Escherichia coli Alkaline Phosphatase RELAXATION SPECTRA OF LIGAND BINDING

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The temperature-jump technique was used to study the binding equilibrium between the *Escherichia coli* alkaline phosphatase dimer and 2-hydroxy-5-nitrobenzyl phosphonate in 0.1 M-tris buffer, pH 8.0. Three partially discrete relaxations were observed, two of which could be related to the bimolecular associations of ligand with different conformations of the enzyme and the third to the interconversion of these states. Relaxation spectra were also used to analyse the changes in the mechanism of ligand binding to alkaline phosphatase caused by increase in ionic strength. The relaxation spectrum observed after the addition of P_i to the equilibrium mixture of phosphonate and enzyme was also studied. Difference spectroscopy indicated that both of these ligands were bound to the alkaline phosphatase dimer at the same time. These results are related to the catalytic mechanism of this enzyme, with particular reference to the role of two identical subunits in a dimeric enzyme that exhibits only one active site functioning in catalysis at any given time.

Two separate mechanisms have been described for the binding of substrate to the *Escherichia coli* alkaline phosphatase dimer under different experimental conditions. Halford *et al.* (1969) observed that a unimolecular isomerization occurred after the formation of the initial complex during the binding of 2-hydroxy-5-nitrobenzyl phosphonate to alkaline phosphatase in 0.1 M-tris–1.0 M-NaCl, pH 8.0. The rate constant measured from the rearrangement of the enzyme-inhibitor complex was in excellent agreement with the predicted value for a proposed rearrangement of the enzyme-substrate complex. During the reaction with substrate at pH 8.0, this conformation change could not be directly observed but its existence had been inferred by Trentham & Gutfreund (1968).

Halford (1971) obtained direct kinetic evidence for a conformation change during the hydrolysis of 2,4-dinitrophenyl phosphate by alkaline phosphatase at pH 5.65 in ionic strengths of both 0.1 and 1.1. However, in these cases, the conformation change occurred before substrate binding. Clearly a more general mechanism is required to account for substrate binding to alkaline phosphatase. The two schemes mentioned above must represent special pathways for particular conditions. The solution conditions under which this general mechanism could be identified were found to be 0.1 M-tris, pH 8.0.

Heppel *et al.* (1962) observed that the catalytic centre activity (k_{cat}) of alkaline phosphatase was increased by increments in the ionic strength of the

assay solution. As this effect was achieved with a range of different salts, it appears to be a general feature of the catalytic mechanism rather than a specific salt effect. After extrapolation of turnover rates measured at substrate concentrations below 0.50 mM to an infinite substrate concentration, k_{cat} is evaluated as $20 s^{-1}$ in 0.1 M-tris, pH 8.0. Under these conditions, alkaline phosphatase obeys Michaelis-Menten steady-state kinetics only at substrate concentrations below 0.50 mM (Heppel *et al.*, 1962). The above value for k_{cat} contrasts with that of $60 s^{-1}$ obtained from experiments in 0.1 M-tris–1.0 M-NaCl, pH 8.0. The kinetics of substrate hydrolysis by alkaline phosphatase require two rate parameters: one to describe the rate of formation of an enzyme-orthophosphate intermediate and the other for the rate of decomposition of this intermediate (Fernley & Walker, 1966). The first parameter is determined by some isomerization, the actual breaking of the phosphate ester bond being fast (Trentham & Gutfreund, 1968). The effect of ionic strength on k_{cat} at alkaline pH values is due to alterations in the phosphorylation rate parameter without modification of the dephosphorylation rate (Fernley & Walker, 1968).

The experiments in the present paper were designed to explore the alterations in mechanism responsible for the dependence of k_{cat} on ionic strength, as well as leading towards a general mechanism for substrate binding to the alkaline phosphatase dimer. Relaxation methods are ideally suitable for the analysis of multi-step equilibria. An application of the relaxation technique is the identification of different equilibria by separating the corresponding

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parts of a thermodynamic function along the time axis (Eigen, 1957). Though the equations relating relaxation times and reactant concentrations are unique to each particular mechanism, it is not always experimentally possible to distinguish between mechanisms by relaxation spectrometry. Hence a survey of the predicted relaxation spectra of various mechanisms has been included here to investigate the circumstances under which the distinctions can be made.

Experimental

Methods and materials

The temperature-jump apparatus was designed by Dr. L. C. de Maeyer and built by Messanlagen G.m.b.H. (Göttingen, Germany). The reaction mixture (5.5 ml) was equilibrated at 22°C in a cell through which a potential of 30 kV was discharged. For a solution of 0.1 M-tris, pH 8.0, the re-equilibration to the new temperature was completed within 40 μ s. When the solution also contained 1.0 M-NaCl, the heating was completed within 10 μ s, and the temperature-jump was measured as 4°C. The relaxation of the chemical system to the new equilibrium position was followed spectrophotometrically on a Tektronix storage oscilloscope. Relaxation records were photographed and analysed after projection on to graph paper. Each reported relaxation time is an average from at least three separate perturbations. *E. coli* alkaline phosphatase was indefinitely stable during repeated temperature-jumps: after 200 discharges, the enzyme exhibited both the same difference spectrum with 2-hydroxy-5-nitrobenzyl phosphonate and the same specific activity as a control sample.

The synthesis of trisodium 2-hydroxy-5-nitrobenzyl phosphonate heptahydrate was described by Halford *et al.* (1969). All other methods and materials were described by Halford (1971). *E. coli* C90 containing high activities of alkaline phosphatase were provided by Dr. K. Sargeant (Microbiological Research Establishment, Porton, Wilts., U.K.).

Analysis of relaxation spectra

Single-step mechanisms. Eigen & de Maeyer (1963) have derived the equations relating relaxation times with individual rate constants and reactant concentrations for a number of reaction schemes. The only limitation to these derivations is that the perturbation from equilibrium must be small so that the rate equations can be linearized. For mechanism (1):

$$\frac{1}{\tau} = k_{-1} + k_{+1} ([E] + [I]) \quad (1.1)$$

In this equation, τ represents the relaxation time and $[E]$ and $[I]$ the respective concentrations of the free reactants. After a small stepwise perturbation of an equilibrium position, the exponential approach to re-equilibration defines the reciprocal relaxation time.

Two-step mechanisms. Several relaxations arising from the perturbation of a multi-step equilibrium are coupled, and have been called a relaxation spectrum (Eigen & de Maeyer, 1963). Mathematically, the relaxation times are the eigen values of a system of coupled differential equations. For small perturbations, there will be one linear differential equation to describe the time-course of re-equilibration of each independent concentration variable. Hence there will be as many relaxations as there are independent concentration variables, though some of the relaxations may not be observed in an experiment. The number of independent concentration variables will be equal to or smaller than the number of steps in the mechanism, depending on the conservation conditions operating over it.

Eigen (1968) has shown that in mechanism (2):

$$\frac{1}{\tau_1} + \frac{1}{\tau_2} = k_{-1} + k_{+1} ([E] + [I]) + k_{-2} + k_{+2}$$

and

$$\frac{1}{\tau_1} \cdot \frac{1}{\tau_2} = k_{-1} k_{-2} + k_{+1} (k_{-2} + k_{+2}) ([E] + [I])$$

If the relaxation times are well separated, these equations can be uncoupled to relate one relaxation time with a single step. If mechanism (2) consists of a fast binding step followed by a slow rearrangement ($\tau_1 \ll \tau_2$), the binding step can be treated as an isolated process (namely mechanism 1), but a concentration factor must be included with the rearrangement term to account for its coupling to the faster step. Thus for mechanism (2):

$$\frac{1}{\tau_1} = k_{-1} + k_{+1} ([E] + [I]) \quad (2.1)$$

and

$$\frac{1}{\tau_2} = \frac{\tau_1}{\tau_1 \cdot \tau_2} = k_{-2} + \frac{k_{+2}}{1 + \frac{K_1}{[E] + [I]}} \quad (2.2)$$

where $K_1 = k_{-1}/k_{+1}$. It can be seen from eqn. (2.2) that:

$$\frac{1}{\tau_2} \rightarrow k_{-2} \text{ when } ([E] + [I])/K_1 \rightarrow \infty$$

and

$$\frac{1}{\tau_2} \rightarrow k_{-2} + k_{+2} \text{ when } ([E] + [I]) \gg K_1$$

A further increase in $([E] + [I])$ will not alter $1/\tau_2$ from its maximum value, thus allowing a relaxation process to be ascribed to such a rearrangement rather than a bimolecular association for which the reciprocal relaxation time, $1/\tau_1$, would increase linearly on increasing $([E] + [I])$. Useful information can be derived from eqn. (2.2) only when it is experimentally possible to vary $\{K_1/([E] + [I])\}$. For example, if K_1 is very small, relaxation times might be measurable only when $([E] + [I]) > K_1$ and the relaxation time would then appear to be independent of concentration. Eqn. (2.2) is symmetrical with respect to $[E]$ and $[I]$: the same alteration to the concentration of either reactant will have the same effect on $1/\tau_2$.

By solving the linearized differential rate equations for mechanism (3):

$$\frac{1}{\tau_0} + \frac{1}{\tau_1^*} = k_0 + k_0^* + k_{-1}^* + k_{+1}^* ([E^*] + [I])$$

and

$$\frac{1}{\tau_0 \tau_1^*} = k_{-1}^* (k_0 + k_0^*) + k_{+1}^* \{k_0^* ([E^*] + [I]) + k_0 ([E^*])\}$$

When $\tau_0 \gg \tau_1^*$, uncoupling leads to:

$$\frac{1}{\tau_1^*} = k_{-1}^* + k_{+1}^* ([E^*] + [I]) \quad (3.1)$$

and

$$\frac{1}{\tau_0} = \frac{\tau_1^*}{\tau_0 \tau_1^*} = k_0^* + \frac{k_0}{1 + \frac{[I]}{[E^*] + K_1^*}} \quad (3.2)$$

where $K_1^* = k_{-1}^*/k_{+1}^*$, and $[E^*]$ is the free concentration of enzyme in the E^* conformation. The same relationships were derived by a different method by Czerlinski (1966). Inspection of eqn. (3.2) reveals that:

$$\frac{1}{\tau_0} \rightarrow k_0^* + k_0 \text{ when } [I] \ll ([E^*] + K_1^*)$$

$$\frac{1}{\tau_0} \rightarrow k_0^* \text{ when } [I]/([E^*] + K_1^*) \rightarrow \infty$$

Thus an increment in $[I]$ will decrease the reciprocal relaxation time, $1/\tau_0$. This is in direct contrast with the concentration-dependence of the reciprocal relaxation time from the unimolecular step in mechanism (2). The relaxation technique can therefore distinguish between a mechanism containing a rearrangement after rapid ligand binding and one in which the isomerization occurs before the binding step. In addition, mechanism (3) can always be differentiated from the corollary in which the isomerization before association occurs in the ligand rather than the enzyme. In eqn. (3.2), the same change to either $[E^*]$ or $[I]$ does not cause the same change in

$1/\tau_0$, so the dependence of $1/\tau_0$ on the concentration of each of the reactants can identify the species undergoing the isomerization.

Multi-step mechanisms. Three independent concentration variables are required for the description of mechanism (4), an extension of the two-step models to the case when isomerization steps are included both before and after ligand binding.

[Note that the nomenclature has been changed since scheme (3), for ligand binding now occurs on to the E conformation.] For mechanism (4), it can be shown that:

$$\frac{1}{\tau_0} \cdot \frac{1}{\tau_1} \cdot \frac{1}{\tau_2} = k_{+1} (k_{+2} + k_{-2}) \{k_0 ([E] + [I]) + k_0^* [E]\} + k_{-1} k_{-2} (k_0 + k_0^*)$$

If the rearrangement after ligand binding is by far the slowest step ($\tau_2 \gg \tau_0, \tau_1$), an expression for $1/\tau_0 \cdot 1/\tau_1$ derived as in the two-step mechanism (3) can be used in:

$$\frac{1}{\tau_2} = \frac{\tau_0 \tau_1}{\tau_0 \tau_1 \tau_2}$$

to give:

$$\frac{1}{\tau_2} = k_{-2} + \frac{k_{+2}}{1 + \frac{K_1}{[E] + \frac{[I]}{K_0 + 1}}} \quad (4.1)$$

where $K_1 = k_{-1}/k_{+1}$ and $K_0 = k_0^*/k_0$. Expressions for $1/\tau_0$ and $1/\tau_1$ can now be obtained as in mechanism (3).

Similarly, if the rearrangement before ligand binding is the slowest step ($\tau_0 \gg \tau_1, \tau_2$):

$$\frac{1}{\tau_0} = k_0 + \frac{k_0^*}{1 + \frac{[I]}{[E] + \frac{K_1 \cdot K_2}{1 + K_2}}} \quad (4.2)$$

where $K_2 = k_{-2}/k_{+2}$. Equations for the faster relaxations are then available from mechanism (2).

The reciprocal relaxation times in eqns. (4.1) and (4.2) both show the same qualitative dependence on reactant concentrations as their two-step counterparts, eqns. (2.2) and (3.2) respectively. It is often difficult to distinguish between simple and complex mechanisms by analysis of only one relaxation. Normally the existence of additional relaxations is the evidence for a complex mechanism, in which

case the concentration dependence of all the relaxations must be determined for the designation of mechanism. But it is possible that a complete relaxation remains unobserved. This is often caused by a relaxation involving intermediates that do not contribute to the signal with which the relaxations are monitored. If a slow relaxation involving no signal is coupled to a fast process that provides the signal, then coupling ensures that both relaxations can be observed. But if the intermediates in the slow relaxation contribute to the signal whereas those in the fast step do not, then only the slow relaxation will be observed.

Relaxation spectra from mechanisms similar to (5) have been analysed by Eigen (1967):

In this scheme, $K_1 = k_{-1}/k_{+1}$ and $K_1^* = k_{-1}^*/k_{+1}^*$. Mechanism (5) differs from (4) in that ligand binding can now also take place on to the E^* conformation, but this does not change the number of independent concentration variables. Thus the establishment of an extra equilibrium does not create an additional relaxation, though it alters the spectrum. When the binding steps are faster than the conformation changes, there will be one relaxation related to each of the binding steps but the two conformation changes will be described by a single relaxation. We consider the case when the relaxation of ligand binding to E (τ_1) is faster than that to E^* (τ_1^*), and that $K_1 \gg K_1^*$. The fastest relaxation is uncoupled from all subsequent events and is given by the equation:

$$\frac{1}{\tau_1} = k_{-1} + k_{+1} ([\text{E}] + [\text{I}]) \quad (5.1)$$

The binding to E^* is coupled to the fastest process through the common ligand. Hence:

$$\frac{1}{\tau_1^*} = k_{-1}^* + k_{+1}^* \left[[\text{I}] + [\text{E}^*] \left(\frac{K_1 + [\text{I}]}{K_1 + [\text{I}] + [\text{E}]} \right) \right] \quad (5.2)$$

Eigen (1967) obtained a qualitative relationship between $1/\tau_0$ and the four rate constants of the conformation changes:

$$\begin{aligned} \frac{1}{\tau_0} &= k_0 + k_0^* \quad \text{when } [\text{I}] \ll K_1^* \ll K_1 \\ \frac{1}{\tau_0} &= k_0^* + k_{-2} \quad \text{when } K_1^* \ll [\text{I}] \ll K_1 \\ \frac{1}{\tau_0} &= k_{-2} + k_{+2} \quad \text{when } K_1^* \ll K_1 \ll [\text{I}] \end{aligned} \quad (5.3)$$

Thus a plot of $1/\tau_0$ against ligand concentration should exhibit some plateau regions from which it is possible to evaluate the individual rate constants, provided ratios of rate constants are known from equilibrium measurements. The experimental difficulty is in determining relaxation times over a sufficiently wide concentration range.

Results and Discussion

Equilibrium studies

The difference spectrum between a mixture of 2-hydroxy-5-nitrobenzyl phosphonate and alkaline phosphatase in 0.1M-tris buffer, pH8.0 and the separate solutions had a peak at 430nm and zero extinction difference at 385nm (Fig. 1). This difference spectrum is qualitatively identical with that observed under the conditions of 0.1M-tris-1.0M-NaCl buffer, pH8.0 (Halford *et al.*, 1969). At the lower ionic strength, the molar difference extinction coefficient was $3 \times 10^3 \text{ cm}^{-1}$ at 430nm. The addition of P_i to the mixture of enzyme and phosphonate resulted in a new difference spectrum with a trough at 430nm (Fig. 1). The same negative-difference extinction at 430nm was observed after adding 2-hydroxy-5-nitrobenzyl phosphonate to both a mixture of alkaline phosphatase and orthophosphate in the sample beam and the free orthophosphate solution in the reference beam. No difference extinction was

Fig. 1. Difference spectra

A, Extinction difference between 0.020mm-alkaline phosphatase and 0.25mm-2-hydroxy-5-nitrobenzyl phosphonate in 0.1M-tris buffer, pH8.0, in one solution and these components in separate solutions. B, As A, except both the mixed and the unmixed solutions were made either 0.25mm or 25.0mm with respect to orthophosphate, the same difference spectrum being obtained at either concentration of orthophosphate.

observed between a mixture of enzyme and orthophosphate and the unmixed references in either the u.v. or the visible regions of the spectrum. If the phosphonate and orthophosphate were in direct competition for the same binding site on alkaline phosphatase, the addition of a large excess of orthophosphate would merely displace the enzyme-phosphonate difference spectrum down to the base line. These spectra therefore suggest that a ternary complex is formed in which both phosphonate and orthophosphate are simultaneously bound to the dimeric alkaline phosphatase.

Measurements of the dissociation constant of 2-hydroxy-5-nitrobenzyl phosphonate from alkaline phosphatase and on the number of ligand binding sites were made by recording the extinction change at 430nm on the addition of the phosphonate to the enzyme. The apparatus for these spectral titrations was designed by Dr. J. J. Holbrook and has been described by McMurray & Trentham (1969). Titrations were done in both 0.1M-tris buffer, pH8.0 and 0.1M-tris-1.0M-NaCl buffer, pH8.0 and also at intermediate NaCl concentrations. In all cases, analysis of the binding curve yielded a value of $27 \pm 7 \mu\text{M}$ for the equilibrium dissociation constant (K_{eq}) and a stoichiometry of 1.0 ± 0.3 phosphonate binding sites/alkaline phosphatase dimer.

Relaxation studies

The binding equilibrium of 2-hydroxy-5-nitrobenzyl phosphonate and alkaline phosphatase in 0.1M-tris buffer, pH8.0, was studied by the temperature-jump technique. A total of three relaxation processes were observed in these experiments, though they were not sufficiently well separated to remain discrete at all reactant concentrations studied in this system. All three relaxations involved an increase in extinction and exhibited maximum amplitude at 430nm. Relaxation times were measured over a range of enzyme and phosphonate concentrations and it was found that the simplest model that could accommodate the results was mechanism (5). The two faster relaxations could be related to the bimolecular associations of phosphonate with different conformations of the enzyme and the slowest relaxation to the unimolecular isomerizations between these conformations.

An example of the slowest relaxation is given in Fig. 2. The variation of its reciprocal relaxation time ($1/\tau_0$) plotted against the total phosphonate concentration at one enzyme concentration is shown in Fig. 3. For enzyme saturated with phosphonate, the asymptote value of $1/\tau_0$ at 6s^{-1} was found to be independent of the enzyme concentration in the range 0.05–0.16mM. Therefore the slow relaxation refers to an isomerization process. Further analysis of the slow relaxation requires information on the nature

Fig. 2. Spectrophotometric record at 430nm of the slowest relaxation observed on a temperature-jump perturbation of the binding equilibrium of 2-hydroxy-5-nitrobenzyl phosphonate (0.30mM) and alkaline phosphatase (0.16mM) in 0.1M-tris buffer, pH8.0

of the faster relaxations. But to analyse the faster relaxations, the equilibrium position between the two conformations of the free enzyme, E and E*, must be known. An iterative procedure was used: the ratio of $[E]/[E^*]$ was taken to be 4/1 in the analysis of the fast relaxations, before re-examination of the slow phase to check on the initial estimate. The concentrations of free phosphonate ($[I]$) and free enzyme ($[E] + [E^*]$) were calculated from the overall dissociation constant, K_{eq} , for each initial concentration of reactants.

The record of the fastest relaxation observed with this system is shown in Fig. 4. In one experiment where the reciprocal relaxation time of this process ($1/\tau_1$) was measured at different phosphonate concentrations but with a constant enzyme concentration of 0.16mM, $1/\tau_1$ was minimum at a total phosphonate concentration of 0.10mM and was larger at both lower and higher phosphonate concentrations. The relationship observed between $1/\tau_1$ and $([E] + [I])$ is compatible with the fastest relaxation being related to the association of phosphonate with the E conformation of the enzyme. This relationship is given by eqn. (5.1) which has been plotted in Fig. 5. Values for the individual rate constants were taken from the gradient and the intercept of the line in Fig. 5, and are reported in Table 1. The microscopic dissociation constant, K_1 , was determined as $2.5 \times 10^{-4}\text{M}$.

Fig. 6 provides an example of the middle relaxation, with intermediate reciprocal relaxation time ($1/\tau_1^*$). The concentration dependence of $1/\tau_1^*$ showed that this phase could be assigned to the binding of phosphonate by the E* conformation of the enzyme.

Fig. 3. Relationship between the reciprocal relaxation time of the slowest relaxation ($1/\tau_0$) and the 2-hydroxy-5-nitrobenzyl phosphonate concentration

The results were obtained from temperature-jump perturbations of the binding equilibrium of the phosphonate and alkaline phosphatase in 0.1M-tris buffer, pH8.0. A constant enzyme concentration of 0.16mM was used. The values of $1/\tau_0$ were calculated from records such as Fig. 2, and the phosphonate concentration refers to its total concentration in the equilibrium mixture.

Evidence for this was that the minimum value of $1/\tau_1^*$ was at a smaller total phosphonate concentration than that for $1/\tau_1$, so the middle relaxation must refer to the association of phosphonate with the minor component of the enzyme. Eqn. (5.2) was used to define the reciprocal relaxation time of the slower of two association reactions coupled by a common ligand (Fig. 7). From the plot in Fig. 7, values were obtained for the individual rate constants (Table 1). The microscopic dissociation constant, K_1^* , was determined as 5.0×10^{-6} M.

At 2-hydroxy-5-nitrobenzyl phosphonate concentrations in excess of 0.10mM, the middle relaxation could no longer be detected. The maximum relaxation amplitude of an isolated association reaction occurs close to the point of symmetrical equilibrium (Eigen & de Maeyer, 1963). Therefore a decrease in the amplitude of the middle relaxation is expected when

Fig. 4. Spectrophotometric record at 430nm of the fastest relaxation observed on a temperature-jump perturbation of the binding equilibrium of 2-hydroxy-5-nitrobenzyl phosphonate (0.30mM) and alkaline phosphatase (0.16mM) in 0.1M-tris buffer, pH8.0

Fig. 5. Analytical plot for the reciprocal relaxation time of the fastest relaxation ($1/\tau_1$) observed in temperature-jump perturbations of the binding equilibrium of 2-hydroxy-5-nitrobenzyl phosphonate and alkaline phosphatase in 0.1M-tris buffer, pH8.0

The values of $1/\tau_1$ were calculated from records such as Fig. 4, and the concentrations of free phosphonate and free enzyme (in the E conformation) were calculated for the abscissa function by the method described in the text. The line drawn gives values of $2.8 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ for k_{+1} from the gradient and 7000 s^{-1} for k_{-1} from the ordinate intercept.

$[I] > K_1^*$, though the amplitude of the fast relaxation will not decrease until $[I] > K_1$. In addition, $1/\tau_1^*$ has

Table 1. Rate constants evaluated for the binding of 2-hydroxy-5-nitrobenzyl phosphonate to alkaline phosphatase

Experiments were done in 0.1M-tris buffer, pH8.0. The values are for the mechanism:

$$k_0^* = 5.4 \text{ s}^{-1}$$

$$k_0 = 24 \text{ s}^{-1}$$

$$k_{+1} = 2.8 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1} \quad k_{+1}^* = 7 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$$

$$k_{-1} = 7000 \text{ s}^{-1} \quad k_{-1}^* = 350 \text{ s}^{-1}$$

$$k_{+2} = 5.4 \text{ s}^{-1}$$

$$k_{-2} = 0.6 \text{ s}^{-1}$$

$$K_1 = \frac{k_{-1}}{k_{+1}} = 2.5 \times 10^{-4} \text{ M} \quad K_1^* = \frac{k_{-1}^*}{k_{+1}^*} = 5 \times 10^{-6} \text{ M}$$

a more marked concentration-dependence than $1/\tau_1$ because k_{+1}^* is larger than k_{+1} , so the values of the two reciprocal relaxation times would become similar at higher phosphonate concentrations. Because of its decreasing amplitude and increasing reciprocal relaxation time, the middle relaxation is then incorporated into the electronic noise on the records on the faster process. (For this reason the record of the middle relaxation in Fig. 6 was not taken from an experiment at the same reactant concentrations as Figs. 2 and 4.)

In mechanism (5), the overall dissociation constant ($K_{eq.}$) from equilibrium binding studies is related to the microscopic dissociation constants by the equations:

$$K_{eq.} = K_1 \frac{k_{-2}}{k_{+2} + k_{-2}} = K_1^* \frac{k_0 + k_0^*}{k_0^*}$$

By using these expressions in conjunction with eqn. (5.3), the concentration dependence of the slow relaxation was analysed to evaluate the individual rate constants in the isomerizations of both the free enzyme and the enzyme-phosphonate complex (Table 1). Fig. 3 is compatible with these evaluations, even though an unequivocal estimation of $(k_0 + k_0^*)$ could not be made from $1/\tau_0$ when $[I] \ll K_1^* \ll K_1$. But no attempt was made to computer-fit a theoretical line for the dependence of $1/\tau_0$ on the phosphonate concentration, for this would necessitate excessive weighting of the reciprocal relaxation times measured at the lowest phosphonate concentrations, where

Fig. 6. Spectrophotometric record at 430nm of the middle relaxation observed on a temperature-jump perturbation of the binding equilibrium of 2-hydroxy-5-nitrobenzyl phosphonate (0.03 mM) and alkaline phosphatase (0.09 mM) in 0.1 M-tris buffer, pH8.0

small amplitudes lead to inaccurate rate measurements. The k_0/k_0^* ratio obtained by this method validates the iterative procedure used in the analysis of the faster relaxations.

As three relaxations were observed after perturbation of the binding equilibrium between 2-hydroxy-5-nitrobenzyl phosphonate and alkaline phosphatase in 0.1 M-tris buffer, pH8.0, only mechanisms containing four or more chemical intermediates need to be considered. Mechanism (5) is the only four-intermediate scheme that can accommodate the results, though further complications cannot be excluded. For example, we can dismiss mechanism (4) which consists of one binding step and two isomerizations: the slow relaxation could be related to the conformation change before ligand binding but the middle relaxation cannot refer to the conformation change after ligand binding. In general, an isomerization can be distinguished from an association reaction by examining whether or not the reciprocal relaxation time tends towards a constant value at saturating ligand concentrations. But in this case, the distinction can be made by the grossly dissimilar concentration dependence of the middle reciprocal relaxation times in mechanisms (4) (see eqn. 2.2) and (5) (eqn. 5.2) when the total phosphonate concentration is varied in the range between $[E^*]$ and $[E]$. But the rate constants that have been evaluated for the individual steps are not unique solutions. They are only internally consistent. The major ambiguity is in the values of the dissociation rate constants, k_{-1} and k_{-1}^* . The scatter of results in Figs. 5 and 7 are such that the errors in the association rate constants are about $\pm 25\%$.

Fig. 7. Analytical plot of the reciprocal relaxation time of the middle relaxation ($1/\tau_1^*$) observed in temperature-jump perturbations of the binding equilibrium of 2-hydroxy-5-nitrobenzyl phosphonate and alkaline phosphatase in 0.1 M-tris buffer, pH 8.0

The values of $1/\tau_1^*$ were calculated from records such as Fig. 6, and the concentrations of free phosphonate and free enzyme (in both the E^* and the E conformations) were calculated for the abscissa function by the method described in the text. The value of K_1 was taken as 2.5×10^{-4} M. The line drawn gives values of $7 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ for k_{+1}^* from the gradient and 350 s^{-1} for k_{-1}^* from the ordinate intercept.

A similar relaxation spectrum to that described above was observed in the binding of 2-hydroxy-5-nitrobenzyl phosphonate to alkaline phosphatase in 0.01 M-tes [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid]–0.10 M-NaCl buffer, pH 8.3. But under these conditions, the relaxations of the association equilibria (τ_1 and τ_1^*) involved much smaller changes in extinction. The extinction increases at 430 nm during the two faster relaxations in 0.1 M-tris, pH 8.0, show that both the concentrations of EI and E^*I are increased by the temperature-jump. This can be explained by the decrease in pH caused by heating a solution of tris. It has been found that the initial dissociation constants of substrate from alkaline phosphatase are smaller at lower pH values (Halford, 1971). The fact that tes has a smaller heat of ionization than tris might account for the decreased amplitude under the latter conditions.

Effect of sodium chloride

In the present paper and that by Halford (1971) the reactions of *E. coli* alkaline phosphatase are described

with a minimum of two enzyme conformations. These have always been denoted as ' E ' and ' E^* '. The ' E^* ' terminology indicates the conformation required for phosphate ester cleavage: this form has a higher affinity for substrate or substrate-analogue than the E conformation. If substrate should bind to E , a conformation change is required before the ester can be hydrolysed. The two essential conformations of the enzyme will always be described on this basis, even though further properties of each structure are dependent on environmental conditions. For example, it is suggested below that the E^* conformation is incapable of direct association with phosphonate in 0.1 M-tris–1.0 M-NaCl buffer, pH 8.0, even though this process can occur with E^* in 0.1 M-tris buffer, pH 8.0. This implies that the two E^* conformations, noted as such solely on the basis of the thermodynamic affinity for this ligand, are not completely equivalent under different conditions.

We can now examine the effect of NaCl on the binding mechanism of 2-hydroxy-5-nitrobenzyl phosphonate to alkaline phosphatase. Halford *et al.* (1969) reported that phosphonate binding in 0.1 M-tris–1.0 M-NaCl buffer, pH 8.0, proceeded by mechanism (2) in which E^*I was the only intermediate with a larger extinction coefficient at 430 nm than free phosphonate. This pathway differs from that in 0.1 M-tris buffer, pH 8.0, under which conditions the enzyme can also isomerize to E^* before binding the phosphonate. The mechanisms also differ with respect to the spectral characteristics of the intermediates. At the lower ionic strength, the relaxation amplitudes show that both EI and E^*I contribute to the positive difference extinction between enzyme-bound and free phosphonate at 430 nm, though their difference extinction coefficients are not identical. This conclusion is supported by results from stopped-flow experiments in which 2-hydroxy-5-nitrobenzyl phosphonate was mixed with alkaline phosphatase in 0.1 M-tris buffer, pH 8.0: 80% of the extinction change expected on the basis of the difference extinction coefficient at 430 nm occurred within the dead-time of the stopped-flow apparatus (rapid formation of binary complexes) and the remaining 20% was realized in a slower phase of measurable rate (EI to E^*I rearrangement). When this experiment was done in 0.1 M-tris–1.0 M-NaCl, pH 8.0, all the extinction change at 430 nm occurred at the slow rate determined by the isomerization (Halford *et al.*, 1969).

These differences were examined in temperature-jump experiments done with constant concentrations of alkaline phosphatase and 2-hydroxy-5-nitrobenzyl phosphonate in 0.1 M-tris buffer, pH 8.0: relaxation spectra were recorded after adding NaCl at concentrations in the range between zero and 1.0 M (Fig. 8). At these reactant concentrations, only two relaxations were observed in 0.1 M-tris, pH 8.0 (the middle relaxation having become imperceptible), and

Fig. 8. Relaxation spectra observed at 430nm on temperature-jump perturbations of the binding equilibrium of 2-hydroxy-5-nitrobenzyl phosphonate (0.4mM) and alkaline phosphatase (0.080mM) in 0.1M-tris buffer, pH8.0

Experiments were done in the presence of various NaCl concentrations: (a) 0M; (b) 0.2M; (c) 0.3M; (d) 0.6M; (e) 1.0M. Amplitudes have been normalized to give a uniform extinction change for the complete re-equilibration at each NaCl concentration.

one in 0.1M-tris–1.0M-NaCl buffer, pH8.0. But at intermediate NaCl concentrations, three relaxations were observed. The new process can be differentiated from the previous middle relaxation of phosphonate binding to the E^* state. If a relaxation from phosphonate binding to E^* exists, even though it cannot be detected, perturbation of this equilibrium in 0.20M-NaCl would produce a total of four relaxations. As it is impossible to have four relaxations from a four-intermediate mechanism, the establishment of the new relaxation implies either that the mechanism is more complicated than previously assessed, or that it has been altered in such a way that a previous relaxation is no longer a possible route for re-equilibration. Though the former possibility cannot be excluded, the results in Fig. 8 can be accommodated by the mechanistic alteration:

It is being proposed that the addition of 0.20M-NaCl to alkaline phosphatase in 0.1M-tris, pH8.0, alters the E^* state in a way that prevents the direct association of phosphonate with this conformation. This creates a new relaxation spectrum, for now the rearrangements between both the unliganded and the liganded conformations can relax separately (mechanism 4).

The fastest relaxation observed in 0.20M-NaCl had the same reciprocal relaxation time as $1/\tau_1$ in the absence of NaCl, so it refers to the binding of phosphonate by the E conformation. The amplitude of this phase was decreased by increasing the ionic strength. This is compatible with the above proposal on the effect of salt on the extinction coefficient of the EI intermediate at 430nm. The concentration dependence of the two slower reciprocal relaxation times were examined by a further temperature-jump experiment in which various concentrations of 2-hydroxy-5-nitrobenzyl phosphonate were employed with a fixed amount of alkaline phosphatase in 0.1M-tris–0.30M-NaCl buffer, pH8.0. From this experiment the middle relaxation in 0.30M-NaCl was assigned to the isomerization after ligand binding and the slowest relaxation under these conditions to the isomerization before ligand binding. The distinction was made by application of eqns. (2.2) and (4.2) respectively, and it was impossible to relate either of these relaxations to the other conformational change in mechanism (4) or to any bimolecular process.

The single relaxation observed in 0.1M-tris–1.0M-NaCl buffer, pH8.0, describes a conformation change after ligand binding (Halford *et al.*, 1969). Therefore the same process determines both the middle relaxation in 0.30M-NaCl and the single relaxation in 1.0M-NaCl. At NaCl concentrations above 0.60M, the relaxation from the pre-equilibrium between E^* and E was no longer observed. On the above model, E^*I is then the only intermediate with a larger extinction coefficient than free phosphonate at 430nm. When τ_0 is greater than τ_2 , coupling ensures that the re-equilibration between E^* and E will affect the concentration of E^*I and hence achieve a relaxation signal but this will not be the case when τ_0 is less than τ_2 .

Effect of inorganic phosphate

The catalytic-centre activity of alkaline phosphatase in 0.1M-tris buffer, pH8.0, is 20s^{-1} after extrapolation to infinite substrate concentration from readings taken at concentrations below 0.5mM. All the relaxation experiments reported here were done with phosphonate concentrations of less than 0.5mM. If the mechanism for the binding of 2-hydroxy-5-nitrobenzyl phosphonate is used to simulate the binding of substrate, then a rate of 27s^{-1} is required

for the formation of E^*I , the analogue of the intermediate required for substrate hydrolysis. For this evaluation, the breaking of the phosphate ester bond in E^*S was assumed to be very fast (Trentham & Gutfreund, 1968) and the dephosphorylation rate to be 90s^{-1} (Aldridge *et al.*, 1964). Thus it is a cause for concern that the limiting rate constant in the formation of E^*I is only 5.4s^{-1} . This rate constant was determined for the isomerization of both the free enzyme and the enzyme-phosphonate complex.

The above scheme for phosphonate binding relates to the association of one equivalent of 2-hydroxy-5-nitrobenzyl phosphonate with the alkaline phosphatase dimer. This stoichiometry was obtained in the spectral titrations. In equilibrium dialysis experiments at pH 8.0, Reynolds & Schlesinger (1969) found only one binding site for inorganic phosphate-alkaline phosphatase dimer. At higher concentrations of orthophosphate, the alkaline phosphatase dimer can bind more than one equivalent of this ligand, but the stoichiometry of this additional binding is uncertain and its dissociation constant is much larger than that of the primary interaction (Applebury *et al.*, 1970). The difference spectroscopy suggests that a ternary complex is formed on addition of P_i to the enzyme-phosphonate complex (Fig. 1). Given the above stoichiometries, it seems probable that this ternary complex contains one equivalent of phosphonate and one of orthophosphate both bound to the dimeric enzyme at the same time. It has been shown that the enzyme can bind both substrate and orthophosphate at the same time under conditions when 1:1 stoichiometry holds (Halford, 1971).

Relaxation spectra were studied in the presence of P_i . After a temperature-jump perturbation to a system of 0.16 mM-alkaline phosphatase, 0.4 mM-2-hydroxy-5-nitrobenzyl phosphonate and 0.1 mM- P_i in 0.1 M-tris buffer, pH 8.0, three separate relaxations were observed at 430 nm (Fig. 9). The reciprocal relaxation times of these steps were 16000, 30 and 9s^{-1} respectively. This is in contrast with a system that was identical except that it did not contain P_i , in which only two relaxations were observed, with reciprocal relaxation times of 16000 and 6s^{-1} . The slow relaxation refers to isomerizations between two conformational states, and this relaxation is split into two components by the addition of orthophosphate. This finding is incompatible with schemes in which P_i acts solely to displace the phosphonate from the enzyme. The experimental observation in Fig. 9 supports the conclusion reached by difference spectroscopy on the simultaneous association of the two ligands with the enzyme.

The additional relaxation indicates that an extra concentration variable is needed to describe the binding of the phosphonate to the enzyme in the presence of P_i in 0.1 M-tris buffer, pH 8.0. Consequently, the binding of 2-hydroxy-5-nitrobenzyl phosphonate to

Fig. 9. Spectrophotometric record at 430 nm of the two slower relaxations observed on a temperature-jump perturbation of the binding equilibrium of 2-hydroxy-5-nitrobenzyl phosphonate (0.4 mM), P_i (0.1 mM) and alkaline phosphatase (0.16 mM) in 0.1 M-tris buffer, pH 8.0

The fastest relaxation observed with this system is not detected in the record at this sweep speed.

the alkaline phosphatase-orthophosphate complex must proceed by a mechanism different to that observed during phosphonate binding to the free enzyme. The rate of this new relaxation is compatible with the magnitude of the catalytic-centre activity of the enzyme under these conditions. However, the complete analysis of this relaxation spectrum was not achieved. The fact that so many relaxations are observed with this system demands the existence of a very complicated mechanism.

An alternative mechanism for alkaline phosphatase has recently been proposed by Lazdunski *et al.* (1971). In this scheme, the dephosphorylation step was proposed to be rate-limiting at alkaline pH values. However, strong evidence against dephosphorylation being rate-limiting at alkaline pH values is provided by the work of Aldridge *et al.* (1964), who measured the rate of dephosphorylation of the phosphoryl-enzyme at pH 8.30 by the quenched-flow technique and found it to be considerably faster than the catalytic-centre activity under the same conditions. Lazdunski *et al.* (1971) reported that a covalent phosphoryl-enzyme containing 1 mol of phosphate/mol of dimeric enzyme could be isolated after the addition of acid to a mixture of enzyme and substrate at pH 8.0. But the same experiment had previously been done by Reid & Wilson (1971) who found that less than 10% of enzyme active sites were phosphorylated during the steady state at alkaline pH values.

As Reid & Wilson (1971) used a rapid quenching device, the difference between the two sets of results must be due to the speed with which the pH was lowered. It is well known that the covalent phosphorylation of alkaline phosphatase is pH-dependent, so a slow decrease in pH from 8 to 1 could readily lead to anomalously high extents of phosphorylation.

Lazdunski *et al.* (1971) were not able to confirm previous observations on the liberation of only one mol of product/mol of dimeric enzyme in the transient phase during substrate hydrolysis by alkaline phosphatase at low pH values. The inherent dangers in measuring amplitudes of transient-product formation by back-extrapolation of the steady state, rather than direct observation of the transient by the stopped-flow method, were discussed by Halford (1971). Equilibrium measurements on the binding of P_i are also in accord with the stoichiometry of only one active site/dimer functioning at any instant (Applebury *et al.*, 1970).

In the present paper it is demonstrated that the rate of the ligand-induced conformation change is very sensitive to changes in ionic strength. It is, therefore, expected that the rate of the conformation change measured in 1.0M-NaCl (Halford *et al.*, 1969) differs from the rate for this process in 0.4M-NaCl (Lazdunski *et al.*, 1971). A serious criticism of the work done in this laboratory comes from the suggestion of Lazdunski *et al.* (1971) that the observations of Halford *et al.* (1969) might be due to the low specific activity of the enzyme preparation. This is not the case. The experiments of Halford *et al.* (1969) are reproducible in quantitative detail with *E. coli* alkaline phosphatase samples of maximum specific activity, either prepared by the method of Halford (1971) and stored in $(NH_4)_2SO_4$, or a freeze-dried sample given by Dr. M. J. Schlesinger.

Conclusions

It has been found that 2-hydroxy-5-nitrobenzyl phosphonate can bind to two conformations of *E. coli* alkaline phosphatase in 0.1M-tris buffer, pH8.0. Isomerizations are permitted between either the free enzyme conformations or the different enzyme-phosphonate complexes. Under these conditions, the E^* conformation has a 50-fold greater affinity for the phosphonate than the E conformation. It is noteworthy that this difference in affinity is due to the dissociation rate constant, k_{-1}^* , being 20 times smaller than k_{-1} whereas the association rate constants differ by a factor of 2.5. A bimolecular rate constant of $7 \times 10^7 M^{-1} \cdot s^{-1}$ is close to the diffusion-controlled limit for the association rate of a small ligand with a large protein. This is not the only case in which different stabilities in a series of ligand-poly peptide complexes are reflected predominantly

through changes in dissociation rate constants (Diebler *et al.*, 1969; Brunori, 1971).

Based on findings under different solution conditions, two linear pathways have been proposed for substrate binding to alkaline phosphatase. One involved a ligand-induced conformation change after substrate binding (Halford *et al.*, 1969) and the other a conformation change in the enzyme before substrate binding (Halford, 1971). The general mechanism observed in 0.1M-tris buffer, pH8.0, shows that these linear pathways represent the two extreme routes out of a single multi-step mechanism. All these mechanisms pertain to the alkaline phosphatase dimer: the addition of excess of zinc is a pre-requisite for tetramer formation (Reynolds & Schlesinger, 1969). The addition of NaCl to alkaline phosphatase in 0.1M-tris buffer, pH8.0, altered the relaxation spectrum of phosphonate binding. It was suggested that this alteration was due to the prevention of direct association of this ligand with the E^* conformation. As such, this accounts for the transition between the different mechanisms of phosphonate binding observed in 0.1M-tris buffer, pH8.0 and in 0.1M-tris-1.0M-NaCl buffer, pH8.0. The change in mechanism means that certain properties of the chemical intermediates are altered by ionic strength. This can also be seen by the changes in the absorption spectrum of the EI intermediate at different ionic strengths. Two distinct enzyme-phosphonate complexes differing in their thermodynamic affinity for this ligand were identified under both sets of conditions, but these pairs of structures are not completely equivalent. One might expect that the fine structure of the protein is dependent on the composition of the solution in which it is dissolved.

From the experiments in 0.1M-tris buffer, pH8.0, the rate constants of the rearrangements from either E to E^* or from EI to E^*I are too small to permit direct extrapolation from the mechanism of phosphonate binding to substrate binding. This contrasts with the situation in 0.1M-tris-1.0M-NaCl buffer, pH8.0, where there was excellent agreement between the measured rate constant of the enzyme-phosphonate rearrangement and the predicted value for the equivalent process with substrate (Halford *et al.*, 1969). But further complications ensue from the addition of orthophosphate to alkaline phosphatase and 2-hydroxy-5-nitrobenzyl phosphonate in 0.1M-tris buffer, pH8.0. Though the mechanistic alterations in the presence of orthophosphate have not been analysed, it is possible that the rearrangement of the enzyme-phosphonate complex then occurs at a rate sufficient to describe the rate-limiting step in substrate hydrolysis under these conditions. P_i is both a competitive inhibitor and the last product of substrate hydrolysis to dissociate from the enzyme.

This suggestion can be related to the observation that only one active site in the alkaline phosphatase

dimer functions in catalysis at any given time (for references see Halford, 1971), despite the fact that the two subunits appear to be identical (Rothman & Byrne, 1963). If a conformation change is an integral step in the turnover of an enzyme, then that turnover must also include a second conformation change. But one possible mode of operation is that the conformation change for substrate binding at one subunit is linked to the second conformation on the other subunit as orthophosphate dissociates from that site. This results in only one conformation change operating over the whole dimer/molecule of substrate hydrolysed. The coupling between the two conformation changes could account for the enhancement of the rate of the substrate-induced conformation change. Should orthophosphate fail to dissociate from the enzyme, the failure of the product-release conformation change would prohibit the substrate-binding conformation change, so competitive inhibition could arise even though it does not involve direct competition for the same binding site.

This postulated requirement for the reaction product during substrate binding is somewhat similar to certain anomalies in the steady-state kinetics of malate dehydrogenase, for which Harada & Wolfe (1968) proposed a reciprocating subunit mechanism. This scheme was first suggested for alkaline phosphatase after doing experiments in 0.1 M-tris-1.0 M-NaCl buffer, pH 8.0 (Halford *et al.*, 1969). But whereas the scheme is compatible with the experimental findings at low ionic strength, it is unable to accommodate the relaxation results for the simultaneous association of phosphonate and orthophosphate obtained at the high ionic strength (Halford, 1970). Just like the mechanism of substrate binding to alkaline phosphatase, environmental conditions may also affect the mechanism by which the number of active sites involved in catalysis at any given time is limited to one/dimer.

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