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Equilibrium Kinetic Study of Bovine Liver Glutamate Dehydrogenase at High pH†

Emanuel Silverstein

ABSTRACT: The kinetics at equilibrium of bovine liver glutamate dehydrogenase have been studied by isotopic exchange of substrate and coenzyme at 25° with glutamate and alanine systems in 126 and 136 mM Veronal buffer at pH 8.8 and in 138 mM Veronal buffer at pH 9.5. At pH 8.8 increase in substrate concentration (alanine:pyruvate or glutamate:α-ketoglutarate) at equilibrium from below K_m to more than tenfold greater resulted in marked and progressive suppression of the $NAD^+ \leftrightarrow$ NADH equilibrium reaction rate (measured with tracer [^{14}C]NADH) while the alanine $\leftrightarrow$ pyruvate (measured with [^{14}C]pyruvate) and glutamate $\leftrightarrow$ α-ketoglutarate (measured with [^{14}C]-α-ketoglutarate) rates simultaneously rose progressively toward maximum values. In similar experiments at pH 9.5 only a slight suppression in the $NAD^+ \leftrightarrow$ NADH equilibrium rates was observed with increasingly saturated substrate concentration while alanine $\leftrightarrow$ pyruvate and glutamate $\leftrightarrow$ α-ketoglutarate rates, respectively, rose toward maximum values. Increase in NAD^+:NADH concentration from highly unsaturating to near saturating resulted in a rise in alanine $\leftrightarrow$ pyruvate and glutamate $\leftrightarrow$ α-ketoglutarate rates to plateau values without any inhibition. These results are compatible with a compulsory binding order mechanism for bovine liver glutamate dehydrogenase with both glutamate and alanine substrate systems at pH 8.8, and an alternative order mechanism (partially compulsory) at pH 9.5, in contrast to an alternative order (random) mechanism previously demonstrated at pH 8.0 (Silverstein and Sulebele, 1973, 1974). Minimum estimates for some dissociation constants were obtained from the kinetic data. These findings further confirm the general similarity in the mechanism of bovine liver glutamate dehydrogenase with respect to glutamate and alanine substrate systems despite marked differences between them with respect to rate, pH optima, and modulation of activity by allosteric effectors, and suggest that similar major alterations in kinetic mechanism by changes in pH and perhaps other factors may obtain for other enzyme systems as well.

Bovine liver glutamate dehydrogenase (L-glutamate: NAD(P) oxidoreductase (deaminating), EC 1.4.1.3) is a six subunit allosteric enzyme important in amino acid and carbohydrate metabolism which catalyzes the oxidative deamination of glutamate, alanine, and other amino acids to the corresponding α-keto acids and ammonium ion (Frieden, 1963; Eisenberg, 1970). While the catalysis with glutamate and alanine differs markedly with respect to rate, pH optima, and modulation of activity by allosteric effectors (Struck, Jr., and Sizer, 1960; Frieden, 1959, 1963, 1964, 1971; Tomkins *et al.*, 1961), we have shown in equilibrium kinetic studies of the catalytic and allosteric mechanisms of regulatory enzymes that the enzyme

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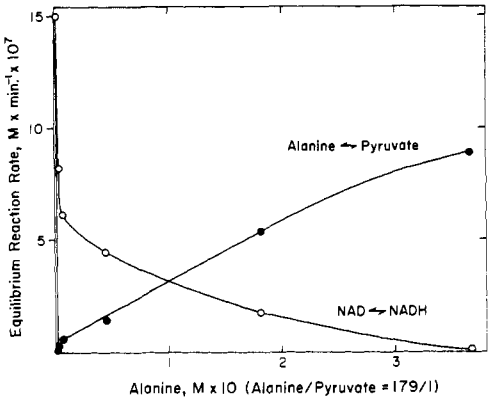

FIGURE 1: Effect of alanine:pyruvate concentration on alanine ↔ pyruvate and NAD^+ ↔ NADH reaction rates at equilibrium catalyzed by bovine liver glutamate dehydrogenase at pH 8.8. Reaction mixtures contained 4.3 mM NAD^+, 0.22–0.33 mM NADH, alanine and pyruvate as indicated, 3.30 mM NH_4^+, and 2.5 μM enzyme in 126 mM Veronal buffer at 25°: (●) alanine ↔ pyruvate; (○) NAD^+ ↔ NADH.

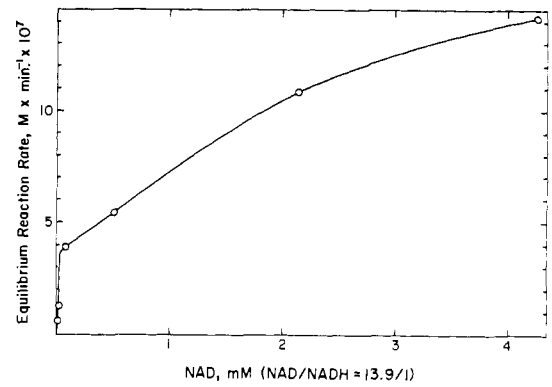

FIGURE 2: Effect of NAD^+ and NADH concentration on alanine ↔ pyruvate reaction rates at equilibrium catalyzed by bovine liver glutamate dehydrogenase at pH 8.8. Reaction mixtures contained 365 mM alanine, 2.04 mM pyruvate, NAD^+ and NADH as indicated, 3.30 mM NH_4^+, and 2.5 μM enzyme in 126 mM Veronal buffer at pH 8.8 and 25°.

nevertheless has a similar alternative order (random) mechanism with respect to both substrates at pH 8.0 (Silverstein and Sulebele, 1973, 1974).

Observation in allosteric mechanism studies at pH 8.8 of reversal of the elevated ratio of NAD ↔ NADH to alanine ↔ pyruvate equilibrium rates found at pH 8.0 prompted reexamination of the kinetic mechanism at pH 8.8 and 9.5. The results suggest that the mechanism changes for both alanine and glutamate substrates from alternative order (random) at pH 8.0 to compulsory order (coenzyme binding prior to substrate) at pH 8.8 and back toward a random mechanism at pH 9.5 (alternative order, partially compulsory). A brief account of this work has been given (Silverstein, 1973).

Materials and methods have been described (Silverstein and Sulebele, 1973, 1974).

Results

Alanine System at pH 8.8. Under conditions of highly unsaturating substrate the NAD^+ ↔ NADH equilibrium reaction rate was markedly higher than the alanine ↔ pyruvate rate (Figure 1) in conformity with results at pH 8.0 (Silverstein and Sulebele, 1974). However, with increasing concentration of alanine:pyruvate to saturation the NAD^+ ↔ NADH rate progressively decreased to near zero while the alanine ↔ pyruvate rate rose to equality with the NAD^+ ↔ NADH rate at about 1 mM alanine and then increasingly surpassed it (Figure 1) in marked contrast to previous results at pH 8.0 (Silverstein and Sulebele, 1974). The marked suppression of the NAD^+ ↔ NADH rate but not the alanine ↔ pyruvate rate at saturating substrate concentrations is compatible with a compulsory order mechanism for the alanine dehydrogenase reaction at pH 8.8 with coenzyme binding prior to substrate (Boyer and Silverstein, 1963; Silverstein and Boyer, 1964).

This conclusion is further supported by the lack of suppression of the alanine ↔ pyruvate rate at NAD^+:NADH concentration near saturating, ruling out a compulsory binding order with substrate binding prior to coenzyme. The alanine ↔ pyruvate rate rose sharply and then gradually in response to increasing NAD^+:NADH concentration from highly unsaturating to near saturating levels (Figure 2).

Glutamate System at pH 8.8. In view of the general similarity of the kinetic mechanism of bovine liver glutamate dehydrogenase with glutamate and alanine at pH 8.0, the finding of evidence for a compulsory pathway with alanine at pH 8.8 prompted similar investigation with the glutamate system. Increasing glutamate:α-ketoglutarate concentration from highly unsaturating to saturating resulted in a rise followed by a progressive and marked fall to near zero in the NAD^+ ↔ NADH rate and a rise to a plateau value in the glutamate ↔ α-ketoglutarate rate followed by a fall, only at the highest substrate level studied (398 mM glutamate), to about half-maximal (Figure 3). This result strongly suggests that catalysis of glutamate oxidative deamination at pH 8.8 by bovine liver glutamate dehydrogenase involves a compulsory pathway with coenzyme binding prior to substrate. The fall in the glutamate ↔ α-keto-

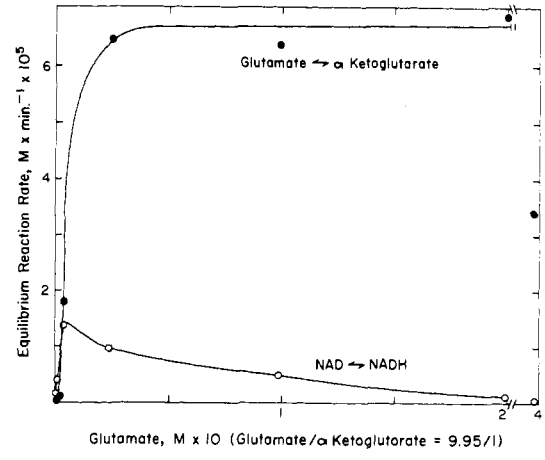

FIGURE 3: Effect of glutamate:α-ketoglutarate concentration on glutamate ↔ α-ketoglutarate and NAD^+ ↔ NADH reaction rates at equilibrium catalyzed by bovine liver glutamate dehydrogenase at pH 8.8. Reaction mixtures contained 3.9 mM NAD^+, 0.14–0.26 mM NADH, glutamate and α-ketoglutarate as indicated, 3.64 mM NH_4^+, and 124 nM enzyme in 136 mM Veronal buffer at 25°: (●) glutamate ↔ α-ketoglutarate; (○) NAD^+ ↔ NADH.

[1] An alternative order mechanism is a branched sequence mechanism in which reactant liquids may bind to and dissociate from enzyme in any order. It includes a *random* mechanism in which the rate of ligand binding to and dissociation from enzyme is independent of other bound ligands, and mechanisms in which the rate of reactant ligand binding and dissociation is not independent of other reactant ligands, such as a *partially compulsory* pathway (Boyer and Silverstein, 1963).

[2] Alanine:pyruvate concentration signifies alanine and pyruvate concentration at constant ratio. Similar designation is used for other reactant concentrations as well.

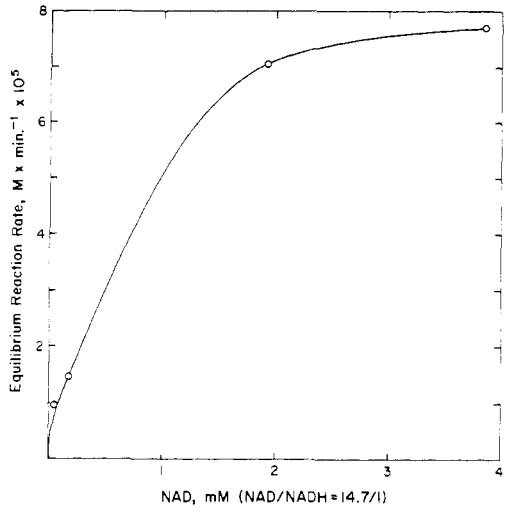

FIGURE 4: Effect of NAD^+:NADH concentration on the glutamate ↔ α-ketoglutarate reaction rate at equilibrium catalyzed by bovine liver glutamate dehydrogenase at pH 8.8. Reaction mixtures contained 99.1 mM glutamate, 10 mM α-ketoglutarate, 3.64 mM NH_4^+, NAD^+ and NADH as indicated and 138 nM enzyme in 136 mM Veronal buffer at 25°.

glutarate rate at 398 mM glutamate may be due to binding of glutamate and/or α-ketoglutarate at a weak binding site other than the catalytic center, resulting in diminution in substrate dissociation rate or chemical transformation rate. The glutamate ↔ α-ketoglutarate rate rose to plateau values in response to increase in NAD^+:NADH concentration from unsaturating to near saturating (Figure 4), ruling out the possibility of the reverse binding order, substrate prior to coenzyme.

Alanine System at pH 9.5. Raising the pH from 8.8 to 9.5 drastically altered the kinetics in the direction of that observed at pH 8.0 (Silverstein and Sulebele, 1973). Increasing alanine: pyruvate concentration from highly unsaturating to saturating resulted in a sharp rise followed by a gradual decrease of only about 20%. The alanine ↔ pyruvate rate simultaneously rose progressively, surpassing the NAD^+ ↔ NADH rate at about 50 mM alanine, and amounting to about twice the NAD^+ ↔ NADH rate at the highest substrate concentration (138 mM alanine) (Figure 5). This result is compatible with an alternative order (partially compulsory) mechanism at pH 9.5 in which dissociation of coenzyme from substrate-containing enzyme complex is slightly less rapid than from enzyme complex lacking bound substrate. There was no evidence for a compulsory binding mechanism with substrate binding first since the

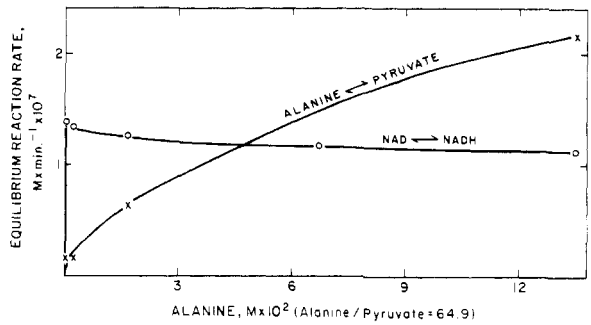

FIGURE 5: Effect of alanine:pyruvate concentration on alanine ↔ pyruvate and NAD^+ ↔ NADH reaction rates catalyzed by bovine liver glutamate dehydrogenase at pH 9.5. Reaction mixtures contained 0.85–1.0 mM NAD^+, 0.20–0.35 mM NADH, alanine and pyruvate as indicated, 3.66 mM NH_4^+, and 2.7 μM enzyme in 138 mM Veronal buffer at 25°: (X) alanine ↔ pyruvate; (O) NAD^+ ↔ NADH.

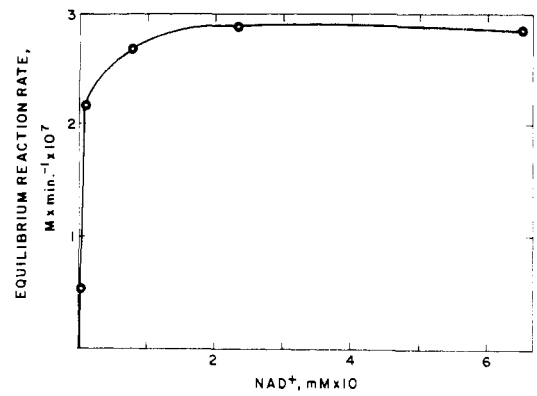

FIGURE 6: Effect of NAD^+:NADH concentration on the alanine ↔ pyruvate reaction at equilibrium catalyzed by bovine liver glutamate dehydrogenase at pH 9.5. Reaction mixtures contained 135 mM alanine, 1.9–2.0 mM pyruvate, 3.3 mM NH_4^+, NAD^+ as indicated, and 5 μM enzyme in 130 mM Veronal buffer at 25°. NAD^+/NADH ranged from 0.13 at the lowest coenzyme concentration to 1.5 at the highest.

alanine ↔ pyruvate rate rose to a plateau in response to increasing coenzyme concentration (Figure 6).

Glutamate System at pH 9.5. The kinetic pattern indicative of a compulsory pathway was also not observed with the glutamate system at pH 9.5 (Figure 7). In response to increasing concentration of glutamate:α-ketoglutarate at equilibrium the NAD^+ ↔ NADH rate rose to a plateau value, falling only slightly at the highest substrate concentration (about 0.4 M glutamate), while the glutamate ↔ α-ketoglutarate rate rose progressively. The results suggest that coenzyme dissociation may be slightly faster from enzyme complexes lacking bound substrates as compared to enzyme complexes containing substrate (alternative order (partially compulsory) mechanism). The glutamate ↔ α-ketoglutarate rate rose to a plateau level in response to increasing concentration of pyridine nucleotide (Figure 8), ruling out a compulsory pathway with substrate binding prior to coenzyme.

Minimum estimates for some *dissociation constants* calculated from $1/R$–$1/S$ plots of the equilibrium kinetic data (Boyer and Silverstein, 1963) were: at pH 8.8, pyruvate, 7.1×10^{-3}; α-ketoglutarate, 2.7×10^{-3}; NADH, 8.4×10^{-7} (0.64–39 μM NADH) and 7.2×10^{-5} (39–310 μM NADH) (alanine system), and 2.4×10^{-6} (1.3–13 μM NADH) and 8.3×10^{-5} (13–260 μM NADH) (glutamate system); at pH 9.5, pyruvate, 7.9×10^{-4}; α-ketoglutarate, 4.7×10^{-4}; NADH, 8.1×10^{-5} (alanine system) and 3.3×10^{-6} (4.8–12 μM NADH), and 4.5×10^{-4} (27–240 μM NADH) (glutamate system).

Discussion

The main point of the present equilibrium kinetic investigation is the demonstration of evidence for a compulsory binding order for bovine liver glutamate dehydrogenase at pH 8.8 with coenzyme binding prior to substrate. This mechanism changes to a partially compulsory, almost random one at pH 9.5. The previous observation of basic similarity in mechanism between alanine and glutamate substrates at pH 8.0 (Silverstein and Sulebele, 1973, 1974) holds consistently also at pH 8.8 and 9.5 despite the striking differences in action of the enzyme with regard to these two substrates already alluded to. The enzyme thus apparently undergoes progressive change in mechanism with change in hydronium ion concentration from alternative order (random) at pH 8.0, to compulsory order at pH 8.8 and back toward random (alternative order; partially compulsory) at pH 9.5.

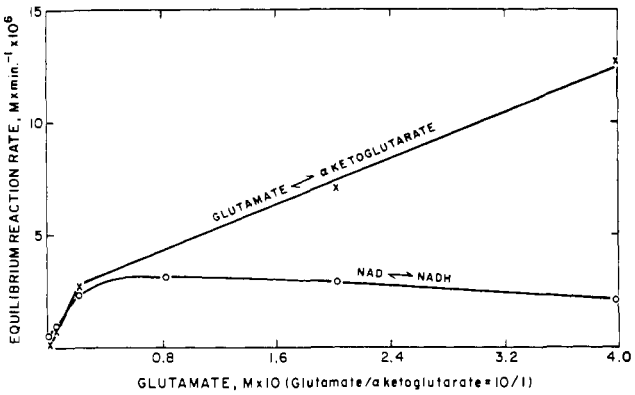

FIGURE 7: Effect of glutamate:α-ketoglutarate concentration on glutamate ↔ α-ketoglutarate and NAD^+ ↔ NADH reaction rates at equilibrium catalyzed by bovine liver glutamate dehydrogenase at pH 9.5. Reaction mixtures contained 0.68–0.79 mM NAD^+, 0.14–0.26 mM NADH, glutamate and α-ketoglutarate as indicated, 3.8 mM ammonium acetate, and 274 nM enzyme in 138 mM Veronal buffer at 25°: (X) glutamate ↔ α-ketoglutarate; (O) NAD^+ ↔ NADH.

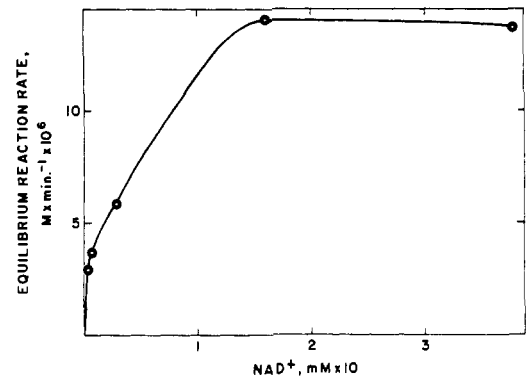

FIGURE 8: Effect of NAD^+:NADH concentration on the glutamate ↔ α-ketoglutarate reaction rate at equilibrium catalyzed by bovine liver glutamate dehydrogenase at pH 9.5. Reaction mixtures contained 199 mM glutamate, 20 mM α-ketoglutarate, 3.98 mM NH_4^+, NAD^+ as indicated, and 604 nM enzyme in 122 mM Veronal buffer at 25°. NAD^+/NADH ranged from 1 at the lowest to 1.6 at the highest coenzyme concentration.

It is not clear what causes these profound changes in mechanism, but the explanation probably includes changes in enzyme and perhaps substrate conformation resulting in critical changes in the character of substrate binding to the catalytic center. At pH 8.0 the catalytic center presumably encompasses the substrate binding site of correct steric configuration which contains suitable binding groups based on charge, and perhaps noncharge interaction to allow substrate binding in the absence of bound coenzyme. Apparently the configuration and perhaps charge of the enzyme, and possibly of the substrate, changes sufficiently at pH 8.8 so that substrate can no longer bind to the catalytic center in the absence of coenzyme. Binding of coenzyme presumably alters the enzyme conformation and catalytic center so as to enable binding of substrate to its binding site at the catalytic center. Perhaps the substrate binding site in the presence of enzyme bound NAD^+ is restored to the condition in which it exists at pH 8.0. However, the major alteration in enzymic activity (decrease for glutamate, increase for alanine) on change in pH from 8.0 to 8.8 suggests that there may be some difference between the substrate binding sites at pH 8.0 and 8.8 which is not fully eliminated by the binding of coenzyme.

Additional deprotonation of enzymic groups at pH 9.5 apparently further changes enzyme conformation and perhaps binding groups at the catalytic center in again allowing for substrate binding in the absence of NAD^+, as at pH 8.0. However, the presence of bound substrate at pH 9.5 somewhat decreases the rate of dissociation of NAD^+ from its binding site. It would be of great interest to compare the three-dimensional structure obtained from X-ray crystallographic studies at pH 8.0, 8.8, and 9.5 to attempt to discern the structural correlate of the observed kinetic effects.

Glutamate dehydrogenase is now the third dehydrogenase which has been shown by isotopic exchange kinetics at equilibrium to have a compulsory pathway under certain conditions. Previously bovine heart and rabbit muscle lactate dehydrogenases (Silverstein and Boyer, 1964) and porcine and bovine heart malate dehydrogenases (Silverstein and Sulebele, 1969a,b) have been shown to have such a mechanism. Curiously, lactate and malate dehydrogenases were found to have compulsory pathway mechanisms at pH 7.9 (lactate dehydrogenase) or 8.0 (malate dehydrogenase) and only partially compulsory mechanisms at more alkaline pH (9.7, lactate dehydrogenase; 9.0, malate dehydrogenase), somewhat the reverse of the case with bovine liver glutamate dehydrogenase where the mechanism is random at pH 8.0, compulsory order at pH 8.8 and partially compulsory at pH 9.5.

The present findings suggest that other enzymes as well may have alterations in kinetic mechanism with change in pH and perhaps in other conditions as well. Clearly, the presence or absence of a compulsory pathway mechanism at one pH may not hold under other conditions.

The suggestion of tighter coenzyme binding at low coenzyme concentrations than at high in these experiments is consistent with similar findings with NAD^+ and $NADP^+$ binding to enzyme–glutarate complexes based on equilibrium dialysis and with initial rate kinetic studies. This phenomenon may be due to negative interactions between oligomer subunits in the binding of coenzyme in ternary complexes or to heterogeneity of the subunits of the oligomer (Dalziel and Egan, 1972; Dalziel and Engel, 1968; Engel and Dalziel, 1969; Dalziel, 1972).

Acknowledgments

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Negative Cooperativity and Half of the Sites Reactivity. Alkaline Phosphatases of *Escherichia coli* with Zn^{2+}, Co^{2+}, Cd^{2+}, Mn^{2+}, and Cu^{2+} in the Active Sites†

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ABSTRACT: Evidence for the nonequivalence of the active sites in dimeric alkaline phosphatase of *Escherichia coli* has been obtained both at acidic and alkaline pH with Zn^{2+}, Co^{2+}, Cu^{2+}, and Cd^{2+} enzymes. (1) Reaction of all metallophosphatases with 2,4-dinitrophenyl phosphate proceeds through a transient phase (a burst) at pH 4.1 and 5°. A biphasic burst of about 2 mol of dinitrophenol/mol of dimer was obtained with Zn^{2+}, Co^{2+}, Cu^{2+}, and Cd^{2+} phosphatases. The biphasicity of the burst indicates that the first site is phosphorylated more rapidly than the second site. The first part of the burst (1 mol of phenol/mol of enzyme) is too fast to be followed by the stopped-flow technique with Co^{2+} and Cu^{2+} phosphatases ($k_{01} > 1000$ sec^{-1}). The second part is much slower and can be followed easily ($k_{02} = 0.8$ and 0.07 sec^{-1} for the Co^{2+} and Cu^{2+} phosphatases, respectively). Both steps can be followed with the Cd^{2+} phosphatase ($k_{01} = 1$ sec^{-1}, $k_{02} = 0.03$ sec^{-1}). Only "native" Zn^{2+} phosphatase contains noncovalent phosphate (one phosphate per mol of enzyme). This endogeneous phosphate can be removed; it affects transient kinetics but neither the total amplitude nor the biphasicity of the burst. (2) The Mn^{2+} alkaline phosphatase gives a monophasic burst of 2 mol of dinitrophenol/mol of enzyme at pH 4.1, 5°. The two sites exhibit independent catalytic behavior in this metalloenzyme of low catalytic activity. (3) Half of the sites reactivity was demonstrated near pH 8.0, 5°, for the Zn^{2+}, Co^{2+}, and Cu^{2+} enzymes. Only one of the two active sites can be phosphorylated at any given time in all these enzymes. (4) Half of the sites reactivity was also demonstrated in the Co^{2+} enzyme. Stopped-flow measurement of the spectral changes at the Co^{2+} chromophore, induced by inorganic phosphate and β-glycerophosphate binding to the Co^{2+} phosphatase, was followed at 640 nm. These measurements demonstrated the exclusive formation of the 1:1 complex, namely the binding of only one substrate molecule per dimer at a time. All these results clearly confirm that site-site interactions are involved in the alkaline phosphatase mechanism. This enzyme is an interesting model for the analysis of "catalytic" cooperativity between subunits.

Considerable work has been devoted in recent years to the analysis of the role of subunit interactions in enzyme catalysis. It has been found that both positive and negative cooperativity may occur for substrate or effector binding and that sometimes both occur simultaneously in the same enzyme.

In the particular case where absolute negative cooperativity is observed at the catalytic level of substrate transformation (*i.e.*, when only one of the sites of a functional dimer functions at a given time) the enzyme is said to display a half of the sites reactivity. This half of the sites reactivity has now been found in a number of enzymes (Lazdunski *et al.*, 1971; Levitzki *et al.*, 1971; Lazdunski, 1972). Particularly good models of this behavior include dehydrogenases such as alcohol dehydrogenases and glyderaldehyde-3-phosphatedehydrogenase and alkaline phosphatases. The alkaline phosphatase of *Escherichia coli* is a dimer comprising identical subunits. It is a metalloenzyme with zinc in its active site (for a review see Lazdunski, 1972). We present in this work a stopped-flow analysis of intersubunit catalytic cooperativity in a series of alkaline phosphatases which contain different metals in the active site: Zn^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+}, and Cd^{2+}.

Methods

Preparation of Metallophosphatase. The zinc alkaline phosphatase was prepared from *Escherichia coli* CW 3747 as previously described (Lazdunski and Lazdunski, 1967). The stock solution of the enzyme is stored at pH 8.0 (Tris-Cl buffer) and −20°. The maximal activity of the enzyme is 37 μmol min^{-1} mg^{-1} at pH 8.5, 0.4 M NaCl, 25°.

The apoenzyme was obtained by two different techniques. (1) One method was by overnight incubation of a Zn^{2+} phosphatase solution (1 mg/ml) with 50 mM EDTA at pH 6.5 and 25°, followed by chromatography on a Sephadex G-25 column (18 × 1.5 cm) equilibrated with 50 mM EDTA at pH 6.5 and 25°. Pooled fractions were concentrated to 30–40 mg/ml and then passed through a column of Sephadex G-25 equilibrated

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