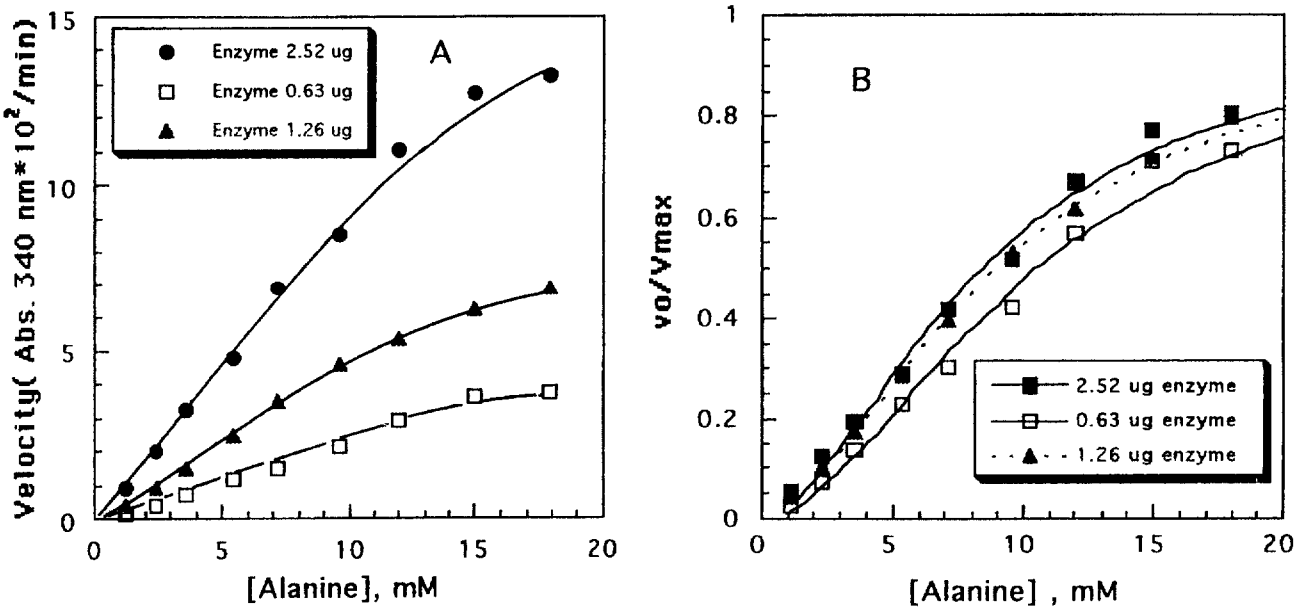

Fig. 4. Inhibition of AlaDH by Zn^{2+} as a function of L-alanine concentration at three different enzyme concentrations. Assay conditions are the same as in Fig. 1. Double kinetic assays were measured at enzyme concnetration as indicated. (A) Direct plot of velocity versus L-alanine concentration. Lines are best fit lines. (B) Plot in terms of v_0/V_{max} vs L-alanine concentration. Data are fitted to Hill equation and lines are generated using best fit parameters of Hill equation.

were reexamined under the same reaction condition used for the effect of Zn^{2+} binding (Fig. 5). The double reciprocal plots in the presence 0.2 mM Cu^{2+} or 0.2 mM Ni^{2+} result in straight lines and Co^{2+} does not inhibit the reaction in appreciable degree at the same concentration. Hg^{2+} strongly inhibited enzyme at its micromolar concentration and the inhibition was irreversible. Addition of EDTA does not reverse the activity of AlaDH inhibited by Hg^{2+}. Thus, Zn^{2+} appears to be a specific reversible inhibitor inducing conformational change of AlaDH at its low micromolar concentration. Zn^{2+} is an allosteric competitive inhibitor of L-alanine. Enzyme cooperativity is induced only by the binding of the inhibitor. The phenomenon of kinetic cooperativity only in the presence of a competitive inhibitor is unusual, and only a few instances are known at present. For phosphorylase b, the cooperative property of glucose-1-phosphate binding is not visible unless ATP, a competitive inhibitor, is added. ATP binds to the enzyme cooperatively (Maden and Shechosky, 1967). For ornithine transcarbamoylase, Zn^{2+}, being an allosteric inhibitor, induces kinetic cooperativity in the ornithine binding (Kuo *et al.*, 1982). And for mannitol-1-phosphate dehydrogenase from *A. parasticus* (Foreman and Niehaus, 1985), Zn^{2+} and other divalent metal ions cause the cooperativity of binding of fructose-6-phosphate. Kuo (1983) analyzed the theoretical basis of allosteric cofactor-mediated enzyme cooperativity. Although all cofactor-induced cooperativities exhibit similar sigmoidal saturation curves, their mechanisms for generating cooperativity can be different. For one mechanism, the substrate and the cofactor bind to separate sites and both binding of substrate and ligand are cooperative by intersubunit interaction and both ligands exert allosteric effects mutually. But the preference for two states in the MWC allosteric model (Monod *et al.*, 1965) of cofactor is much stronger than that of substrate so that only cofactor can bind cooperatively. The cooperativity of substrate, however, can be enhanced and so visualized by the equilibrium shift by the binding of a cofactor ligand. Phosphorylase b and mannitol-1-phosphate dehydrogenase belong to this class. In another mechanism, substrate and cofactor compete for the same binding site. Substrate binding is not cooperative, i.e. substrate binds to both states (R and T) with the same affinity and only cofactor shows the preference for R/T state and exhibits cooperativity in binding. Apparent cooperativity of substrate binding in the presence of cofactor is therefore the result of exclusion of the cooperative cofactor from its binding site and not by the heterotropic allosteic effect of cofactor through conformational change. Kuo (1983) concluded that for ornithine transcarbamoylase, the apparent cooperativity of substrate binding is the effect of competitive binding of Zn^{2+} and L-ornithine.

The results from this work indicate that AlaDH from *B. subtilis* is an allosteric enzyme of the latter type of

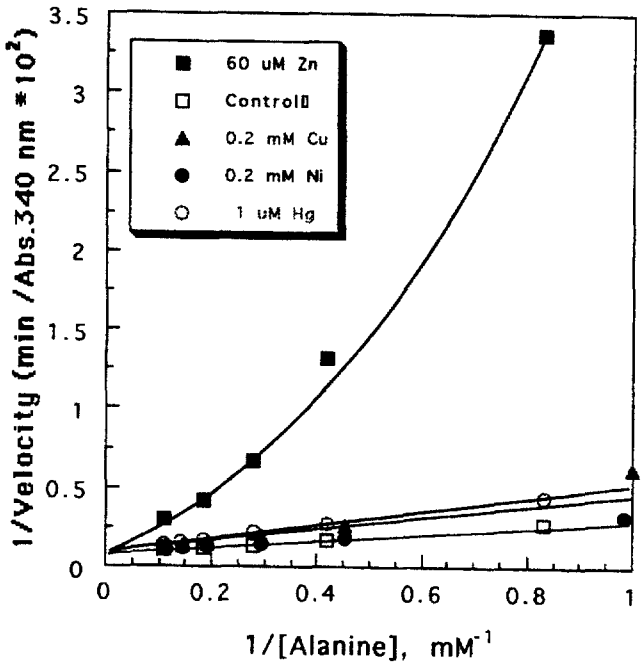

Fig. 5. Effect of various divalent metal ions on the AlaDH catalyzed reactivity. Double reciprocal plot of the initial velocity data measured in the absence and presence of metal ion, Hg^{2+}, Cu^{2+}, Ni^{2+}, and Zn^{2+}. Total metal ion concentrations are indicated. Assay conditions are the same as in Fig. 1.

allosteric mechanism. It is the only known enzyme besides ornithine transcarbamoylase, a biosynthetic enzyme, which requires much higher concentration of Zn^{2+} than in AlaDH in order to induce the cooperativity of binding. This means that AlaDH would be a good target enzyme for the investigation of the "non-cooperative substrate–cooperative cofactor allosteric system." The experimental results are consistent with both MWC and Koshland's allosteric model. The exclusive binding of substrate or inhibitor is compatible with the two state concerted conformational change in MWC model, but the competitive binding between substrate and cofactor ligand molecule can be fitted to the sequential conformational change proposed by Koshland *et al.* (1966). The physiological function of the Zn^{2+}-induced cooperativity is not clear yet. For the *E. coli* ornithine transcarbamoylase, Kuo *et al.* (1990) suggested, on the basis of their kinetic and fluoresence spectroscopic studies, that the effect of Zn^{2+} binding could be comparable with the function of the catalytically inactive regulatory subunits of aspartate transcarbamoylase which is a well defined allosteric enzyme. They also demonstrated that the ornithine transcarbamoylase can be induced to express with the intrinsic substrate cooperativity (Kuo *et al.*, 1989; Lee *et al.*, 1990). The combined results reveal the significance of the silent substrate cooperativity together with cofactor-induced cooperativity of this enzyme in the evolution of the complex, regulatory enzymes.

More works on the quaternary structure and structure of the metal ligand binding site of the AlaDH should be done before the significance of this distinct mechanistic property of this catabolic enzyme in the overall metabolism of the cell can be elucidated.

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