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Escherichia coli Alkaline Phosphatase

KINETIC STUDIES WITH THE TETRAMERIC ENZYME

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1. The stability of the tetrameric form of *Escherichia coli* alkaline phosphatase was examined by analytical ultracentrifugation. 2. The stopped-flow technique was used to study the hydrolysis of nitrophenyl phosphates by the alkaline phosphatase tetramer at pH 7.5 and 8.3. In both cases transient product formation was observed before the steady state was attained. Both transients consisted of the liberation of 1 mol of nitrophenol/2 mol of enzyme subunits within the dead-time of the apparatus. The steady-state rates were identical with those observed with the dimer under the same conditions. 3. The binding of 2-hydroxy-5-nitrobenzyl phosphonate to the alkaline phosphatase tetramer was studied by the temperature-jump technique. The self-association of two dimers to form the tetramer is linked to a conformation change within the dimer. This accounts for the differences between the transient phases in the reactions of the dimer and the tetramer with substrate. 4. Addition of P_i to the alkaline phosphatase tetramer caused it to dissociate into dimers. The tetramer is unable to bind this ligand. It is suggested that the tetramer undergoes a compulsory dissociation before the completion of its first turnover with substrate. 5. On the basis of these findings a mechanism is proposed for the involvement of the alkaline phosphatase tetramer in the physiology of *E. coli*.

When isolated from *Escherichia coli*, alkaline phosphatase is a dimer of molecular weight 86000. The two identical subunits each contain two Zn^{2+} ions (Reynolds & Schlesinger, 1969*a*). However, Reynolds & Schlesinger (1969*b*) found that, in the presence of excess of Zn^{2+}, the molecular weight of alkaline phosphatase increased as a function of pH to a maximum of 172000 at pH 8.0. This self-association was rapid and reversible, and no higher aggregates were observed in the ultracentrifuge. It was found that each subunit in the tetramer bound two extra Zn^{2+} ions and lost up to a maximum of two protons as compared with the dimer. Reynolds & Schlesinger (1969*b*) also reported that this self-association led to an alteration in the u.v.-absorption spectrum of the enzyme, probably due to the removal of one tryptophan residue/chain from contact with the aqueous medium.

In the present paper we describe some studies on the hydrolysis of nitrophenyl phosphate esters by the alkaline phosphatase tetramer and on its ability to bind certain inhibitors. Unimolecular isomerizations determine the initial rate of product formation during substrate hydrolysis by the alkaline phosphatase dimer (Halford *et al.*, 1969; Halford, 1971): i.e. the rate of formation of the enzyme–P_i intermediate is determined by these conformation changes rather than by the chemical processes in the breaking of the phosphate ester bond (Trentham & Gutfreund, 1968). Whether or not the final step, dephosphorylation, determines the overall catalytic-centre activity depends on the pH (Fernley & Walker, 1966). As these conformation changes are readily separated from other events during the turnover of alkaline phosphatase with substrate, the formation of the tetramer provides a system in which we can examine the relationship between the tertiary and quaternary structures of a polymeric enzyme. Conditions were found for the preparation of two solutions of alkaline phosphatase that were identical in all respects with the exception of the free Zn^{2+} concentration: one solution contained the dimer and the other the tetramer. Thus, in experiments comparing their reactivities, the dimer and the tetramer could be studied under very similar experimental conditions and at the same protein concentration.

Materials and Methods

Alkaline phosphatase (EC 3.1.3.1) was prepared from either *E. coli* C90 (Halford, 1971) or *E. coli* CW 3747 (Schlesinger & Olson, 1970). *E. coli* C90 cells were provided by Dr. K. Sargeant (Microbiological Research Establishment, Porton, Wilts.,

U.K.). The molecular weights of the alkaline phosphatase dimer and tetramer have been taken to be 86000 and 172000 respectively.

The reported concentrations of Zn^{2+} solutions are those of free Zn^{2+} and have been corrected for the uptake of Zn^{2+} by alkaline phosphatase during tetramer formation. The amount of Zn^{2+} initially present on the dimer (up to two Zn^{2+} ions/subunit) was calculated from the specific activity, and each subunit in the tetramer contains four Zn^{2+} ions (Reynolds & Schlesinger, 1969*a*,*b*). 2-Hydroxy-5-nitrobenzyl phosphonate chelates Zn^{2+} (Halford *et al.*, 1969), so when this inhibitor was added to a solution of the alkaline phosphatase tetramer an equimolar amount of $ZnCl_2$ was added with it.

Buffers of tes [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid] and tricine [*N*-tris-(hydroxymethyl)methylglycine] were adjusted to the required pH with 5M-KOH.

In stopped-flow records of substrate hydrolysis by a mixture of alkaline phosphatase dimers and tetramers at pH5.7, product formation was observed to occur in three separate phases: a very rapid phase of transient product formation completed within the instrument dead-time, a slower transient phase the rate of which could be measured with the stopped-flow apparatus, and finally a steady-state phase. To simplify the nomenclature, the very rapid transient is described in this paper as an 'instant phase', and the term 'burst' is restricted to the transient whose rate could be measured with the stopped-flow apparatus.

Sedimentation velocities were determined in a Beckman model E analytical ultracentrifuge at a rotor speed of 59780rev./min. The ultracentrifuge was equipped with a schlieren optical system. All other methods and materials have been described by Halford (1971, 1972).

Results and Discussion

Ultracentrifuge experiments

An alkaline phosphatase solution of 5mg/ml in 50mM-KCl, pH8.10, was found to have a sedimentation coefficient, $s^{0.5\%}_{20,w}$, of 6.3S, but when this solution also contained 0.10mM-$ZnCl_2$ a value of 9.5S was obtained. These sedimentation coefficients characterize the dimeric and tetrameric forms of alkaline phosphatase (Reynolds & Schlesinger, 1969*b*). But the self-association is critically dependent on pH, so it was necessary to use a buffer for kinetic experiments. However, most of the buffers that can be used close to pH 8.0 are amines, which have a high affinity for Zn^{2+}. When 0.1ml of a 0.1M solution of tes, tris, tricine or veronal buffers at pH8.10 was added to 1.0ml of the tetramer solution as above, a partial dissociation of the tetramer was observed. This was indicated by the single peak in the schlieren pattern sedimenting at a velocity between those measured for tetramer and dimer. Of these buffers, tes caused the least dissociation, whereas tricine and veronal were the most damaging to the stability of the tetramer.

The linked function equations of Wyman (1964) predict that the dimer–tetramer equilibrium should be driven towards the associated form by increasing the pH above 8.10 and the Zn^{2+} concentration above 0.10mM. In 10mM-tes–50mM-KCl buffer, pH8.30, values for $s^{0.5\%}_{20,w}$ of 9.4 and 6.3S were obtained when the enzyme solutions contained either 0.30mM-$ZnCl_2$ or no extra Zn^{2+} respectively. Thus under these conditions the tetramer is stable in the presence of tes. The difference in the sedimentation behaviour was not caused by Zn^{2+} alone. A sample of enzyme in 10mM-tes – 50mM-KCl – 0.30mM-$ZnCl_2$ buffer, pH6.50, sedimented at the rate of the dimeric enzyme. As concluded by Reynolds & Schlesinger (1969*b*), tetramer formation requires both high Zn^{2+} concentrations and pH values above 8.10.

The effects of competitive inhibitors on the self-association equilibrium were also studied in the ultracentrifuge. The sedimentation coefficient of the alkaline phosphatase tetramer was not altered when the solution was made 1.0mM with respect to 2-hydroxy-5-nitrobenzyl phosphonate. But when a solution of the tetramer was made 1.0mM with respect to P_i, a dissociation into dimers was observed. These effects have been studied in further detail (see Figs. 2 and 6 below).

Studies with substrate at pH5.7

The stopped-flow technique has been used to study the hydrolysis of 2,4-dinitrophenyl phosphate by dimeric and tetrameric alkaline phosphatase at pH5.7 (Figs. 1*a* and 1*b*). Fig. 1(*a*) is the record of the reaction after mixing alkaline phosphatase in 10mM-tes–50mM-KCl buffer, pH8.30, with 2,4-dinitrophenyl phosphate in 0.1M-sodium acetate buffer, pH5.5. The final pH of the reaction mixture was 5.7. The concentrations of reactants used for Fig. 1(*b*) were identical except that the enzyme solution also contained 0.30mM-$ZnCl_2$. The difference between these reactions was not solely due to the Zn^{2+} concentration. If the enzyme solution with Zn^{2+} was adjusted to pH5.5 some time before mixing with substrate or if Zn^{2+} was added only to the substrate solution, then reactions of the type shown in Fig. 1(*a*) were observed. Comparison with the ultracentrifuge results shows that the enzyme was present as the dimer for the reaction in Fig. 1(*a*) and as the tetramer at the start of the reaction in Fig. 1(*b*). The rate of dissociation of the tetramer at pH5.7 must be lower than the first turnover of this form of the enzyme, for otherwise no difference would be observed between Figs. 1(*a*) and 1(*b*).

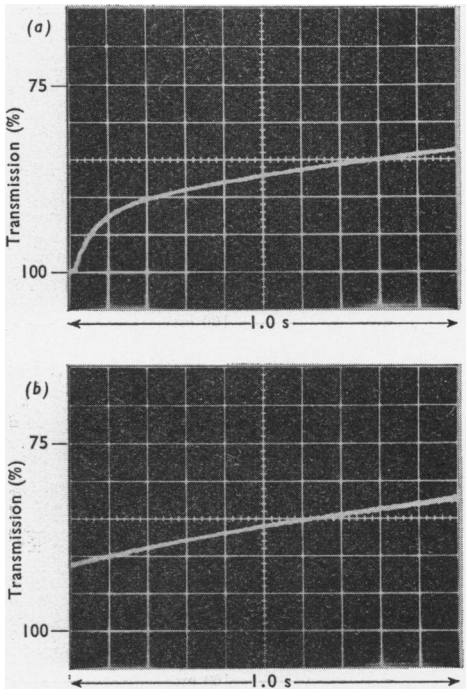

Fig. 1. *Spectrophotometric records at 360nm of stopped-flow observations after mixing alkaline phosphatase in the presence and in the absence of Zn^{2+} with 2,4-dinitrophenyl phosphate*

(*a*) After mixing alkaline phosphatase (0.83mg/ml) in 10mM-tes–50mM-KCl buffer, pH8.30, with 0.14mM-2,4-dinitrophenyl phosphate in 0.1M-sodium acetate buffer, pH5.5. The final pH of the reaction mixture was 5.7. Two reaction traces are recorded. (*b*) As (*a*), except that the enzyme solution also contained 0.30mM-$ZnCl_2$.

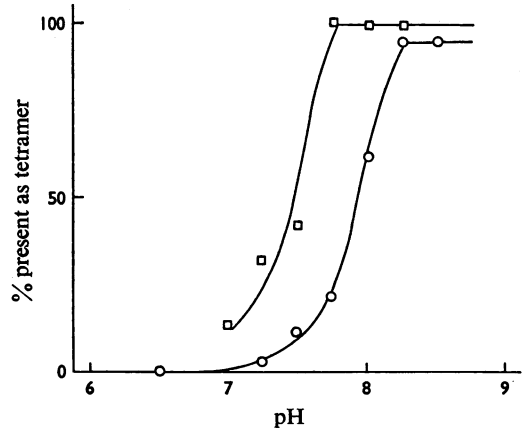

Fig. 2. *Relationships between percentage of tetramer present in solutions of alkaline phosphatase and pH in the presence and in the absence of 2-hydroxy-5-nitrobenzyl phosphonate*

o, Alkaline phosphatase (0.98mg/ml) in 10mM-tes–50mM-KCl–0.30mM-$ZnCl_2$ buffers. The stopped-flow procedure used to calculate the percentage of tetramer is described in the text. □, As above, except that the enzyme solution also contained 0.20mM-2-hydroxy-5-nitrobenzyl phosphonate.

During the hydrolysis of 2,4-dinitrophenyl phosphate by the alkaline phosphatase dimer, a transient release of 2,4-dinitrophenol was observed before the establishment of the steady state (Fig. 1*a*). But with the tetramer the release of the first product was too fast to measure with the stopped-flow apparatus, whereas the rate of the subsequent steady state was identical with that of the dimer (Fig. 1*b*). After extrapolation of the steady-state phases of these reactions back to zero time and correction for the E_{360} of the enzyme solutions, it was found that the two forms of the enzyme generated identical extinction changes in the approach to the steady state.

The amplitude of the burst phase observed during substrate hydrolysis at pH5.7 shows that only one active site in the alkaline phosphatase dimer functions in catalysis at any given time. As identical amplitudes of transient product formation were observed with the dimer and the tetramer, the stoicheiometry of one effective active site/two polypeptide chains is unaltered by this aggregation. As with the observed burst of the dimer, the amplitude of the instant phase from the tetramer was independent of the substrate concentration over the range from 25μM to 0.50mM.

An examination of Figs. 1(*a*) and 1(*b*) shows that the amplitudes of the burst and instant phases provides a sensitive measurement of the equilibrium position in the protein association reaction. By increasing the sensitivity of the transmission scale after making the appropriate positional adjustment to the 100% transmissionl ine, the existence of a small fraction of dimer present in a solution that is principally tetramer is readily identified by the observation of a burst. Estimations of the amount of dimer present when this is the predominant species are less accurate because it is difficult to measure a small difference between the amplitudes of two large bursts with a single-beam stopped-flow apparatus. Further examination of the reaction in Fig. 1(*b*) on a more sensitive transmission scale revealed the existence of a small burst that occurred after the completion of the instant phase but before the steady state. A rate constant of $15s^{-1}$ was evaluated for this burst, the same as that determined from the burst rate during substrate hydrolysis by the dimer under identical

conditions. From the amplitude, it was calculated that 0.83mg of alkaline phosphatase/ml in 10mM-tes–50mM-KCl–0.30mM-$ZnCl_2$ buffer, pH8.30, was present as 5% (by weight) dimer and 95% tetramer.

The above procedure was used to study the stability of the tetramer as a function of pH, both in the absence and in the presence of 2-hydroxy-5-nitrobenzyl phosphonate (Fig. 2). The shape of the plots in Fig. 2 is identical with that obtained by Reynolds & Schlesinger (1969*b*), but the additional components in the enzyme solution cause displacements along the pH axis. The linkage between the association of alkaline phosphatase dimers and the pH is co-operative: a change of 0.25 pH unit can increase the fraction of enzyme in the tetrameric state from 50% to 100%. The phosphonate stabilized the tetramer: addition of this ligand to the enzyme at pH7.75 increased the fraction present as tetramer from 25 to 100%. The previous addition of ligands such as 2-hydroxy-5-nitrobenzyl phosphonate or P_i to the enzyme solution did not alter the total amplitude of the pre-steady-state phases in the reaction after mixture of that enzyme solution with substrate at pH5.5. The effect of these ligands was only on the relative proportions of instant and burst phases within an overall pre-steady-state of constant amplitude.

*Studies with substrate at pH*8.30

The only difference between the reactions of the alkaline phosphatase dimer and tetramer with substrate at pH5.7 was the rate of transient-product formation. With the dimer this rate is determined by a conformation change in the enzyme before binding of substrate, the subsequent steady-state rate being limited by the dephosphorylation step (Halford, 1971). At alkaline pH values the steady-state rate of substrate hydrolysis by the dimer is partially determined by an isomerization of the enzyme–substrate complex (Halford *et al.*, 1969), for dephosphorylation is faster than the catalytic-centre activity under these conditions (Aldridge *et al.*, 1964). The evidence for this change in the rate-limiting step between acidic and alkaline conditions was first presented by Fernley & Walker (1966) and also by Trentham & Gutfreund (1968).

When alkaline phosphatase in 10mM-tes–50mM-KCl buffer, pH8.30, was mixed in the stopped-flow apparatus with 4-nitrophenyl phosphate in the same buffer, the dimer caused the liberation of 4-nitrophenol, starting at the steady-state rate from 100% transmission at zero time (Fig. 3*a*). On repetition of this experiment but after the enzyme solution had been made 0.30mM with respect to $ZnCl_2$ to form the tetramer, a very rapid release of 4-nitrophenol occurred within the dead-time of the stopped-flow apparatus (Fig. 3*b*). The amplitude of transient product formation with the tetramer at pH8.30 gave a stoicheiometry of 1 functional active site/2 enzyme subunits. This result was independent of the substrate concentration across the range 0.05 to 1.0mM. The dimer and the tetramer always exhibited the same steady-state rates of substrate hydrolysis at pH8.30. This was observed even when the substrate was dissolved in the Zn^{2+}-containing buffer used for tetramerization, under which conditions it might have been expected that the tetramer would not dissociate.

These experiments at pH8.30 pose the following paradox. No transient is observed during substrate hydrolysis by the alkaline phosphatase dimer because the rate-limiting step for the complete turnover precedes the appearance of the chromo-

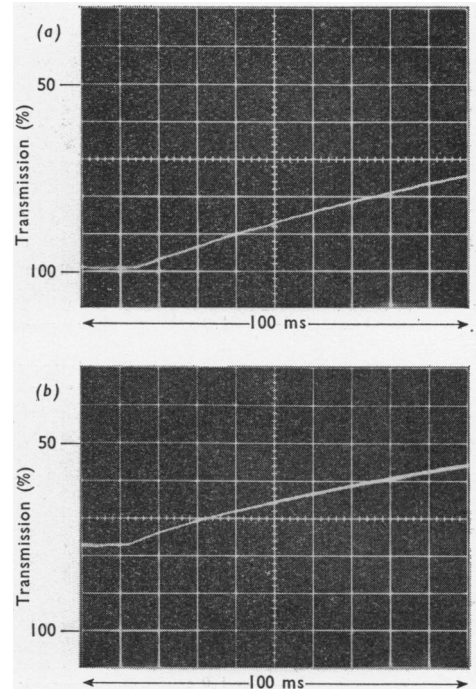

Fig. 3. *Spectrophotometric records at 400nm of stopped-flow observations after mixing alkaline phosphatase in the presence and in the absence of Zn^{2+} with 4-nitrophenyl phosphate*

(*a*) After mixing alkaline phosphatase (0.98mg/ml) in 10mM-tes–50mM-KCl buffer, pH8.30, with 0.20mM-4-nitrophenyl phosphate in the same buffer. Two reaction traces are recorded. (*b*) As (*a*), except that the enzyme solution also contained 0.30mM-$ZnCl_2$. The observed curvature of the reaction record is caused by the ordinate being in units of percentage transmission. The change in extinction at 400nm is linear with time.

phore, 4-nitrophenol: transient product formation is observed with the tetramer, so in this case the rate-limiting step is located in the mechanism at some point after the initial liberation of 4-nitrophenol: yet the steady-state rates of substrate hydrolysis by the dimer and the tetramer are identical. The remaining experiments described in this paper were designed to seek a solution of this paradox.

Studies with 2-hydroxy-5-nitrobenzyl phosphonate

The binding of 2-hydroxyl-5-nitrobenzyl phosphonate to the alkaline phosphatase tetramer has been studied. The complex of 2-hydroxy-5-nitrobenzyl phosphonate and the alkaline phosphatase dimer exhibits a difference spectrum, with a peak at 430nm, when compared with the separate solutions (Halford *et al.*, 1969). In addition a difference spectrum is observed between the complex of this phosphonate with Zn^{2+} and the separate solutions, but this had a peak at 390nm (Halford *et al.*, 1969). By examining a particular reaction of the phosphonate in the presence of alkaline phosphatase and excess of Zn^{2+} at both 430 and 390nm, it can readily be shown whether the reaction is caused by the association of the phosphonate with the enzyme or with free Zn^{2+}. The peak of the difference spectrum between a mixture of 2-hydroxy-5-nitrobenzyl phosphonate and alkaline phosphatase at pH8.30 was still found at 430nm when both the mixture and reference solutions contained Zn^{2+} in excess of the phosphonate.

Equilibrium binding curves were obtained from records of the extinction change at 430nm on the addition of the Zn^{2+}–2-hydroxy-5-nitrobenzyl phosphonate complex to alkaline phosphatase in 10mM-tes–0.10M-KCl–0.30mM-$ZnCl_2$ buffer, pH8.30. It was found that the alkaline phosphatase tetramer had 0.5 ± 0.1 ligand binding site/protein subunit and that the equilibrium dissociation constant, $K_{eq.}$, of the phosphonate from the tetramer was 5×10^{-6}M. Both the experimental technique and the method of analysis were described by Halford (1972). The value for this dissociation constant is considerably smaller than that of 2.7×10^{-5}M obtained for the dimer under these conditions but in the absence of Zn^{2+}. However, the stoicheiometry of this binding reaction was not affected by the protein association if one considers the number of sites/subunit.

The individual steps in the association of 2-hydroxy-5-nitrobenzyl phosphonate with the alkaline phosphatase tetramer were analysed by the temperature-jump technique. Only one relaxation was observed with this system at 430nm (Fig. 4). An examination of this process at different wavelengths revealed that the maximum amplitude occurred at 430nm, so it is related to the association of the phosphonate with the enzyme rather than its chelation of excess of Zn^{2+}. The reciprocal relaxation

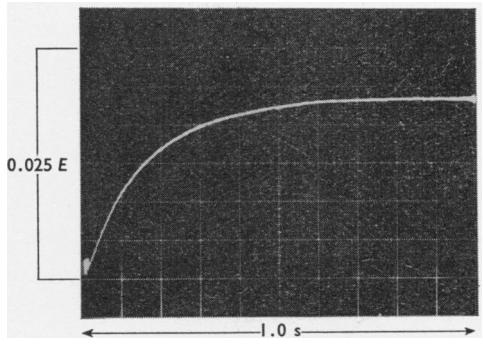

Fig. 4. *Spectrophotometric record at 430nm of a temperature-jump perturbation of the binding equilibrium of 2-hydroxy-5-nitrobenzyl phosphonate and alkaline phosphatase*

The binding of 2-hydroxy-5-nitrobenzyl phosphonate (0.20mM) and alkaline phosphatase (10.3mg/ml) in 10mM-tes–0.10M-KCl–0.30mM-$ZnCl_2$ buffer, pH8.30, was followed.

time of this phase was measured at a fixed concentration of tetrameric enzyme in the presence of various concentrations of the phosphonate (Fig. 5). This reciprocal relaxation time was independent of the phosphonate concentration over the range from 10μM to 0.30mM, and had a value of $7s^{-1}$. The time-constant of the slowest relaxation observed in temperature-jump studies on phosphonate binding to the alkaline phosphatase dimer in 10mM-tes–0.10M-KCl buffer, pH8.30, was also measured over a range of phosphonate concentrations (Fig. 5).

The concentration-independence of the relaxation time with the tetramer indicates that it describes the re-equilibration of some unimolecular step. Provided that the rearrangement is slower than the binding step in Scheme (3). The numbers and nomenclature of reaction schemes is that used by Halford (1971, 1972):

$$E \underset{k_0}{\overset{k_0^*}{\rightleftharpoons}} E^*;\ E^* + I \underset{k_{-1}^*}{\overset{k_{+1}^*}{\rightleftharpoons}} E^*I \qquad (3)$$

the reciprocal relaxation time of the conformational equilibrium is given by:

$$\frac{1}{\tau_0} = k_0^* + \frac{k_0}{1+\dfrac{[I]}{K_1^* + [E^*]}}$$

where [I] and [E*] are the concentrations of free phosphonate and enzyme in the E* conformation and $K_1^* = k_{-1}^*/k_{+1}^*$. In the temperature-jump experiment with the tetramer (Fig. 5) the concentration of

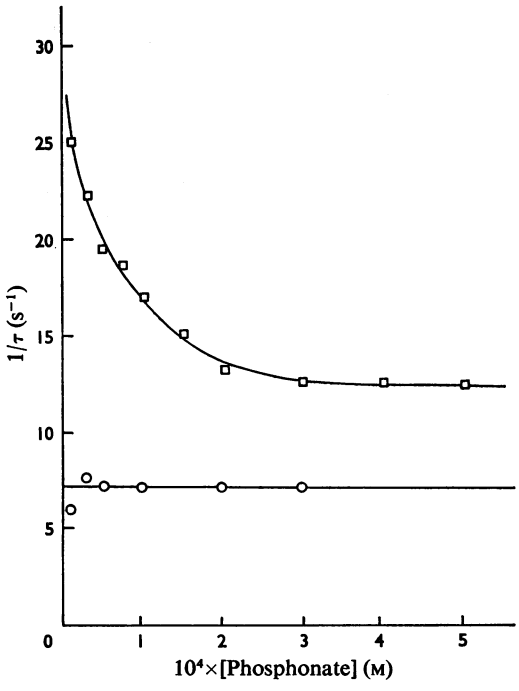

Fig. 5. *Relationships between the reciprocal relaxation times and the 2-hydroxy-5-nitrobenzyl phosphonate concentration, obtained from temperature-jump perturbations of the binding equilibrium of the phosphonate with fixed concentrations of alkaline phosphatase*

Reciprocal relaxation times ($1/\tau$) were determined from records such as Fig. 4, and the phosphonate concentration refers to its total concentration in the equilibrium mixture. o, Alkaline phosphatase (10.3mg/ml) in 10mM-tes – 0.10M-KCl – 0.30mM-$ZnCl_2$ buffer, pH8.30 (tetramer). □, Alkaline phosphatase (7.2mg/ml) in 10mM-tes–0.10M-KCl buffer, pH8.30 (dimer).

2-hydroxy-5-nitrobenzyl phosphonate was varied in the proximity of both the enzyme concentration and the value of the overall dissociation constant, yet the reciprocal relaxation time did not vary. However, this concentration-independence can still be accommodated in Scheme (3) if $k_0^\star \gg k_0$. Then $k_0^\star = 7s^{-1}$, whereas k_0 is very much smaller than this value. On the basis of this analysis the equilibrium position between the E and $E^\star$ conformations within the tetramer must be strongly in favour of E in the absence of ligand.

Scheme 3 can also accommodate the relaxation results for phosphonate binding to the dimer (Fig. 5). Provided that the above condition of $k_0^\star \gg k_0$ is not maintained, this model predicts a decrease in the reciprocal relaxation time as the phosphonate concentration is increased. From Fig. 5 it can be seen that $k_0^\star$ is smaller than k_0 in the dimer, even though relaxation times were not measured at phosphonate concentrations low enough to permit an unequivocal evaluation of k_0. Hence the equilibrium position between the E and $E^\star$ conformations in the dimer is in favour of the E state in the absence of ligand. We therefore conclude that the self-association of alkaline phosphatase dimers to form the tetramer is linked to a conformation change from the E to the $E^\star$ structures within individual units. This conclusion does not require modification should additional pathways exist for the formation of $E^\star I$ from E, provided that the kinetically preferred route involves the conformation change in the free enzyme rather than in the enzyme–ligand complex.

The conformations of the subunits within the different aggregations can explain the stabilization of the tetramer by 2-hydroxy-5-nitrobenzyl phosphonate (Fig. 2). Under conditions similar to those in which the tetramer has been studied (I 0.05 and pH8.0; Halford, 1972) the equilibrium concentrations of the intermediates in the binding of phosphonate to the dimer obey the following relationships: $[E] > [E^\star]$ but $[EI] > [E^\star I]$. If the tetramer is present as $E^\star$, the addition of phosphonate to produce $E^\star I$ will lock the enzyme into this conformation. The tetramer is then stabilized through the linkage between conformational and aggregation states. Further, the overall dissociation constant, $K_{eq.}$, of phosphonate from the tetramer should equal the microscopic dissociation constant, $K_1^\star$, if the tetramer is already in the $E^\star$ conformation. In Scheme 3:

$$K_{eq.} = K_1^\star [(k_0 + k_0^\star)/k_0^\star]$$

so, if $k_0^\star \gg k_0$, then $K_{eq.}$ equals $K_1^\star$. The overall dissociation constant was determined as 5×10^{-6}M from the equilibrium binding curve, and the same value was taken for $K_1^\star$ in relaxation experiments of phosphonate binding to the $E^\star$ conformation of the dimer (Halford, 1972).

The relationship between conformation and self-association also accommodates the differing reactivities of the dimer and the tetramer in the first turnover with substrate. Both at acidic and at alkaline pH values a conformation change limits the initial rate of appearance of the alcohol in stopped-flow observations of phosphate ester hydrolysis by the alkaline phosphatase dimer (Halford *et al.*, 1969; Halford, 1971). In both cases there is a step in the pathway from E to $E^\star S$ with the dimer whose rate is measurable with the stopped-flow apparatus. It has been suggested that the subsequent chemical catalysis within the $E^\star S$ intermediate is extremely rapid (Trentham & Gutfreund, 1968). However, if the tetramer is already in the $E^\star$ conformation, $E^\star S$ will be the first intermediate formed after the addition

of substrate to the tetramer. The rate of formation of E^*S will then be determined by a very rapid bimolecular step. Consequently the hydrolysis of nitrophenyl phosphate by the tetramer should proceed with the liberation of 1 mol of nitrophenol/mol of effective active sites within the dead-time of the stopped-flow apparatus, provided that the decomposition of the ester in the E^*S intermediate is rapid. This kinetic behaviour has been observed (Figs. 1*b* and 3*b*). On the analysis from mechanism 3 the very fast rate of transient-product formation with the tetramer is experimental evidence that the bond-breaking step in phosphate monoester hydrolysis by alkaline phosphatase is extremely fast and plays no part in determining the catalytic-centre activity of this enzyme.

The above scheme is not unique in its ability to produce concentration-independent reciprocal relaxation times. For example, provided that the isomerization is slower than the binding step in Scheme (2):

$$E+I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EI \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} E^*I \qquad (2)$$

the second reciprocal relaxation time is given by:

$$\frac{1}{\tau_2} = k_{-2} + \frac{k_{+2}}{1+\dfrac{K_1}{[E]+[I]}}$$

where [E] and [I] are the concentrations of free reactants and $K_1 = k_{-1}/k_{+1}$. If this reciprocal relaxation time is to remain invariant while the concentrations of free reactants are varied from below to above the value of K_1, then $k_{-2} \gg k_{+2}$. With Scheme (2), k_{-2} is evaluated as $7\,s^{-1}$ from the concentration-independent reciprocal relaxation time in Fig. 5, and k_{+2} must be very much smaller than that value. Hence the rate of formation of E^*I with the tetramer is extremely low if Scheme (2) applies. This prediction is incompatible with the stopped-flow observations on the very rapid rate of transient product formation during substrate hydrolysis by the alkaline phosphatase tetramer. A mechanism in which the rate of the conformation change in Scheme (2) is very much faster in the tetramer than in the dimer could accommodate the stopped-flow results on transient product formation presented in this paper, but not the temperature-jump observations.

The only simple mechanism that can accommodate the results from both stopped-flow and temperature-jump experiments is that there is a pre-equilibrium between two conformations of the enzyme, and that self-association to form the tetramer is linked to a shift in the equilibrium position between these conformations. The conformation in the tetramer is that required for catalytic activity. However, as the stoicheiometry of transient-product formation during substrate hydrolysis by the tetramer is 1 mol of product/2 mol of enzyme subunits, only one subunit in the dimer can be participating in this conformation change linked to self-association.

The different pre-steady-states in the hydrolysis of 2,4-dinitrophenyl phosphate by the alkaline phosphatase dimer and tetramer at pH 5.7 have been used to determine the position of the self-association equilibrium. But a rigorous correlation between the amplitudes of the fast and the slow phases in this reaction and the molecular weight of the enzyme has not been done. Studies on haemoglobin have shown that such correlations are not necessarily simple (Kellett & Gutfreund, 1970, and references cited therein). In Fig. 1 the relative amplitudes of the observed burst and the instant phase in fact correspond to the fractions of enzyme present in the E and E^* conformations respectively, rather than to the proportions of dimer and tetramer. But, within the limits of experimental error, the sole conformation of the dimer at pH 5.7 in low ionic strength is the E state in the absence of ligand (Halford, 1971), whereas the maximum amplitude of the instant phase indicates a complete isomerization of two out of the four subunits into the E^* conformation for the tetramer. Thus under these specific conditions the correlation is valid.

Studies with P_i

Although the above relationship between subunit conformation and protein association can explain the different rates of transient product formation during substrate hydrolysis by the alkaline phosphatase dimer and tetramer, there remains the problem of the identical steady-state rates. At alkaline pH values the conformation change induced by substrate binding determines the catalytic-centre activity (Halford *et al.*, 1969). Thus in by-passing the conformation change it might have been expected that the tetramer would display an enhanced catalytic-centre activity at pH 8.30, but this is not the case (Fig. 3*b*).

However, it was noted in the ultracentrifuge studies that the addition of P_i to the alkaline phosphatase tetramer caused its dissociation into dimers. It has also been observed that the addition of P_i to both the alkaline phosphatase tetramer and dimer abolished the u.v. difference spectrum that Reynolds & Schlesinger (1969*b*) had observed between the two aggregates. The u.v.-absorption spectrum of the dimer was not altered by the addition of P_i. This dissociation of the tetramer has been studied as a function of the P_i concentration. Small portions of P_i were added to solutions of 0.75 mg of alkaline phosphatase tetramer/ml in 10 mM-tes–50 mM-KCl–0.30 mM-$ZnCl_2$ buffer, pH 8.30, and these solutions were then mixed with 2,4-dinitrophenyl phosphate in

0.1M-sodium acetate buffer, pH5.5, in the stopped-flow apparatus. From the amplitudes of the observed bursts, the amount of dimer formed at each P_i concentration could be calculated (Fig. 6). Equivalent results were obtained when dimer formation was measured by u.v. difference spectroscopy.

The results in Fig. 6 were analysed as an equilibrium binding curve. The dissociation constant, K_0, between the dimer–P_i complex and the ligand-free enzyme was evaluated as $7.0 (\pm 1.4) \times 10^{-5}$M. For this analysis the molarity of the ligand-free enzyme was expressed in dimeric units of 86000 daltons, and it was assumed that there was one P_i-binding site/dimer. The binding curve could not define a unique stoicheiometry for this reaction. Reynolds & Schlesinger (1969*a*,*b*) found, however, only 1equiv. of P_i bound to the alkaline phosphatase dimer with high affinity: the dissociation constant was determined to be 3×10^{-6}M. As we have observed the formation of the dimer on the addition of P_i to the tetramer, the final product must be the dimer–P_i complex containing 1equiv. of P_i. In the previous study on the binding of P_i to the tetramer, done by equilibrium dialysis, the molecular weight of the enzyme–phosphate complex was not determined (Reynolds & Schlesinger, 1969*b*).

It is noteworthy that the dissociation constant of P_i is larger from a sample of alkaline phosphatase that was originally in the tetrameric state than it is from the dimer. This result can be accommodated by the following Scheme, in which P_i can bind to only the dimeric form of the enzyme:

$$E_T \rightleftharpoons E_D \qquad K_D = \frac{[E_D]^2}{[E_T]}$$

$$E_D + P_i \rightleftharpoons E_D \cdot P_i \qquad K_S = \frac{[E_D][P_i]}{[E_D \cdot P_i]}$$

$[E_D]$ and $[E_T]$ are the molarities of dimeric and tetrameric alkaline phosphatase, $[P_i]$ that of P_i and $[E_D \cdot P_i]$ the dimer–P_i complex. For this mechanism the overall dissociation constant, K_0, is defined by:

$$K_0 = \frac{[E][P_i]}{[E_D \cdot P_i]}$$

where [E] is the total concentration (in units of 86000 daltons) of enzyme not bound to ligand; i.e.:

$$[E] = [E_D] + 2[E_T]$$

For this scheme it can be shown that the above definitions of K_0 and K_S are related by:

$$K_0 = K_S \left(\frac{2[E_T]}{[E_D]} + 1 \right)$$

From the values of 7×10^{-5}M and 3×10^{-6}M for K_0 and K_S respectively, $[E_T]/[E_D] = 11.2$. It should be noted that, in the absence of P_i, 5% (by weight) of the enzyme was present as the dimer. With the total alkaline phosphatase concentration of 0.75mg/ml, the protein dissociation constant, K_D, was calculated to be 3.3×10^{-8}M.

The above scheme for the binding of P_i to the tetramer was tested by repeating this experiment under identical conditions but with a total enzyme concentration of 1.75mg/ml. The dissociation constant, K_0, between the dimer–P_i complex and the free enzyme was then evaluated as 1.2×10^{-4}M. With this value, K_D was calculated to be 2.6×10^{-8}M, a value in good agreement with that determined at the lower protein concentration. Further, the amount of dimer obtained by the addition of a fixed amount of P_i to a solution of the tetramer was markedly dependent on the pH of the tetramer solution, there being less dimer formed at higher pH values. Because the dissociation constant, K_S of P_i from the dimer was invariant over the pH range 7–9 (Reynolds & Schlesinger, 1969*b*), this variation in the extent of dimer production must be caused by the pH-dependence of the protein dissociation constant, K_D, of the alkaline phosphatase tetramer. It was noted above that this constant is indeed very sensitive towards pH changes (Fig. 2).

Frieden (1967) pointed out that saturation curves such as that obtained for the binding of P_i to a mixture of alkaline phosphatase dimers and tetramers should be sigmoidal rather than hyperbolic if

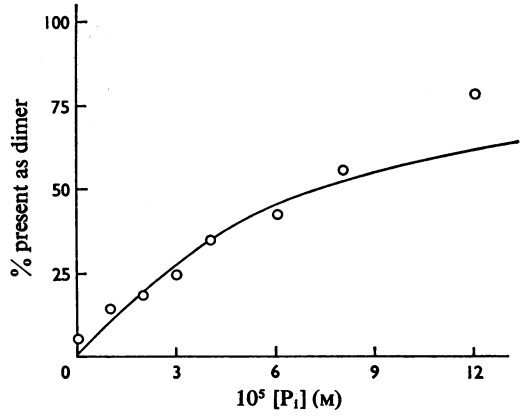

Fig. 6. *Relationship between percentage of dimer in solutions of alkaline phosphatase and concentration of added P_i*

Alkaline phosphatase (0.75mg/ml) was in 10mM-tes–50mM-KCl–0.30mM-$ZnCl_2$ buffer, pH8.30, and P_i was added as indicated. The line drawn is that predicted for $K_0 = 7.0 \times 10^{-5}$M. The stopped-flow procedure used to measure the percentage of dimer and the definition of K_0 are both described in the text.

the two forms of the enzyme have different affinities for the ligand. But, with the above constants, it was found that the difference between the sigmoidal curve calculated from the equation of Frieden (1967) and the hyperbolic curve from the above analysis was smaller than the experimental error. All the experimental points in Fig. 6 except that at the highest P_i concentration fell within the range given by $K_0 = 7.0\ (\pm 1.4) \times 10^{-5}$ M. But, as mentioned above, the stopped-flow procedure for measuring the fraction of enzyme present as dimer is not accurate when this species is predominant. If the molar ratio, $[E_T]/[E_D]$, had a much greater value than 11.2, the sigmoidicity would have been more apparent.

The dissociation of the alkaline phosphatase tetramer caused by the addition of P_i is compatible with a scheme in which the only complex formed between P_i and this enzyme is the dimeric intermediate. If this is the case, then there will be a compulsory dissociation of the tetramer during its first turnover with substrate because microscopic reversibility demands that P_i be released from the dimer. As substrate can bind at a rate that is close to being diffusion-controlled (Halford *et al.*, 1969), the next substrate will bind to the dimer before protein reassociation can take place. Therefore, under the conditions used for the experiments in Figs. 1(*b*) and 3(*b*), alkaline phosphatase will remain as a dimer after the first turnover with substrate. No simpler mechanism can account for the identical steady-state rates of substrate hydrolysis by the dimer and the tetramer. But, if the protein were present in large excess over substrate, as might be the case for certain situations *in vivo* (see below), then the reassociation of dimers might occur at a rate comparable with that of substrate binding.

Conclusions

It has been demonstrated that the conformation of the alkaline phosphatase dimer is changed on self-association to form the tetramer. An isomerization is also a kinetically important prerequisite for substrate hydrolysis by the dimer. The properties of the tetramer provide further evidence that the unimolecular step coupled to substrate binding by the dimer is a conformation change of the protein. By undergoing the necessary conformation change during the self-association reaction rather than during substrate binding, the alkaline phosphatase tetramer becomes poised for catalytic activity in the absence of substrate. This is the simplest mechanism for the tetramer that can accommodate both the fast transient observed by the stopped-flow technique and the slow relaxation noted in temperature-jump studies. There are a number of alternative mechanisms that could account for either the fast transient or the slow relaxation, but not both. This illustrates the complementary nature of transient and relaxation methods and the necessity of using both approaches in the study of enzyme mechanisms.

The transient liberation of 2,4-dinitrophenylate during the hydrolysis of 2,4-dinitrophenyl phosphate by the alkaline phosphatase tetramer at pH5.7 is so rapid that its rate constant cannot be measured by the stopped-flow technique (Fig. 1*b*). But the amplitude of this reaction is so large that, even if the first five half-times were completed within the dead-time of the stopped-flow apparatus, the rate of transient-product formation could still be measured. We can therefore set a lower limit of $10^3\ s^{-1}$ for the rate of the bond-breaking step in the catalytic mechanism of the alkaline phosphatase tetramer.

A complete kinetic analysis of the bond-breaking step will be necessary before distinctions can be made between the various possible mechanisms. These studies will have to be done by observations of the first turnover of tetrameric alkaline phosphatase, because the slow isomerization coupled to substrate binding obstructs kinetic analysis of the chemical events during substrate hydrolysis by the dimer (Trentham & Gutfreund, 1968).

The amplitudes of transient-product formation show that both the alkaline phosphatase dimer and the tetramer have only one active site/two subunits functioning at any instant in catalysis. These observations impose limitations on the symmetry properties of the enzyme or enzyme–substrate complex. In the absence of any ligands the subunits of the dimer are related by a crystallographic twofold rotation axis (Hanson *et al.*, 1970). It appears that the alkaline phosphatase dimer can bind both P_i and 2,4-dinitrophenyl phosphate at the same time, under conditions when only one site is operating in the reaction with substrate alone (Halford, 1971) so the half site reactivity cannot be due to charge repulsion or steric hindrance between the ligands. However, a conformation change is required for catalytic action by the dimer. This conformation change might occur in only one subunit at a time. On the basis of this scheme, the molecular symmetry of the dimer will be lost when the substrate-induced conformation change occurs. But the kinetic methods used in the present work only report on events linked to the catalytic mechanism, and thus do not provide any direct information about the conformational state of the subunit that is not participating in the hydrolysis of substrate. Additional complexities are observed in studies of the simultaneous binding of two different ligands (Halford, 1972). In fact, properties of the tetramer demonstrate the ability of alkaline phosphatase to distinguish between the binding of phosphate esters and of P_i. Lazdunski *et al.* (1971) postulated that substrate and P_i induced the same conformation change on binding to the dimer, but this hypothesis is incompatible with the dissimilar

effects of the different ligands on the dimer–tetramer equilibrium described here (Figs. 2 and 6).

However, the tetramer is in the conformation required for catalytic activity in the absence of substrate. Even so, the stoicheiometric limitation was still observed with this form of the enzyme. Thus the four chemically identical subunits cannot be in identical conformations, even in the absence of ligand. We therefore suggest that the tetramer possesses an inherent structural asymmetry to match the observed functional asymmetry. Non-equivalence of active sites within a polymeric enzyme of identical subunits can be an automatic consequence of certain subunit geometries (Hanson, 1968). On the basis of this scheme the alkaline phosphatase tetramer could be considered as a union of two ($E^*\cdot E$) dimers, asymmetrical in themselves though two such units could perhaps be related by one twofold symmetry axis in the tetramer, as are the $\alpha\beta$ pairs in haemoglobin.

We now speculate on a role for the tetramerization of alkaline phosphatase *in vivo*. Alkaline phosphatase is specifically localized in the periplasmic space of *E. coli*, exterior to the cell membrane but within the outer cell wall (Heppel, 1967). This periplasmic environment is essential for the final steps in the biosynthesis of the dimeric enzyme consisting of the association of monomers and activation by Zn^{2+} (Schlesinger *et al.*, 1969). Under de-repressed conditions for the biosynthesis of alkaline phosphatase the concentration of enzyme confined to this space can be very high (Reynolds & Schlesinger, 1969*b*). Tetramer formation *in vitro* requires specific conditions of Zn^{2+} concentrations and pH values, but very little information is currently available about the chemical constitution of the periplasmic medium. In our speculations we assume that this special environment is suitable for tetramerization.

It is unlikely that the different kinetics of substrate hydrolysis by the dimer and the tetramer are physiologically important. The kinetic alteration was restricted to the pre-steady-state production of the alcohol from the phosphate monoester, after which the tetramer dissociated into dimers before the release of P_i. But the function of alkaline phosphatase in *E. coli* is to provide the bacterial cell with P_i, not a supply of carbon, by the hydrolysis of organic phosphate esters (Torriani & Rothman, 1961). It would be advantageous for this supply role if the dissociation constant of P_i from the alkaline phosphatase dimer could be raised from 3×10^{-6}M. But the apparent dissociation constant of P_i from the enzyme is dependent on the equilibrium position of the self-association reaction between the dimer and the tetramer.

We have examined the dissociation of the tetramer after the addition of P_i (Fig. 6) and have related this observation to a reaction scheme of two reversible steps, protein dissociation followed by ligand association with the dimer. But an alternative sequence of events could be the release of P_i from the dimer when free dimers associate to form the tetramer. Thus the tetramerization of alkaline phosphatase could provide thermodynamic assistance for its physiological role. Moreover, the self-association reaction is very dependent on pH (Reynolds & Schlesinger, 1969*b*; this paper, Fig. 2). A small increase in pH can lead to a marked increase in tetramerization with concomitant loss of affinity for P_i. This relationship between tetramer formation and the pH could regulate the cytoplasmic uptake of the P_i produced by the catalytic action of alkaline phosphatase in the periplasm. Differences of pH across membranes have been implicated with the transport of ions in many systems (Mitchell, 1967).

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