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A Substrate-Induced Conformation Change in the Reaction of Alkaline Phosphatase from *Escherichia coli*

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1. Benzyl phosphonates were prepared and their potentialities as chromophoric reagents for the exploration of the substrate-binding site of *Escherichia coli* alkaline phosphatase were investigated. 4-Nitrobenzylphosphonate is a competitive inhibitor of the enzyme. 2-Hydroxy-5-nitrobenzylphosphonate changes its spectrum on binding to the enzyme. This spectral change is reversed when the phosphonate is displaced from the enzyme by substrate. 2. The kinetics of the reaction of 2-hydroxy-5-nitrophenylphosphonate were studied by the stopped-flow and the temperature-jump techniques. It was found that the combination of the phosphonate with the enzyme occurred in two successive and reversible steps: enzyme–phosphonate complex-formation followed by rearrangement of the complex. The spectral change is associated with the rearrangement. At pH 8 in 1 M-sodium chloride at 22° the rate constant is 167 sec.$^{-1}$ for the rearrangement of the initially formed binary complex and is 18 sec.$^{-1}$ for the reverse process. 3. It has previously been proposed that the reactions of phosphatase with its substrates include a distinct step between enzyme–substrate combination and chemical catalysis. The rate constant involved could be predicted but not measured from experiments with substrates. The value for the rate constant measured from the rate of the enzyme–phosphonate rearrangement is in excellent agreement with the predicted value. A model for the reaction mechanism is proposed that includes a conformation change in response to phosphate ester binding before phosphate transfer from substrate to enzyme.

Trentham & Gutfreund (1968) have proposed that during the reaction of *Escherichia coli* phosphatase with its substrates a kinetically distinct step occurs after initial complex-formation and before chemical catalysis. These findings suggested that the simplest model that can be used to describe the mechanism involves a first-order rearrangement of the enzyme–substrate complex, which is slow compared with the subsequent transfer of phosphate, P, from substrate, ROP, to enzyme, E; the final step is the liberation of phosphate from an enzyme–phosphate intermediate:

$$E + ROP \underset{EP + ROH}{\overset{k_1}{\rightleftharpoons}} EROP \overset{k_2}{\rightleftharpoons} E^*ROP \xrightarrow{k_3}$$

$$EP \xrightarrow{k_4} E + P$$

It was predicted that the step characterized by k_2 is rate-determining for the formation of EP and that its rate is insensitive to the nature of the substrate. This would mean that the step characterized by k_3 is very fast and that the concentration of E*ROP would always be small compared with the other forms of the enzyme. We therefore investigated the possibility of studying the kinetics of the step EROP→E*ROP with a competitive inhibitor that might produce the same response on binding to the enzyme and that contained a chromophore which would provide a signal during changes in its surroundings.

The present paper describes studies on the use of phosphonates for the exploration of the active site. The experiments were designed to provide kinetic data for the interaction of phosphonates with *E. coli* phosphatase. The results of rapid-flow and chemical-relaxation measurements allowed the evaluation of individual rate constants that can be used to test the above model for the overall reaction.

MATERIALS

Synthesis of disodium 4-nitrobenzylphosphonate. 4-Nitrobenzylphosphonic acid was synthesized from diethyl benzylphosphonate (Kagan, Birkenmeyer & Strube, 1959). The phosphonate diester was synthesized from benzyl chloride and triethyl phosphite by the method of Kosalopoff (1945). The acid was crystallized as its disodium salt from

acetone–water (8:1, v/v) in 25% overall yield from benzyl chloride. The p.m.r. (proton-magnetic-resonance) spectrum of the sodium salt in D_2O showed a four-proton *AB* quartet characteristic of a *para*-substituted benzene ring. $H_{(3)}$ and $H_{(5)}$ were assigned to peaks τ 1·86, J 9·0 cyc./sec., and $H_{(2)}$ and $H_{(6)}$ were assigned to peaks τ 2·50, J 9·0 cyc./sec. The latter peaks showed fine splitting, J 2·6 cyc./sec. A two-proton doublet, τ 6·99, J 21 cyc./sec., was assigned to the methylene group. The solvent of crystallization was water alone. Titration of the acid and its sodium salt showed the presence of two ionizing groups of equal neutralizing capacity, with pK values 7·5 and less than 3. The disodium salt, when dried *in vacuo* (6mm. Hg) for 6hr. at room temperature, was the hexahydrate (mol.wt. 369), on the basis of its titration with standard HCl. The salt was stored at −20°, since it became coloured at room temperature. For elemental analysis, the salt was dried *in vacuo* (1mm. Hg) at room temperature overnight, when it became slightly hygroscopic. *Disodium 4-nitrobenzylphosphonate* had m.p. 245° (decomp.), uncorrected (Found: C, 28·2; H, 3·4; N, 4·7; P, 10·0; $C_7H_6O_5NNa_2P,2H_2O$ requires C, 28·3; H, 3·4; N, 4·7; P, 10·4%).

Synthesis of trisodium 2-hydroxy-5-nitrobenzylphosphonate. 2-Hydroxy-5-nitrobenzylphosphonic acid was synthesized from 2-hydroxy-5-nitrobenzyl chloride and triethyl phosphite by the method of Kosalopoff (1945). The parent halide was obtained by chloromethylation of 4-nitrophenol (Buehler, Kirchner & Deebel, 1940). The acid was crystallized as its trisodium salt from ethanol–water (6:1, v/v) in 35% overall yield from 4-nitrophenol. The p.m.r. spectrum of the sodium salt in D_2O showed three distinct peaks characteristic of aromatic protons. $H_{(3)}$ and $H_{(4)}$ were assigned to doublet peaks, τ 3·54 and 2·07, J 9·4 cyc./sec. $H_{(6)}$ was assigned to a singlet peak, τ 1·62. The peaks assigned to the aromatic protons showed further fine splitting. A two-proton doublet peak, τ 7·25, J 19 cyc./sec., was assigned to the methylene group. The solvent of crystallization was water alone. The p.m.r. spectra of both the solvent and the solution of the phosphonate were measured in the presence of the same concentration of internal reference compound. Hence the contribution to the HOD peak by the water of crystallization of the phosphonate was measured. If the assumption is made that the intensity of the signals of a methylene and an HOD proton are the same, the sodium salt contained 6·6moles of water/mole. Titration of the acid and its sodium salt showed the presence of three ionizable groups of equal neutralizing capacity with pK values 9·4, 6·3 and less than 3. The trisodium salt, when dried *in vacuo* at room temperature, was the heptahydrate (mol.wt. 425), on the basis of its titration with standard HCl. For elemental analysis, the salt was dried *in vacuo* at 80°, when it became slightly hygroscopic. *Trisodium 2-hydroxy-5-nitrobenzylphosphonate* had m.p. 250° (decomp.), uncorrected, $\lambda_{max.}$ at pH10·2 in 0·1M-EDTA 418nm. (ϵ $2·15\times10^4$) (Found: C, 25·5; H, 2·6; N, 4·2; P, 10·2; $C_7H_5O_6NNa_3P,2H_2O$ requires C, 25·1; H, 2·7; N, 4·2; P, 9·3%. P analysis gave variable results. The ratio of C to P atoms from three analyses was 8·1, 7·5 and 6·45).

Assay of alkaline phosphatase. To determine the concentration of alkaline phosphatase solutions, the enzyme was added to a solution of 1·00mM-4-nitrophenyl phosphate and 1·0M-tris at pH8·0 at 25°. The rate of 4-nitrophenol release was followed at 400nm. The pK_a of 4-nitrophenol was taken as 6·74 so that the molar extinction coefficient of 4-nitrophenol is $1·73\times10^4$ at pH8·0 and I 0·5 (Trentham & Gutfreund, 1968). A unit of enzyme was defined as the quantity required to liberate 1μmole of 4-nitrophenol/hr. The concentration of alkaline phosphatase in the solution could then be calculated by using the value of 3250 units/mg. for the pure enzyme (Malamy & Horecker, 1964*b*). The molecular weight of the enzyme was taken as 86000 (Schlesinger & Barratt, 1965) and $E^{1\%}_{1cm.}$ as 7·2 at 278nm. From these values the molar concentration and specific activity (as units/mg.) could be determined.

Preparation of alkaline phosphatase. A 3·78kg. portion of *E. coli* C90, which had been grown under phosphate-limiting conditions, was provided by Dr K. Sargeant (Microbiological Research Establishment, Porton, Wilts.). The cells were stored on ice for at least 2 weeks. They contained 2·5mg. of alkaline phosphatase/g. wet wt. of cells.

The enzyme was released into solution by suspending the autolysed cells in 0·1M-tris–1mM-EDTA, from which the solid debris was removed by centrifugation. The supernatant was subjected to $(NH_4)_2SO_4$ fractionation. The precipitate collected from the 50–65%-saturated-$(NH_4)_2SO_4$ fraction was resuspended in 1mM-$ZnCl_2$–0·01M-tris, pH7·4. The rest of the preparation, heat treatment in the presence of $MgCl_2$ and batch chromatography on DEAE-cellulose, was the same as that of Schlesinger & Barratt (1965), except that the enzyme was stored at 4° as a suspension in 70%-saturated $(NH_4)_2SO_4$. Enzyme solutions were prepared by resuspending the precipitate obtained from the suspension and dialysing overnight against the required buffered solution.

The enzyme had a specific activity of 1470 ± 100, which is 45% of the specific activity of the pure crystalline enzyme (Malamy & Horecker, 1964*b*). The specific activity could be increased to 1890 either by diluting the enzyme into a buffer containing 1mM-$ZnCl_2$ before the assay, or by assaying the enzyme in the usual medium containing 1mM-$ZnCl_2$. In the latter case maximum activity was reached within 3min.

Within the limits of detection the alkaline phosphatase was homogeneous when analysed by Sephadex G-100 filtration or by isoelectric fractionation. The conditions of Wenn & Williams (1968) for the isoelectric fractionation (LKB ampholyte solution, pH range 5–7) were used. The isoelectric point of *E. coli* alkaline phosphatase was 5·80 in 17·6% (w/v) sucrose.

Substrates and other reagents. Disodium 4-nitrophenyl phosphate tetrahydrate was obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.) as Sigma 104 phosphatase substrate. The mono-2,6-lutidine salt of 2,4-dinitrophenyl phosphate was provided by Dr A. J. Kirby (Kirby & Varvoglis, 1966). All other chemicals used were of analytical or reagent grades and were used without further purification. Water was glass-distilled. Tris buffers were adjusted to the required pH with 5M-HCl, and the final volume was adjusted so that the stated concentration is that of tris.

Spectroscopic measurements. The p.m.r. spectra were determined with a Varian A-60 spectrophotometer with tetramethylsilane, τ 10·00 (for solutions in CCl_4), and sodium 3-(trimethylsilyl)propane-1-sulphonate, τ 10·00 (for solutions in D_2O), as internal standards. Visible and

u.v. spectra were recorded on a Unicam SP.800 scanning spectrophotometer. Non-scanning visible- and u.v.-absorption measurements including enzyme assays were made in the 1 cm. thermostatically controlled cuvette of a Uvispek H700 spectrophotometer (Hilger and Watts Ltd.) provided with a Gilford recording attachment and a Servoscribe RE511 recorder (Kelvin Electronics Co.).

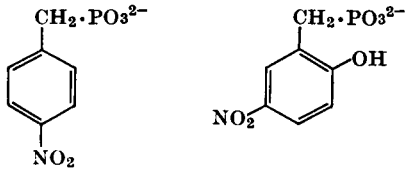
Microanalyses. These were performed by Dr Franz Pascher (Mikroanalytisches Laboratorium, Bonn, Germany).

METHODS AND RESULTS

Kochman, Mastalerz & Wolna (1964) have shown that phosphonates are competitive inhibitors of calf intestinal alkaline phosphatase. 4-Nitrobenzylphosphonate (I) was shown to be a competitive inhibitor of the enzyme from *E. coli* (Fig. 1). 4-Nitrobenzylphosphonate had a half-life of about 1 month in 1·0M-tris at pH 8·0 at room temperature. The decomposition products were characterized as inorganic phosphate and 4-nitrotoluene. Inorganic phosphate was determined by the method of Fiske & Subbarow (1925). 4-Nitrotoluene, which crystallized from solution, was washed with water and characterized by its melting point and p.m.r. spectrum in carbon tetrachloride. Meisters & Swan (1963) noted a similar decomposition of 4-nitrobenzylphosphonate in sodium hydroxide. Solutions of 4-nitrobenzylphosphonate used in the inhibition studies contained negligible inorganic phosphate. Alkaline phosphatase did not catalyse the rate of decomposition of 4-nitrobenzylphosphonate. Under comparable conditions 2-hydroxy-5-nitrobenzylphosphonate (II) was stable both in the presence and absence of alkaline phosphatase.

The u.v.- and visible-absorption spectra of 2-hydroxy-5-nitrobenzylphosphonate measured at pH 5·0, 7·65 and 10·2 show the absorptions characteristic of the mono-, di- and tri-anions respectively (Fig. 2).

The spectrum of 2-hydroxy-5-nitrobenzylphosphonate changed when alkaline phosphatase or zinc chloride was added to a solution of the phosphonate in 0·1M-tris–1·0M-sodium chloride, pH 8·0. Since alkaline phosphatase is a zinc-containing enzyme careful controls were necessary to distinguish between difference spectra arising from the addition of alkaline phosphatase to the phosphonate and from the addition of zinc chloride. The difference spectrum between the Zn^{2+}–phosphonate complex and the unmixed zinc chloride and phosphonate solutions had a peak at 390nm.



(I) (II)

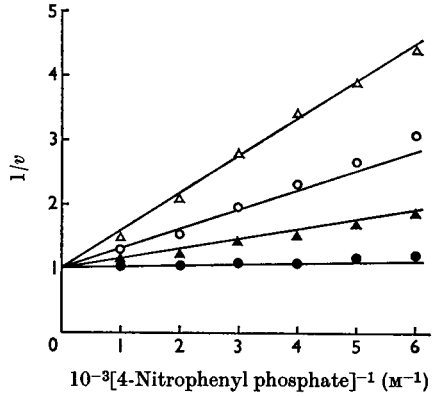


Fig. 1. Lineweaver–Burk plot showing the dependence of the reciprocal relative rate of the enzyme-catalysed hydrolysis of 4-nitrophenyl phosphate with the reciprocal of the substrate concentration in the presence of 4-nitrobenzylphosphonate at 25°. v is the rate of hydrolysis relative to the maximum rate of hydrolysis at infinite substrate concentration. The assay media contained: ●, 4-nitrophenyl phosphate, alkaline phosphatase (0·22 μg./ml.), 1·0M-tris, pH 8·0; ▲, ○ and △, in addition 4-nitrobenzylphosphonate (1·00 mM, 2·00 mM and 4·00 mM respectively). The lines are those predicted if 4-nitrobenzylphosphonate is a competitive inhibitor for which the K_m/K_i ratio is 0·141. The unity point on the ordinate axis was chosen for that family of lines which gave the best fit with experimental results.

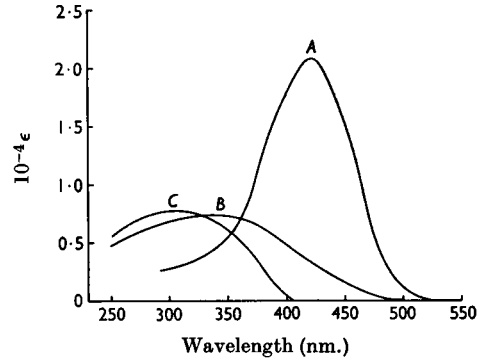


Fig. 2. Spectra of the molar extinction coefficient of 2-hydroxy-5-nitrobenzylphosphonate at different pH values. The solutions used to measure the spectra contained in addition to 2-hydroxy-5-nitrobenzylphosphonate (0·05 mM): spectrum *A*, 0·1M-EDTA at pH 10·2; spectra *B* and *C*, 0·1M-phosphate at pH 7·65 and 5·0 respectively.

The difference spectrum between the enzyme–phosphonate complex and the unmixed enzyme and phosphonate solutions had a peak at 430nm. The molar extinction coefficients of the two difference spectra were approximately the same at their absorption peaks. However, at 430nm. the zinc difference-spectrum extinction was only 12% that of the enzyme difference spectrum.

Studies of the binding of 2-hydroxy-5-nitrobenzylphosphonate to alkaline phosphatase were performed with the prepared enzyme that was partially deficient in zinc (hereafter called the zinc-deficient enzyme). Since the enzyme was always dialysed against Zn^{2+}-free solutions before these experiments the concentration of free Zn^{2+} in solution was much less than the enzyme concentration. Because of the uncertain stoicheiometry associated with Zn^{2+} binding to the enzyme (Reynolds & Schlesinger, 1967; Simpson & Vallee, 1968), no attempt was made to realize the maximum activity of the enzyme by the addition of Zn^{2+}.

The concentration of active enzyme in the zinc-deficient enzyme was determined by measuring the activity and correlating this to the value of 3250 units/mg. for the pure enzyme (Malamy & Horecker, 1964*b*). The validity of this determination was checked by measuring the concentration of 2,4-dinitrophenol released during the first turnover of the enzyme-catalysed hydrolysis of 2,4-dinitrophenyl phosphate at pH 6·0 by using stopped-flow techniques. A 1:1 stoicheiometry was observed between the concentration of 2,4-dinitrophenol and the concentration of active enzyme in the zinc-deficient enzyme as measured above. The 1:1 stoicheiometry agrees with the findings of Ko & Kézdy (1967) and Trentham & Gutfreund (1968).

Zn^{2+}-chelating agents such as EDTA inactivate alkaline phosphatase (Plocke & Vallee, 1962). The phosphonate (II) (0·2mM) was incubated with the enzyme (0·05mM) at pH 8·0 at 25° for 6hr. After suitable dilution of the incubation mixture the enzyme had the same activity as a control enzyme solution.

Estimates of the dissociation constant of the phosphonate (II) to alkaline phosphatase, the number of binding sites on the enzyme and the extinction of the difference spectrum at its peak value, 430nm., were made by recording the extinction change as the phosphonate was added at a constant rate to alkaline phosphatase. 2-Hydroxy-5-nitrobenzylphosphonate (10·0mM) in a motor-driven Agla syringe (Burroughs Wellcome) was added at a constant rate of 5μl./min. to 5·0ml. of a stirred solution of alkaline phosphatase (0·0283mM) in 0·1M-tris–1·0M-sodium chloride, pH 8·0, at 25°. The thermostatically controlled cuvette housing was equipped with a stirrer and light-sealed opening through which solutions could be added to the cuvette. A trace showing the rate of $E_{430}^{1cm.}$ change was recorded. The trace was curved initially and then flattened out to a straight line (within the limits of detection). The constant rate of extinction change as determined from the straight line was 8% greater than the rate of extinction change when the phosphonate was added to the solution in the absence of enzyme. The pH values of the two solutions were carefully measured and were the same. The dissociation constant was 3·0 ($\pm$2·5) $\times 10^{-5}$M as calculated from the curved portion of the trace by the method of Klotz (see Stockell, 1959). The difference molar extinction coefficient was 6000 ($\pm$300). The analysis was complicated by the secondary binding that must occur since the final rate of extinction change did not equal the control rate of extinction change in the absence of enzyme. This secondary binding may be to inactive enzyme or to less specific sites on the active enzyme. This complication introduced uncertainty in the estimate of the stoicheiometry of that binding of phosphonate to enzyme associated with the major chromophoric change. It appeared that 1mole of phosphonate was bound/mole of active enzyme.

Transient kinetics. The rate of binding of 2-hydroxy-5-nitrobenzylphosphonate to alkaline phosphatase was studied by the stopped-flow technique. The stopped-flow apparatus used was similar to that described by Gutfreund (1965). The interchangeable mixer and observation cell consisted of eight mixing jets and a 1cm. light-path. The solution in the observation tube was 3msec. old when flow stopped and the record of change in light-transmission started. Monochromatic light was obtained from a quartz–iodine lamp and a Bausch and Lomb grating monochromator. Transmission of light was recorded on a Tektronix storage oscilloscope, which amplified

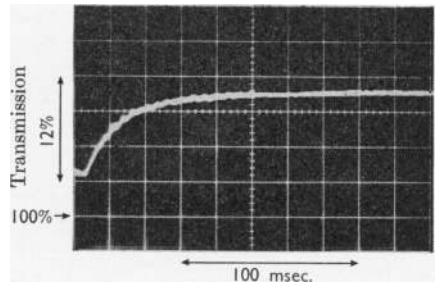


Fig. 3. Spectrophotometric record at 430nm. of a stopped-flow observation of the binding of 2-hydroxy-5-nitrobenzylphosphonate (0·050mM) to alkaline phosphatase (0·0138mM) in 0·1M-tris–1·0M-NaCl, pH 8·0. Two reaction traces are recorded.

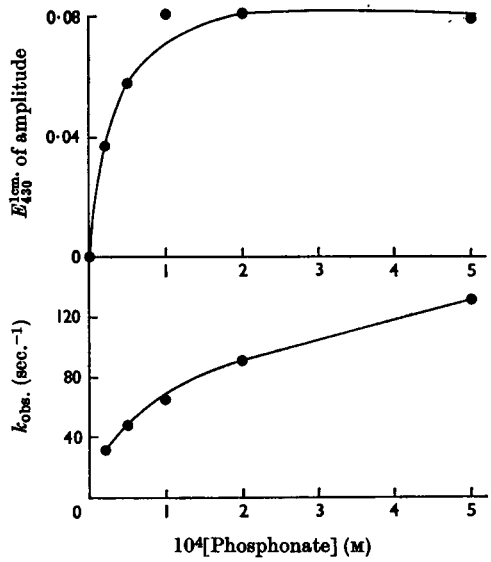


Fig. 4. Relationships between the amplitude and the observed rate constant, $k_{obs.}$, of the binding of 2-hydroxy-5-nitrobenzylphosphonate to alkaline phosphatase as a function of the phosphonate concentration at one enzyme concentration (0·0138mM) in 0·1M-tris–1·0M-NaCl, pH8·0. Both the amplitudes and the first-order rate constants of this reaction were calculated from stopped-flow records as in Fig. 3.

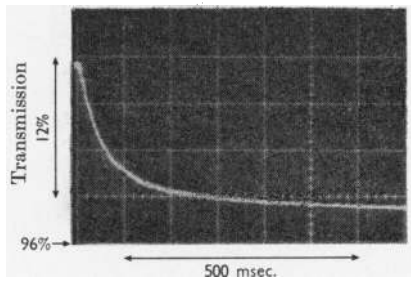


Fig. 5. Spectrophotometric record at 430nm. of a stopped-flow observation of the displacement of 2-hydroxy-5-nitrobenzylphosphonate (0·10mM) from alkaline phosphatase (0·0086mM) by phenyl phosphate (25mM) in 0·1M-tris–1·0M-NaCl, pH8·0.

the output of an EMI 9592B photomultiplier. The oscilloscope pictures were photographed and projected on to graph paper, and extinction was calculated from the percentage transmission data.

Enzyme solutions in 0·1M-tris–1·0M-sodium chloride, pH8·0, at 22° were mixed with a solution of the phosphonate in the same buffer and the subsequent reaction was followed spectrophotometrically at 430nm. (Fig. 3). The dependence of the rate and amplitude of this extinction with substrate concentration at fixed enzyme concentration was measured (Fig. 4). In each case the observed process was cleanly first-order and could be described by a single rate constant. The first-order rate plots were extrapolated back to zero time after mixing of the reagents. There was no difference in extinction between the mixed phosphonate and enzyme solutions at zero time and the two unmixed solutions.

The displacement of 2-hydroxy-5-nitrobenzylphosphonate from alkaline phosphatase by inorganic phosphate and phenyl phosphate was studied by using the stopped-flow technique. An increase in transmission was observed after the mixing of an enzyme solution containing the phosphonate and a solution of either inorganic phosphate or phenyl phosphate (Fig. 5). Both solutions at pH8·0 were in 0·1M-tris–1·0M-sodium chloride. The rate of the first-order process was 18sec.$^{-1}$ and was independent of both the concentration of enzyme and the degree of its saturation with phosphonate. The rate was independent of whether the displacing agent was inorganic phosphate (50mM or 250mM) or phenyl phosphate (25mM).

A slower process whose total amplitude was at least an order of magnitude smaller than the reactions described above (Figs. 4 and 5) was observed in all the stopped-flow experiments. This process may be linked to the less specific binding indicated in the equilibrium binding studies.

Relaxation kinetics. The binding equilibrium of 2-hydroxy-5-nitrobenzylphosphonate with alkaline phosphatase is a suitable system for study by relaxation techniques. Experiments were carried out with a temperature-jump apparatus designed by Dr L. C. de Maeyer and built by Messanlagen G.m.b.H. (Göttingen, Germany). This equipment is illustrated in a recent review (Eigen, 1968), where the theoretical concepts are also defined.

The reaction mixture, containing enzyme and phosphonate in 0·1M-tris–1·0M-sodium chloride, pH8·0, was equilibrated at 22° in a cell through which a potential of 30kv was discharged, resulting in a 5° temperature rise in 5μsec. The relaxation of the chemical system to the new equilibrium position at the higher temperature was followed spectrophotometrically at 430nm.

An instantaneous (for the time-scale being considered) increase in transmission occurred before the observed slower process. The spectrum of the instantaneous change was identical with the phosphonate di- and tri-anion difference spectrum. Protonation of the phosphonate trianion at the higher temperature resulted from the different heats of ionization of tris and the phosphonate in

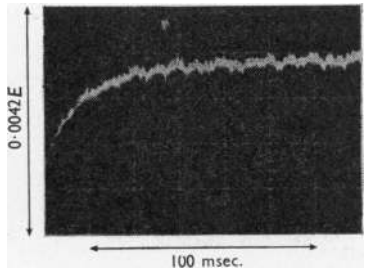


Fig. 6. Spectrophotometric record at 430nm. of a temperature-jump perturbation of the binding equilibrium of 2-hydroxy-5-nitrobenzylphosphonate (0·070mM) to alkaline phosphatase (0·058mM) in 0·1M-tris–1·0M-NaCl, pH8·0.

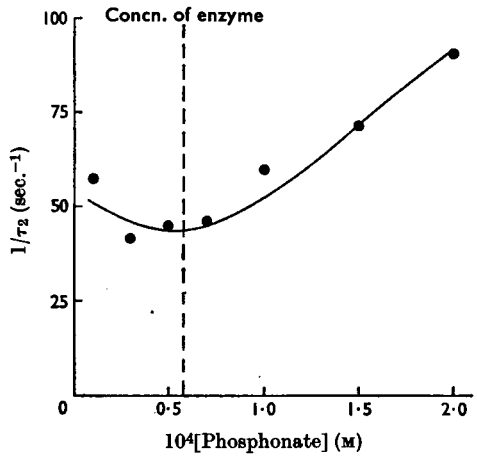


Fig. 7. Relationship between the reciprocal relaxation time and 2-hydroxy-5-nitrobenzylphosphonate concentration of the temperature-jump perturbation of the binding equilibrium of the phosphonate to alkaline phosphatase.

1·0M-sodium chloride. This rapid step is the only relaxation observed in control experiments with enzyme-free solutions of phosphonate in 0·1M-tris–1·0M-sodium chloride, pH8·0.

Apart from this rapid ionization step only one relaxation process was observed over the entire range of enzyme and phosphonate concentrations. This relaxation involved a decrease in transmission (Fig. 6). The relation between the relaxation time and the substrate concentration at fixed enzyme concentration was studied (Fig. 7).

As a control the relaxation spectrum associated with the Zn^{2+}–phosphonate complex was measured. A single relaxation process (τ approx. 30msec.) was observed whose amplitude at comparable concentrations of zinc chloride and enzyme was 25% of the above relaxation. Moreover the relaxation involved an increase rather than a decrease in transmission.

DISCUSSION

The alkaline phosphatase of *E. coli* is liberated in the space between the cell wall and the spheroplast membrane (Malamy & Horecker, 1964*a*). If the growth of cells is limited by the supply of inorganic phosphate in the medium, high local concentrations of phosphatase are induced. Extensive studies have been carried out on the genetic control and other factors involved in the synthesis of the enzyme and of the dimer essential for catalytic activity (Echols, Garen, Garen & Torriani, 1961; Schlesinger & Barrett, 1965; Schlesinger & Andersen, 1968). Such studies and the very broad specificity of the bacterial phosphatase for phosphate esters support the conclusion that one physiological function of this enzyme is to supply inorganic phosphate for the organism from any available source.

There is a striking contrast between the relative aqueous hydrolysis rate (1:10^4) of the dianions of 4-nitrophenyl phosphate and 2,4-dinitrophenyl phosphate and the relative enzyme-catalysed hydrolysis rate (1:1) of these phosphates. This equal catalytic hydrolysis rate occurs both when the two phosphates are mixed together in the same solution and when the cleavage of the enzyme–phosphate complex is not rate-determining. This led Trentham & Gutfreund (1968) to propose a conformation change as the rate-determining step for formation of the enzyme–phosphate complex. A chromophoric competitive inhibitor provides a direct method of probing for the proposed conformation change since k_3 becomes zero and so the enzyme–inhibitor complex analogous to E*ROP will have a measurable concentration, in contrast with E*ROP.

The inhibition studies provide qualitative evidence that 4-nitrobenzylphosphonate is a competitive inhibitor of the substrates of alkaline phosphatase from *E. coli*. The inhibition constant is not of quantitative significance because of uncertainties in the very low K_m value and the fact that the constant derived from transient kinetic studies relates to a different phosphonate.

Although the equilibrium binding studies do not clearly characterize the stoicheiometry of the binding of 2-hydroxy-5-nitrobenzylphosphonate to alkaline phosphatase, they provide a value for the dissociation constant and the molar difference extinction coefficient. These values are based on 1:1 stoicheiometry of the binding, and would require modification if, say, 2 moles of phosphonate bind/mole of enzyme. The binding curve suggested

that 1:1 stoicheiometry was the most plausible. In addition to the secondary binding referred to above, the presence of isoenzymes may contribute to the complicated nature of the binding curve. The model that we propose is consistent with the kinetic results whatever the correct stoicheiometry.

It is shown below that at pH 8 in the presence of 1 M-sodium chloride the simplest model that can accommodate our kinetic data for phosphonates, I, binding to enzyme, E, is that shown in Scheme 1,

$$E + I \underset{k'_{-1}}{\overset{k'_{+1}}{\rightleftharpoons}} EI \underset{k'_{-2}}{\overset{k'_{+2}}{\rightleftharpoons}} EI^*$$

Scheme 1.

where EI and EI* represent binary complexes, and that this model serves to explain some of the catalytic properties of the enzyme with its substrates. If we define $K_I = k'_{-1}/k'_{+1}$ and $K_0 = k'_{-2}K_I/(k'_{+2}+k'_{-2})$, the competitive inhibition constant and the dissociation constant obtained from the binding curve should correspond to K_0.

The simple model of a bimolecular process between E and I to form EI without any further rearrangement may be excluded. Such a model demands that the rate of the binding process of the phosphonate to the enzyme increases linearly with inhibitor concentration when [I] is greater than [E]. This is not the case (Fig. 4). Moreover alkaline phosphatase has a broad specificity so that the rates of the initial bimolecular process would be expected to be diffusion-controlled and hence orders of magnitude more rapid than the observed process (Fernley & Walker, 1969).

The observed rate constant, $k_{obs.}$, for the rate of production of EI* predicted by Scheme 1 is:

$$k_{obs.} = k'_{-2} + \frac{k'_{+2}}{1+K_I/[I]}$$

when $[I] \gg [E]$ and the bimolecular binding process is very rapid. When $[E] \gg [I]$ the same relation holds except that E replaces I. Over the range when $[E] \simeq [I]$ the mathematical analysis to determine $k_{obs.}$ is more complex. However, the equation:

$$k_{obs.} = k'_{-2} + \frac{k'_{+2}}{1+K_I/([E]+[I])}$$

gives a useful working relationship over the whole range of [E] and [I], and is correct when the ratio [I]/[E] is not close to unity. This equation may be rewritten as:

$$\frac{1}{k_{obs.}-k'_{-2}} = \frac{1}{k'_{+2}}\left(1+\frac{K_I}{[E]+[I]}\right)$$

The model (Scheme 1) predicts that the rate of displacement of the phosphonate by excess of substrate or inorganic phosphate will be k'_{-2} provided that $k'_{-2} \ll k'_{-1}$. The rate of displacement was invariant as predicted, so that k'_{-2} was evaluated as 18 sec.$^{-1}$.

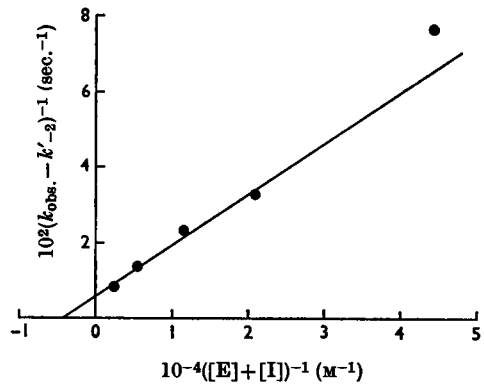


Fig. 8. Secondary plot of the observed rate processes, $k_{obs.}$, associated with the binding of 2-hydroxy-5-nitrobenzylphosphonate to alkaline phosphatase. k'_{-2} was evaluated as 18 sec.$^{-1}$ from the phosphonate displacement reaction (Fig. 5). [E] and [I], the concentrations of free enzyme and phosphonate when equilibrium is established, were evaluated from the variation of $E_{430}^{1cm.}$ with phosphonate concentration (Fig. 4). The line is drawn by regression (except that no weight is given to the point at low [E+I] when $[E] \simeq [I]$, and the linear relation for the model shown in Scheme 1 becomes an approximation).

$(k_{obs.}-k'_{-2})^{-1}$ varied linearly with $([E]+[I])^{-1}$, as is demanded by the model (Fig. 8). From the slope and abscissa intercept of the graph k'_{+2} was evaluated as 167 sec.$^{-1}$ and K_I as $2{\cdot}2 \times 10^{-4}$ M.

From the stopped-flow analysis K_0 is $2{\cdot}15 \times 10^{-5}$ M and is in agreement with the value of $3(\pm 2{\cdot}5) \times 10^{-5}$ M obtained from the spectral titration.

The theory of chemical relaxation of two successive equilibria has been summarized by Eigen (1968). In the model (Scheme 1), if the assumption is made that the bimolecular process is very rapid compared with the rearrangement of EI to EI*, then the relaxation times are given by:

$$\frac{1}{\tau_1} = k'_{+1}([E]+[I]) + k'_{-1}$$

$$\frac{1}{\tau_2} = \frac{k'_{+2}}{1+K_I/([E]+[I])} + k'_{-2}$$

The stopped-flow experiments indicated that no spectral change is associated with the bimolecular binding process, so that the observation of only one relaxation process is to be expected.

The graphical presentation of the results (Fig. 9) shows that there is a reasonable correlation between the results of the stopped-flow experiments and the relaxation studies. There was a 5° temperature

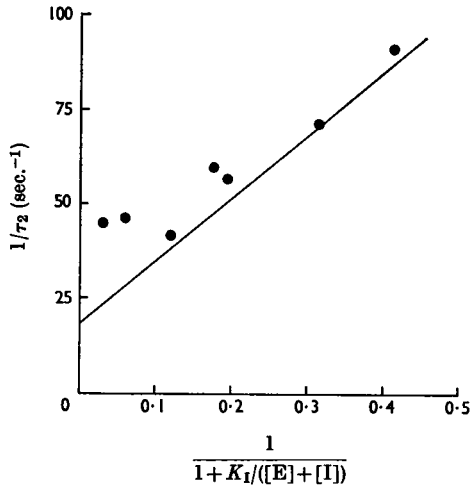


Fig. 9. Secondary plot of the reciprocal relaxation times (see Fig. 6) of the temperature-jump perturbations of the 2-hydroxy-5-nitrobenzylphosphonate and alkaline phosphatase binding equilibrium. K_I ($2{\cdot}2\times10^{-4}$M) was evaluated from Fig. 8. [E] and [I], the concentrations of free enzyme and phosphonate when equilibrium is established, were evaluated from the variation of $E^{1cm.}_{430}$ with phosphonate concentration (Fig. 4). The line drawn is that predicted from the model shown in Scheme 1 by using values of k'_{+2} and k'_{-2} determined from the stopped-flow studies.

differential, which will also introduce a small pH differential, in the two series of experiments. The shallow minimum in $1/\tau_2$ values at equal phosphonate and enzyme concentration is to be expected (Fig. 7). However, the divergence of points from the straight line at small ([E]+[I]) values probably indicates additional complexities in the system.

We are now in a position to examine the significance of the rate of conversion of EI into EI*, which is characterized by k_2 in the catalytic mechanism. At pH8 in the presence of 0·1M-tris and 1M-sodium chloride the catalytic-centre activity, $k_{cat.}$, is 60sec.$^{-1}$ from measurements of the steady-state rate of hydrolysis of 4-nitrophenyl phosphate. The rate constant, k_4, for the decomposition of the enzyme–phosphate complex is approx. 90sec.$^{-1}$ at pH8 (Aldridge, Barman & Gutfreund, 1964). The rate constant, k_4, is unaffected by changes in ionic strength (Fernley & Walker, 1968). In the model outlined in the introduction for the reaction of substrates with phosphatase k_3 is expected to be much greater than k_2 and k_4, since the reaction is insensitive to the nature of ROH. The catalytic-centre activity is a function of k_2 and k_4, and the relative contributions to these two rate constants to $k_{cat.}$ is pH-dependent (Fernley & Walker, 1966; Trentham & Gutfreund, 1968). At pH8, if one takes the value $k_4=90$sec.$^{-1}$ together with $k_2=167$sec.$^{-1}$, $k_{cat.}=nk_2k_4/(k_2+k_4)=58n$sec.$^{-1}$ where n is the number of simultaneously active sites/dimer (mol.wt. 86000) of enzyme.

The value for k_2 is obtained from the reaction of enzyme with phosphonate on the assumption that the interconversion of the enzyme–phosphonate complex occurs at the same rate as the interconversion of the enzyme–substrate complex during steady-state catalysis. The excellent fit of the experimentally determined constant when $n=1$ with the overall rate constant calculated for the behaviour of such a model supports the hypothesis that an important step in the reaction of *E. coli* phosphatase is indeed a rearrangement of the initial enzyme–substrate complex in response to phosphate binding. As such the experiments described are evidence for the induced-fit hypothesis proposed by Koshland (1958).

In view of the fact that the enzyme is a dimer and that it has been suggested that the two monomers are identical (Rothman & Byrne, 1963), it is noteworthy that a number of authors have reported that only one active site/dimer is reactive at any one time (Fernley & Walker, 1966, 1969; Ko & Kézdy, 1967; Trentham & Gutfreund, 1968). Our numerical results support this conclusion since the best fit is obtained when $n=1$.

A conformation change of an enzyme during a single turnover must necessarily involve a second change during that turnover. However, with a dimeric enzyme such as alkaline phosphatase, in which only one site appears to be active at any one time, it may be that the first change on one subunit is coupled to the second change on the other subunit, resulting in only one conformation change operating over the whole dimer/molecule of substrate hydrolysed.

The consistent fit of the individual rate constants with our model for the response of phosphatase to substrate binding is at present restricted to a particular set of conditions of pH, salt concentration and temperature. If the ionic strength is decreased to 0·1 a second, much faster, relaxation time is observed as well as a slow third relaxation time, which has a more complex phosphonate-concentration-dependence. This enzyme undergoes a number of pH- and ionic-strength-dependent transitions and these will superimpose other rate phenomena on those essential for the simplest sequence of catalytic turnover. It is, however, possible that some of these additional steps are involved in alternate activity of two sites of the dimer.

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REFERENCES

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

Buehler, C. A., Kirchner, F. K. & Deebel, G. F. (1940). In *Organic Syntheses*, vol. 20, p. 59. Ed. by Allen, C. F. H. New York: John Wiley and Sons Inc.

Echols, H., Garen, A., Garen, S. & Torriani, A. (1961). *J. molec. Biol.* **3**, 425.

Eigen, M. (1968). *Quart. Rev. Biophys.* **1**, 3.

Fernley, H. N. & Walker, P. G. (1966). *Nature, Lond.*, **212**, 1435.

Fernley, H. N. & Walker, P. G. (1968). *Biochem. J.* **110**, 11P.

Fernley, H. N. & Walker, P. G. (1969). *Biochem. J.* **111**, 187.

Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.

Gutfreund, H. (1965). *An Introduction to the Study of Enzymes*, p. 123. Oxford and Edinburgh: Blackwell Scientific Publications.

Kagan, F., Birkenmeyer, R. D. & Strube, R. E. (1959). *J. Amer. chem. Soc.* **81**, 3026.

Kirby, A. J. & Varvoglis, A. G. (1966). *J. Amer. chem. Soc.* **88**, 1823.

Kirby, A. J. & Varvoglis, A. G. (1967). *J. Amer. chem. Soc.* **89**, 415.

Ko, S. H. D. & Kézdy, F. J. (1967). *J. Amer. chem. Soc.* **89**, 7139.

Kochman, M., Mastalerz, P. & Wolna, E. (1964). *Arch. immunol. therap. exp.* **12**, 106.

Kosalopoff, G. M. (1945). *J. Amer. chem. Soc.* **67**, 2259.

Koshland, D. E. (1958). *Proc. nat. Acad. Sci., Wash.*, **44**, 98.

Malamy, M. H. & Horecker, B. L. (1964*a*). *Biochemistry*, **3**, 1889.

Malamy, M. H. & Horecker, B. L. (1964*b*). *Biochemistry*, **3**, 1893.

Meisters, A. & Swan, J. M. (1963). *Aust. J. Chem.* **16**, 725.

Plocke, D. J. & Vallee, B. L. (1962). *Biochemistry*, **1**, 1039.

Reynolds, J. A. & Schlesinger, M. J. (1967). *Biochemistry*, **6**, 3552.

Rothman, F. & Byrne, R. (1963). *J. molec. Biol.* **6**, 330.

Schlesinger, M. J. & Andersen, L. (1968). *Ann. N.Y. Acad. Sci.* **151**, 159.

Schlesinger, M. J. & Barrett, K. (1965). *J. biol. Chem.* **240**, 4284.

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

Stockell, A. (1959). *J. biol. Chem.* **234**, 1286.

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

Wenn, R. V. & Williams, J. (1968). *Biochem. J.* **108**, 69.