Negative Homotropic Interactions in Binding of Substrate to Alkaline Phosphatase of *Escherichia coli**

Robert T. Simpson† and Bert L. Vallee‡

ABSTRACT: The active site stoichiometry of alkaline phosphatase of *Escherichia coli* has been evaluated by studies of both phosphate binding at equilibrium and substrate concentration dependence of enzymatic activity. In the native enzyme, there are two phosphate binding sites which differ in apparent dissociation constant; the magnitude of this difference depends upon the ionic strength of the solvent, at pH 7.8 and $\Gamma/2 = 1.0$, $K_{P1} = 8 \times 10^{-6}$ and $K_{P2} > 4 \times 10^{-3}$ M; while at $\Gamma/2 = 0.05$, $K_{P1} = 1.7 \times 10^{-5}$ and $K_{P2} = 3 \times 10^{-4}$ M. Nitration of the enzyme with tetranitromethane followed by reduction with dithionite to the aminophenol derivative virtually abolishes the difference between the two phosphate binding sites. Thus, even at the high ionic strength, the two dissociation constants are nearly equal; $K_{P1} = 1.3 \times 10^{-5}$ M and $K_{P2} = 9 \times 10^{-5}$ M. Kinetically, the native enzyme shows substrate activation at low ionic strength, suggesting that the two phosphate binding sites may represent two catalytically active sites. The possibility is discussed that these experimental results represent an allosteric negative homotrophic interaction in the binding of substrates to alkaline phosphatase.

A number of considerations have suggested previously that alkaline phosphatase of *Escherichia coli* might be expected to have two active sites. The protein consists of two subunits which are apparently identical (Rothman and Byrne, 1963; Schlesinger and Barrett, 1965). Further, by equilibrium dialysis, two molecules of phosphate ion have been thought to be bound to each molecule of enzyme (Hede and Levinthal, 1962[1]).

In contrast, several types of investigations have indicated the existence of but one active site. Thus, phosphate labeling of the active site seryl residue at acidic pH values resulted in the incorporation of from 0.6 to 1.3 moles of phosphate per mole of protein (Schwartz and Lipmann, 1961; Engstrom, 1962a,b; Schwartz, 1962; Pigretti and Milstein, 1965). "Active site burst titrations" at acidic pH values have also indicated the presence of but one active site (Fernley and Walker, 1965; Ko and Kezdy, 1967; Trentham and Gutfreund, 1968).[2] More recent investigations of phosphate binding at equilibrium have suggested the presence of but one binding site per mole of enzyme (Reynolds and Schlesinger, 1969). Thus the stoichiometry of active sites for this enzyme has remained uncertain.

The present studies have approached this problem by evaluation of the substrate concentration dependence of hydrolysis of phosphomonoesters and of the binding of phosphate at equilibrium. The data suggest the existence of two phosphate binding (active) sites of the enzyme. The dissociation constant for the most firmly bound molecule of phosphate is relatively invariant when different conditions or enzymes are studied. In contrast, the dissociation constant for the second mole of bound phosphate is highly variable, being a function both of ionic strength and of the nature of the enzyme under investigation. In conjunction with earlier physical studies of the enzyme, the present results may represent an example of a negative homotropic allosteric interaction in binding of substrates.

Experimental Section

The methodology employed for the isolation, measurements, and assay of the enzyme have been described (Simpson *et al.*, 1968; Gottesman *et al.*, 1969), and procedures for nitration and reduction of phosphatase (P. C. Christen and B. L. Vallee, in preparation) are based on methods previously published (Sokolovsky *et al.*, 1967).

Carrier-free [^{32}P]phosphate was obtained from Isoserve Corp. and was diluted into solutions of known nonradioactive phosphate content before use. The isotope was quantitated by counting in a Packard Tri-Carb liquid-scintillation counter, using 10 ml of scintillation fluid, containing 4.0 g of 2,5-diphenyloxazole and 0.2 g of 1,4-bis[2-(5-phenyloxazolyl)]-benzene per l. of 50% toluene, 50% methanol. Standard solutions were counted in the presence of the amount of protein and water was added to each vial to ensure that no detectable quenching occurred consequent to the presence of either of these components.

Equilibrium dialysis was performed with equal volumes of

* From the Biophysics Research Laboratory, Department of Biological Chemistry, Harvard Medical School, and the Division of Medical Biology, Peter Bent Brigham Hospital, Boston, Massachusetts. *Received September 30, 1969.* This work was supported by Grants-in-Aid HE-07297 and GM-15003 from the National Institutes of Health of the Department of Health, Education, and Welfare.

† Special Fellow of the National Institutes of General Medical Sciences, National Institutes of Health. This work was submitted by Robert T. Simpson in partial fulfillment of the requirements of the Ph.D. degree to the Department of Biological Chemistry, Division of Medical Sciences, Harvard University. Present address: Staff Associate, Laboratory of Nutrition and Endocrinology, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland.

‡ Requests for reprints should be addressed to: Biophysics Research Laboratory, Boston, Massachusetts 02115.

[1] Quoted in Levinthal *et al.* (1962).

[2] Values of 1.7 to 2.7 sites per mole of protein were obtained by Fife (1967). However, the results were extrapolated from steady-state measurements without direct observation of the transient kinetic phase.

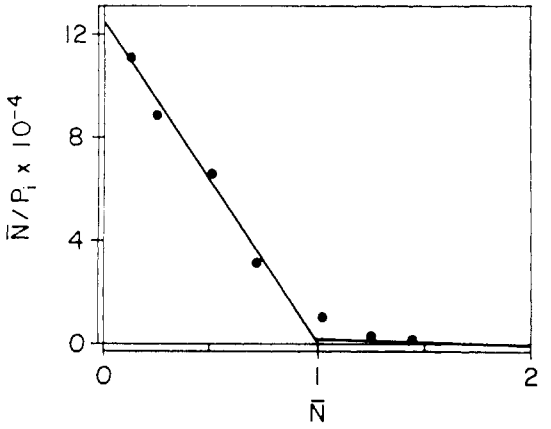

FIGURE 1: Phosphate binding to alkaline phosphatase at high ionic strength. Equilibrium phosphate binding was determined as in the Experimental Section with an enzyme concentration of 6.66 $\times$ 10^{-6} M in 0.01 M Tris-Cl, 1.0 M NaCl, pH 7.8 and 20°.

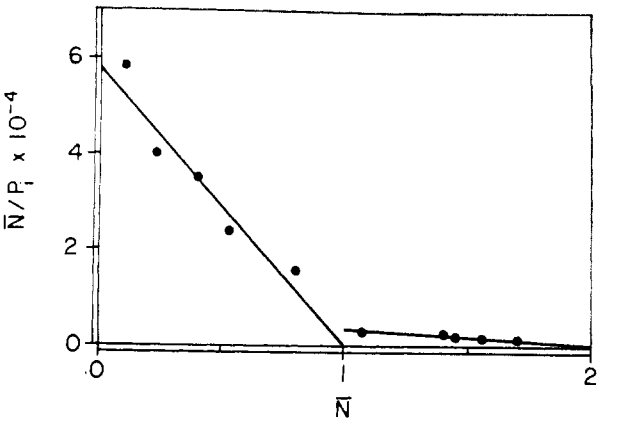

FIGURE 2: Phosphate binding to alkaline phosphatase at low ionic strength. Equilibrium phosphate binding was determined as in the Experimental Section with an enzyme concentration of 1.33 $\times$ 10^{-4} M in 0.1 M Tris-Cl, pH 7.8 and 20°.

protein and buffer solutions in 1-ml capacity dialysis cells (Chemical Rubber Corp.). The cells were rotated on a slanted table during the dialysis procedure.

Initial experiments demonstrated the attainment of equilibrium within 16 hr. Usually, dialysis experiments were carried out for 48 hr to ensure complete equilibration. Equivalent results were obtained irrespective of the side of the membrane on which phosphate was placed at the beginning of dialysis, confirming that the results reflect thermodynamic equilibrium.

At the conclusion of a dialysis experiment, duplicate aliquots of protein, buffer, and standards were removed with a single 50- or 100-μl Carlsberg micropipet for counting of the isotope. In any case in which results between the two duplicate samples differed by more than 0.2%, the experiment was rejected. The total number of counts assayed was between 20,000 and 200,000. Recoveries of added isotope were between 98 and 102%. Protein concentrations were determined at the conclusion of each dialysis experiment.

Phosphate binding data are presented in the form of one of the linearized equations of the law of mass action (Scatchard, 1949) as plots of $\bar{N}$ *vs.* $\bar{N}/[P_i]$, where $\bar{N}$ is the average number of moles of phosphate bound per mole of enzyme, and P_i is the concentration of free phosphate in equilibrium with the enzyme–phosphate complex.

Results

Studies of the interaction of phosphate with alkaline phosphatase have been carried out with the native enzyme at two ionic strengths as well as with an enzyme modified by nitration followed by reduction. Under all conditions examined, nonlinear Scatchard plots are obtained, results which are inconsistent with a simple interaction of 1 mole of phosphate with 1 mole of enzyme.[3] At pH 7.8 and high ionic strength, $\Gamma/2$ = 1.0, the native enzyme binds 1 mole of phosphate with a dissociation constant of 8 $\times$ 10^{-6} M (Figure 1). However, three values obtained at high phosphate concentrations suggest the binding of additional phosphate ion(s) with much higher dissociation constant(s). At the protein concentration employed, 6.66 $\times$ 10^{-5} M, neither the stoichiometry nor the strength of this additional binding can be established readily. If, however, the data are assumed to reflect the interaction of *1* additional mole of phosphate, the minimal value of its dissociation constant can be calculated to be 4 $\times$ 10^{-3} M.

At pH 7.8 and low ionic strength, $\Gamma/2$ = 0.05, two distinct phosphate binding sites would seem to be present (Figure 2). The dissociation constants can be calculated to be 1.7 $\times$ 10^{-5} and 3 $\times$ 10^{-4} M. Relative to that obtained at high ionic strength, the dissociation constant for the first mole of phosphate interacting with the enzyme is increased only by a factor of 2. In contrast, the dissociation constant for the second binding site is at least tenfold lower than the minimal value obtained at the higher ionic strength.

Nitration of phosphatase with tetranitromethane, followed by reduction of the resultant nitrotyrosyl enzyme with sodium dithionite to form an aminotyrosyl enzyme (Sokolovsky *et al.*, 1967), leads to activity changes similar to those previously observed following modification of alkaline phosphatase with *N*-bromosuccinimide (Tait and Vallee, 1966; P. C. Christen and B. L. Vallee, unpublished observations). Relative to the native enzyme, hydrolase activity is apparently unchanged, while phosphotransferase activity is approximately doubled. Binding of phosphate to this chemically modified enzyme differs strikingly from that to the native protein and indicates the existence of two binding sites. Even at high ionic strength, 2 moles of phosphate is firmly bound to the aminotyrosyl enzyme with apparent dissociation constants of 1.3 $\times$ 10^{-5} and 9 $\times$ 10^{-5} M (Figure 3). The first mole of phosphate interacts with the aminotyrosyl enzyme with a binding constant nearly equal to that for the native enzyme under similar conditions. However, the dissociation constant for the second mole of phosphate is decreased to a value nearly two orders of magnitude lower than that observed for the native protein, and is only fivefold greater than that for the first mole of phosphate bound.

[3] No attempt was made to fit these plots by multisite binding equations. Rather, straight lines have been drawn through the points for the binding of each mole of phosphate, and the intercepts of these lines used to calculate approximate binding constants.

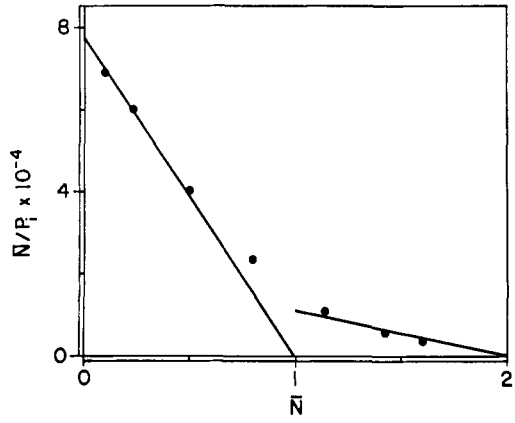

FIGURE 3: Phosphate binding to aminotyrosyl phosphatase. Equilibrium phosphate binding to the aminotyrosyl enzyme was determined as in the Experimental Section at an enzyme concentration of 6.66×10^{-5} M in 0.01 M Tris-Cl, 1.0 M NaCl, pH 7.8 and 20°.

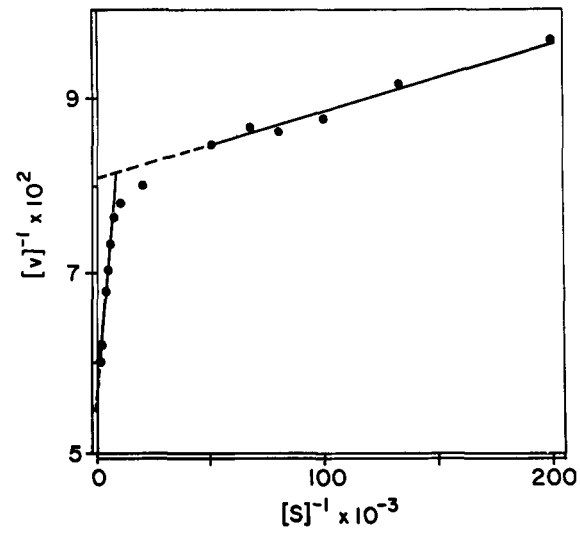

FIGURE 4: Substrate concentration dependence for the activity of native alkaline phosphatase at low ionic strength. The dependence of the activity of alkaline phosphatase on substrate concentration was determined with 4-nitrophenylphosphate as substrate in 0.1 M Tris-Cl, pH 7.8 and 20°. Velocities are expressed as μmoles of substrate hydrolyzed per min per mg of enzyme.

Kinetic investigations of the substrate concentration dependence of hydrolysis of 4-nitrophenylphosphate were performed under conditions of pH and ionic strength identical with those employed for the equilibrium phosphate binding studies. The existence of two phosphate binding sites with differing dissociation constants could reflect the presence of two active catalytic sites. In that case, substrate activation might be expected under some conditions, as will be detailed below.

At high ionic strength, the Lineweaver–Burk plot for the native enzyme is linear up to substrate concentrations of 10^{-3} M, as has been reported (Lazdunski and Lazdunski, 1966). Under these conditions, when 4-nitrophenylphosphate is the substrate, K_m is 5×10^{-6} M, in close agreement with 8×10^{-6} M, the dissociation constant for the first mole of phosphate bound on equilibrium dialysis (*vide supra*). However, substrate activation, reflecting binding of a second mole of substrate, is not seen.

At low ionic strength, the native enzyme does exhibit substrate activation (Figure 4). This phenomenon, already noted by Heppel *et al.* (1962), is observed at substrate concentrations greater than 5×10^{-4} M. The apparent Michaelis constants determined for the two ranges are 1×10^{-5} and 7×10^{-4} M, in good agreement with the phosphate binding constants at this ionic strength, 1.7×10^{-5} and 3×10^{-4} M. In the lower substrate range ($<5 \times 10^{-4}$ M), the apparent maximal velocity is 12 units while in the higher range ($>5 \times 10^{-4}$ M) it is 19.

At high ionic strength, the substrate concentration dependence of the activity of aminotyrosyl phosphatase is linear from 10^{-5} to 10^{-3} M substrate. The apparent K_m for substrate is 2×10^{-5} M, between 1.3×10^{-5} and 9×10^{-5} M, the dissociation constants for the two molecules of phosphate bound. This is consistent with the closely similar numerical values of the binding constants of the 2 moles of phosphate bound (Figure 3) and the theoretical analysis of the substrate concentration dependence of a two-site enzyme which differs in the magnitude of the dissociation constants for the two molecules of substrate bound (*vide infra*).

For all conditions studied, substrate inhibition is observed at substrate concentrations greater than 5×10^{-3} M. This complicating circumstance could account for the fact that substrate activation is not observed at high ionic strength for the native enzyme.

We have calculated the substrate concentration dependence expected for the enzymatic activity of an enzyme with two sites assumed to exhibit a negative homotropic interaction. This situation was selected as a plausible operational model for the features of alkaline phosphatase currently investigated. Thus,

$$E + S \underset{K_1}{\overset{K_1}{\rightleftharpoons}} \begin{matrix} ES \\ SE \end{matrix} + S \overset{K_2}{\rightleftharpoons} SES \qquad (1)$$

with

$$ES \text{ (or } SE) \xrightarrow{k_{cat}} E + P \qquad (2)$$

and

$$SES \xrightarrow{2k_{cat}} E + 2P \qquad (3)$$

where the symbols have their usual meaning, and K_1 and K_2 are assumed to be true dissociation constants. Only affinity for substrate is altered by binding the first molecule of substrate, and catalytic efficiency (per occupied site) is assumed to be constant.

The appropriate rate equation is

$$v_{obsd} = \frac{V_{max}(S)[K_2 + 2(S)]}{K_1K_2 + K_2(S) + (S)^2} \qquad (4)$$

where $V_{max} = k_{cat} \times E_{total}$, *i.e.*, the maximal velocity per active site.

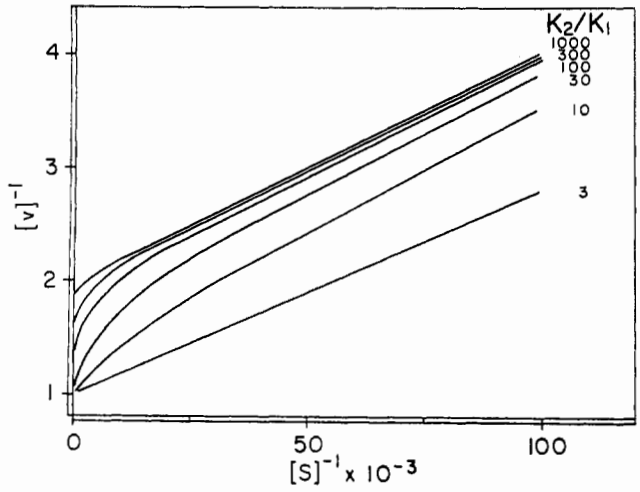

FIGURE 5: Calculated substrate concentration dependence of a two-site enzyme with K_m differing for the 2 moles of substrate (eq 1–4 in text). The calculated values for eq 4 under the conditions detailed in the text are presented as Lineweaver–Burk plots of $[v]^{-1}$ (in arbitrary units) *vs.* $[S]^{-1}$ for the indicated ratios of K_2:K_1.

Using this rate equation and setting K_1 equal to 1×10^{-5} M, an average value for the actual data obtained, the substrate concentration dependence of apparent activity was calculated over a substrate concentration range from 1×10^{-5} to 5×10^{-3} M while values of K_2 were varied from 3×10^{-5} to 1×10^{-2} M (Figure 5). This substrate concentration range is that employed routinely for kinetic investigations of alkaline phosphatase and the range of values for K_2 encompass those obtained experimentally in the phosphate binding studies.

As is apparent, if $10^{-5} < K_2 < 10^{-4}$ (*i.e.*, $K_2/K_1 < 10$), linear double-reciprocal plots are obtained (Figure 5). At values of K_2 greater than 10^{-4}, substrate activation would be apparent. Such substrate activation would become difficult to detect if K_2 were to be greater than 5×10^{-3} M, as K_2 would then be greater than the highest substrate concentration generally employed in kinetic studies of this enzyme. Further, studies at concentrations of this substrate greater than 5×10^{-3} M are not readily feasible since substrate inhibition is observed at higher concentrations. Thus, substrate activation, reflecting two active sites with differing association constants, might be detected kinetically only under certain restricted circumstances.

The experimental results obtained kinetically are consistent with this model for all three cases examined. Thus, for the native enzyme at high ionic strength, $K_{P2}/K_{P1} > 500$; and $1/v$ *vs.* 1/[S] is apparently linear. The apparent K_m, 5×10^{-6} M, is nearly equal to K_{P1}, 8×10^{-6} M.[4] When the native enzyme is evaluated at low ionic strength, $K_{P2}/K_{P1} \simeq 18$, and substrate activation is observed (Figure 4). In the case of the aminotyrosyl enzyme, $K_{P2}/K_{P1} \simeq 5$, and substrate concentration dependence is apparently linear. K_m, 2×10^{-5} M, is between the two phosphate binding constants, 1.3×10^{-5} and 9×10^{-5} M.

A number of physicochemical parameters of alkaline phosphatase were examined in 0.1 M Tris-Cl, pH 7.8, 20°, in the presence and absence of 0.01–0.1 M phosphate. At the indicated enzyme concentrations, no significant changes consequent to addition of phosphate were observed in sedimentation coefficient (8 mg/ml), specific viscosity (17.6 mg/ml), ultraviolet absorption (2 mg/ml), or ultraviolet circular dichroism (1.8 mg/ml).

Discussion

The present studies of phosphate binding to alkaline phosphate at equilibrium are consistent with the presence of two binding sites. The dissociation constant for the first mole of bound phosphate is quite similar when examined for three different conditions: native enzyme at high and low ionic strength, and aminotyrosyl enzyme at high ionic strength. Values of 0.8, 1.7, and 1.3×10^{-5} M were obtained, respectively. In contrast, the second mole of phosphate is bound much more weakly, and the dissociation constant is highly variable, both as a function of the nature of the enzyme and of solvent conditions. For the binding of the second mole of phosphate the dissociation constants are >400, 30, and 9×10^{-5} M, respectively.

Phosphate is a product of the energetically favored hydrolysis of phosphomonoesters, inhibits the enzyme competitively, and also serves as a substrate for the reverse reaction (Lazdunski and Lazdunski, 1966; Meyerhof and Green, 1949). Thus, the stoichiometry and stability of binding of phosphate to the enzyme might reflect the number and stability of active sites for alkaline phosphatase. Kinetic studies, under conditions similar to those employed for equilibrium binding, support the hypothesis that these two phosphate binding sites might be equivalent to two active enzymatic sites. For all conditions studied, the dissociation constants for equilibrium binding of phosphate ion correspond closely to the Michaelis constants for the substrate. Similarly, this hypothesis is supported by the close agreement between the substrate concentration dependence calculated for an enzyme with two active sites but with different dissociation constants and that obtained experimentally for alkaline phosphatase. Throughout, experimental and calculated parameters coincide, suggesting that a scheme such as summarized in eq 1–3 could be pertinent to alkaline phosphatase.

The failure of previous studies to identify two phosphate binding sites (or active sites) may relate to the fact that the majority of the previous investigations were performed at relatively low concentrations of phosphate or phosphate esters. Thus, if a second active site were to be present, but its dissociation constant for substrate were to be more than two orders of magnitude higher than that for the first site, such a site would not be detected under most conditions employed prior to these studies.[5]

The exact cause for the difference in apparent dissociation constants must remain uncertain, although the present and

[4] K_m and K_s should be within a factor of two of each other, if the phosphosyl-enzyme intermediate mechanistic scheme applies, as judged from the known magnitudes of the microscopic rate constants at the pH of the present studies (Aldridge *et al.*, 1964; Fernley and Walker, 1965; Ko and Kezdy, 1967; Trentham and Gutfreund, 1968).

[5] These studies are performed at pH 7.8, whereas phosphate incorporation and active site titrations have been studied at pH values of less than 6. Extension of the present results to those obtained at the acidic pH requires further investigation.

previous studies bear upon the possible basis for this phenomenon. The alternatives which could explain the microscopic basis for such negative homotropic binding or kinetic phenomena have been noted by Levitzki and Koshland (1969). They list "(1) ligand-induced conformational changes which affect subunit interactions, (2) electrostatic repulsion between ligands, (3) nonidentical peptide chains having active sites with different binding constants as in isozymes, (4) two or more polymorphic forms of the same enzymes, (5) geometric arrangements of identical chains which produce nonidentical sites either because of static geometry or because of subunit conformational changes during the association of subunits, and (6) a combination of two or more of these alternatives" (Levitzki and Koshland, 1969). On the basis of considerations to be detailed, alternative (1), *i.e.*, an allosteric[6] interaction, seems a likely explanation for alkaline phosphatase, while alternatives 3–5, requiring a static nonidentity of the active sites, can be invoked less readily.

Alkaline phosphatase is thought to consist of identical polypeptide subunits, as evidenced by genetic considerations and fingerprints of tryptic peptides (Rothman and Byrne, 1963). As much as 1.3 moles of phosphate/mole of protein have been trapped as phosphoseryl-enzyme intermediate, and in those experiments the pattern of phosphorus-containing peptides did not differ from that obtained when less than 1 mole of phosphate/mole of enzyme is incorporated (Pigretti and Milstein, 1965). Restoration of either zinc or cobalt to the apoprotein generates activity directly proportional to the addition of the two metal atoms thought to bind at the active sites (Simpson and Vallee, 1968). Similarly, the visible absorption spectra associated with the two cobalt atoms involved directly in activity seem indistinguishable, unless nonidentical sites (yet with equal metal binding constants) are assumed to exist. Finally, titration of the cobalt enzyme indicates the presence of two geometrically equivalent sites for phosphate interaction (Simpson and Vallee, 1968). Although isozymes exist for alkaline phosphatase, the present investigations were performed with material which was >90% a single electrophoretic species. These considerations, indicating the presence of two intrinsically identical active sites for the enzyme, mitigate against the latter hypotheses, those which require two active sites which differ in intrinsic substrate binding constants.

Further, it is noteworthy that either a decrease in ionic strength for the native enzyme, or its chemical modification, decreases the dissociation constant for the second mole of phosphate bound, while affecting that for the first mole of phosphate but little. Changes in solvent properties, or alterations of binding groups would be expected to affect the interaction of the substrate with both binding sites equally, either increasing or decreasing the association constants. Specifically, increasing the ionic strength should lessen electrostatic repulsion between the ligands. In contrast to this expectation, both lowering the ionic strength and nitration and reduction of the enzyme hardly affect the binding of the first molecule of substrate but increase the affinity for the second substrate molecule. Such findings are most consistent with a protein-mediated interaction as the cause for the observation of the difference in apparent dissociation constants for the two sites. In these terms, the effects of the chemical modification could constitute an allosteric desensitization, reducing the effects of the interaction and thereby making the two binding constants more nearly identical. Thus, an allosteric interaction appears consistent with the occurrence of negative homotropic binding and kinetic phenomena of alkaline phosphatase.

If, indeed, an allosteric interaction formed the basis for the difference in apparent dissociation constant between the two molecules of phosphate which bind to the enzyme, it would be expected that a conformational change in the protein should attend binding of the first molecule of phosphate. Such a change has been detected previously in studying the selective removal of the enzymatically active zinc atoms by 8-hydroxyquinoline-5-sulfonic acid. At low ionic strength this chelating agent rapidly removes two of the zinc atoms of native phosphatase and, concomitantly, activity is lost to less than 10% of the control value (Simpson and Vallee, 1968). Phosphate ion retards the rate of removal of these zinc atoms with concentration-dependent kinetics characteristic of a saturation process. Phosphate concentrations which allow binding of only 1 mole of phosphate 1 mole of protein retard the rate of removal of *both* active site zinc atoms. The apparent dissociation constant for phosphate, calculated from these data, is 2×10^{-5} M, in close agreement with both the equilibrium and the kinetic dissociation constants for the first molecule of phosphate bound to native phosphatase at low ionic strength. Charge alterations consequent to phosphate binding might affect the interaction of protein and chelating agent, but the magnitude of such effects would be expected to be small, relative to those observed experimentally. These data would suggest that the protein has indeed undergone a significant conformational change consequent to interaction with an effector, although more general methods for evaluation of protein conformation do not indicate any gross alterations in structure related to binding of phosphate. Thus, the binding of a molecule of phosphate to the metal atoms at one active site would not only reduce the availability of that metal to chelating agents, but also limit the access of solvent components to the other active metal atom. Such a conformational change on binding of substrate is quite consistent with the preferred interpretation of the results of these studies. It is of interest that the change is detected only when a property unique to the active site is evaluated.

In the context of this interpretation, phosphatase would constitute an example of an allosteric system with a *negative homotropic interaction*, a conclusion also reached independently by Lazdunski and Lazdunski (personal communication). Such an interaction between substrate molecules binding at active sites is consistent with most definitions of allosterism (Changeux, 1964; Monod *et al.*, 1965; Koshland and Neet, 1968; Levitzki and Koshland, 1969), although certain other definitions would not include such an interaction (Stadtman, 1966). The present investigations point to a conformation-mediated difference in substrate binding as the possible basis for the observed anomalies of kinetic and equilibrium binding data. Such negative cooperativity has been demonstrated recently for the binding of DPNH to rabbit muscle glyceraldehyde 3-phosphate dehydrogenase (Conway and Koshland, 1968), and kinetically

[6] The terminology employed in the discussion of allosteric phenomena is that of Changeux (1964), Monod *et al.* (1965), and Koshland and Neet (1968).

for the interaction of GTP and glutamine with CTP synthetase (Levitzki and Koshland, 1969). Such a phenomenon would tend to make the enzyme involved less markedly sensitive to alterations to the concentrations of its substrate or cofactor (Conway and Koshland, 1968). In a system with positive cooperativity, enzyme activity varies so as to maintain a relatively constant concentration of substrate and product. In contrast, in a system with negative cooperative interactions, enzymatic activity would respond to substrate concentration over a wide range.

The functional significance of this interaction for phosphatase is not clear presently, in part due to a lack of knowledge concerning the physiologic role of alkaline phosphatase of *E. coli*, and the possible occurrence of similar phenomena in the phosphatases of higher organisms. The occurrence of such a negative cooperative interaction would support the sequential theory of allosteric interactions (Koshland and Neet, 1968; Conway and Koshland, 1968) as opposed to the symmetry model for allosteric phenomena (Monod *et al.*, 1965).

References

Aldridge, W. N., Barman, T. E., and Gutfreund, H. (1964), *Biochem. J. 92*, 23C.

Changeux, J.-P. (1964), *Brookhaven Symp. Biol. 17*, 232.

Conway, A., and Koshland, D. E., Jr. (1968), *Biochemistry 7*, 4011.

Engstrom, L. (1962a), *Biochim. Biophys. Acta 56*, 606.

Engstrom, L. (1962b), *Ark. Kemi 19*, 129.

Fernley, H. N., and Walker, P. G. (1965), *Biochem. J. 97*, 95.

Fife, W. K. (1967), *Biochem. Biophys. Res. Commun. 28*, 309.

Gottesman, M. M., Simpson, R. T., and Vallee, B. L. (1969), *Biochemistry 8*, 3776.

Heppel, L. A., Harkness, D. R., and Hillmoe, R. J. (1962), *J. Biol. Chem. 237*, 841.

Ko, S. H. D., and Kezdy, F. J. (1967), *J. Amer. Chem. Soc. 89*, 7139.

Koshland, D. E., Jr., and Neet, K. E. (1968), *Ann. Rev. Biochem. 37*, 359.

Lazdunski, C., and Lazdunski, M. (1966), *Biochim. Biophys. Acta 113*, 551.

Levinthal, D., Signer, E. R., and Fetheroff, K. (1962), *Proc. Natl. Acad. Sci. U. S. 48*, 1230.

Levitzki, A., and Koshland, D. E., Jr. (1969), *Proc. Natl. Acad. Sci. U. S. 62*, 1121.

Meyerhof, O., and Green, H. (1949), *J. Biol. Chem. 178*, 655.

Monod, J., Wyman, J., and Changeux, J.-P. (1965), *J. Mol. Biol. 12*, 88.

Pigretti, M. M., and Milstein, C. (1965), *Biochem. J. 94*, 106.

Reynolds, J. A., and Schlesinger, M. J. (1969), *Biochemistry 8*, 588.

Rothman, F., and Byrne, R. (1963), *J. Mol. Biol. 6*, 330.

Scatchard, G. (1949), *Ann. N. Y. Acad. Sci. 51*, 660.

Schlesinger, M. J., and Barrett, K. (1965), *J. Biol. Chem. 240*, 4284.

Schwartz, J. H. (1962), *Proc. Natl. Acad. Sci. U. S. 49*, 871.

Schwartz, J. H., and Lipmann, F. (1961), *Proc. Natl. Acad. Sci. U. S. 47*, 1996.

Simpson, R. T., Tait, G. H., and Vallee, B. L. (1968), *Biochemistry 7*, 4336.

Simpson, R. T., and Vallee, B. L. (1968), *Biochemistry 7*, 4343.

Sokolovsky, M., Riordan, J. F., and Vallee, B. L. (1967), *Biochem. Biophys. Res. Commun. 27*, 20.

Stadtman, E. R. (1966), *Advan. Enzymol. 28*, 41.

Tait, G. H., and Vallee, B. L. (1966), *Proc. Natl. Acad. Sci. U. S. 56*, 1247.

Trentham, D. R., and Gutfreund, H. (1968), *Biochem. J. 106*, 455.