Ionic control of immobilized enzymes

Kinetics of acid phosphatase bound to plant cell walls

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When an enzyme is bound to an insoluble polyelectrolyte it may acquire novel kinetic properties generated by Donnan effects. If the enzyme is homogeneously distributed within the matrix, a variation of the electrostatic partition coefficient, when substrate concentration is varied, mimics either positive or negative co-operativity. This type of non-hyperbolic behaviour may be distinguished from true co-operativity by an analysis of the Hill plots. If the enzyme is heterogeneously distributed within the polyelectrolyte matrix, an apparent negative co-operativity occurs, even if the electrostatic partition coefficient does not vary when substrate concentration is varied in the bulk phase. If the partition coefficient varies, mixed positive and negative co-operativities may occur. All these effects must be suppressed by raising the ionic strength in the bulk phase. Attraction of cations by fixed negative charges of a polyanionic matrix may be associated with a significant decrease of the local pH. The magnitude of this effect is controlled by the pK of the fixed charged groups of the Donnan phase. The local pH cannot be much lower than the value of this pK . This effect may be considered as a regulatory device of the local pH. Acid phosphatase of sycamore (*Acer pseudo-platanus*) cell walls is a monomeric enzyme that displays classical Michaelis–Menten kinetics in free solution. However, when bound to small cell-wall fragments or to intact cells, it has an apparent negative co-operativity at low ionic strength. Moreover a slight increase of ionic strength apparently activates the bound enzyme and tends to suppress the apparent co-operativity. At 10.1, or higher, the bound enzyme has a kinetic behaviour indistinguishable from that of the purified enzyme in free solution. These results are interpreted in the light of the Donnan theory. Owing to the repulsion of the substrate by the negative charges of cell-wall polygalacturonates, the local substrate concentration in the vicinity of the bound enzyme is smaller than the corresponding concentration in bulk solution. The kinetic results obtained are consistent with the view that there exist at least three populations of bound enzyme with different ionic environments: a first population with enzyme molecules not submitted to electrostatic effects, and two other populations with molecules differently submitted to these effects. The theory allows one to estimate the proportions of enzyme belonging to these populations, as well as the local pH values and the partition coefficients within the cell walls.

It has been shown in recent years (Goldstein, 1976; Engasser & Horvath, 1976; Wharton *et al.*, 1968*a,b*) that the attachment of an enzyme to an insoluble polyelectrolyte may produce changes in the value of its kinetic parameters, $V_{\max}$ and K_m , and a shift of its pH–activity profile as well. A simplified but very clear-cut interpretation of these results has been proposed by Goldstein *et al.* (1964). This interpretation is based on the theory of Donnan equilibrium and on the idea that the concentration of

a charged ligand, including the proton, within the charged matrix in the close vicinity of the immobilized enzyme may be different from that in the bulk solution. This situation would generate shifts of both the kinetic parameters ($V_{\max}$ and K_m) and the apparent pK values for the enzyme.

This line of work has been explored in the light of studies on the technology of immobilized enzymes (Goldstein, 1976; Engasser & Horvath, 1976). More recently, Douzou and his colleagues (Maurel &

Douzou, 1976; Douzou & Maurel, 1977a,b) have applied these ideas to biological problems. They have shown that several enzyme systems do, in fact, display shifts of their pH profiles when bound to insoluble biological polyelectrolytes (bacterial cell walls, cell membranes) and they have interpreted their data on the basis of the Goldstein *et al.* (1964) ideas.

Although this does not seem to have been recognized by Goldstein *et al.* (1964), if the substrate bears a charge, one may expect that, under certain conditions, the immobilized enzyme will exhibit a departure from its usual kinetic behaviour, which mimics positive or negative co-operativity. The analytical formulation of these effects has not been developed in depth so far, but Engasser & Horvath (1975) have shown unambiguously, by computer simulation, that the effect may occur.

When the ionic concentration in the bulk phase is varied, one may expect from the Donnan theory that the local pH in the polyelectrolyte matrix must also vary. If the charged groups of the Donnan phase have a pK located within the domain of variation of local pH, the equivalent concentration of fixed charges in the Donnan phase will vary in response to a variation of concentration of the charged substrate in the bulk phase. Indeed, one may therefore expect that this effect controls the departure from the usual kinetic behaviour.

The model of Goldstein *et al.* (1964) implicitly postulates that both the bound enzyme and the fixed charges are homogeneously distributed in the Donnan phase. If it is not so, one may wonder about the kinetic behaviour generated by a heterogeneous distribution of fixed charges and (or) enzyme molecules in the Donnan phase.

The above ideas may be tested with real biological systems. Primary walls of isolated sycamore (*Acer pseudoplatanus* L.) cells in sterile culture contain, among various hydrolases, an acid phosphatase. This enzyme, which has been solubilized and purified to homogeneity, appears to be a monomeric glycoprotein with a mol.wt. of about 100 000 (Crasnier *et al.*, 1980). The cell-wall acid phosphatase is obviously an outer-membrane protein that has been transported through the plasma membrane.

Since cell walls contain polygalacturonic acid, they can be considered as a polyanion with a pK of 3.2 (Morvan *et al.*, 1979; Noat *et al.*, 1980). It has been shown recently (Noat *et al.*, 1980) that negative charges create, in cell walls, a Donnan potential that tends to attract mobile cations and conversely tends to repel mobile anions. Raising the ionic strength in unbuffered media tends to suppress this Donnan potential and therefore tends to equalize bulk and local concentrations. This equalization results in a proton release when the ionic strength of

a suspension of cell-wall fragments is raised from a 'low' to a 'high' value (0.1).

Since substrates of acid phosphatase bear charges, one may wonder whether their electrostatic repulsion may result in non-hyperbolic kinetics of the enzyme bound to unbroken cells or to cell-wall fragments. Acid phosphatase being obtainable either in the soluble state in free solution, or in the bound state, small cell-wall fragments that bear bound enzyme may be suited to test the validity of the above ideas.

The aims of the present paper are fourfold: to study and to characterize the non-hyperbolic kinetics generated by Donnan effects; to understand how a variation of the equivalent concentration of fixed charges in the Donnan phase may control these non-hyperbolic kinetics; to study the effect of heterogeneous distribution of the enzyme within the matrix on its kinetic behaviour; to determine whether the above ideas may be applied to the kinetic behaviour of acid phosphatase bound to plant cell walls.

Material and methods

Sycamore (*Acer pseudoplatanus* L.) cells were cultured in liquid medium as already described (Crasnier *et al.*, 1980). Cells used in the present study were taken in the exponential growth phase. Cell disruption was effected in a French press under a pressure of 19.6 kPa (200 kg/cm²) in the presence of 0.4 M-sucrose. The resulting suspension was centrifuged for 15 min at 1088 g. The supernatant was discarded and the pellet resuspended and centrifuged several times in sucrose solution of increasing concentrations (from 0.6 to 1 M). Cell-wall fragments or unbroken cells were filtered and treated three times with 1 M-NaCl. Cell-wall fragments obtained in this way were free from cytoplasmic contamination. Acid phosphatase was solubilized from either intact cells or cell-wall fragments as described previously (Crasnier *et al.*, 1980).

p-Nitrophenyl phosphate hydrolysis in presence of either purified enzyme, cell-wall fragments, or unbroken cells ('eluted' three times with NaCl as described above), was followed at 30°C and pH 5.6 in non-buffered media, with added NaCl to bring the ionic strength to the selected value. The initial pH was adjusted to 5.6 with a pH-stat, then 0.1 ml of either a cell-wall suspension or unbroken-cell suspension was introduced into the reaction mixture. During the time course of the reaction, pH-stat additions were always negligible. After 15 min, NaOH was added to the reaction mixture and the A_{400} was read in a Beckman Acta VII spectrophotometer. At this wavelength one measures the concentration of *p*-nitrophenol formed. Pre-

liminary assays have shown that, whatever the experimental conditions, formation of *p*-nitrophenol was linear with respect to time for at least 30 min. When unbroken cells or cell-wall suspensions were used, filtration of the reaction mixture was performed before absorbance reading.

Fitting of experimental results was effected by a method derived from those of Cleland (1967), Cornish-Bowden & Koshland (1970), Pettersson & Pettersson (1970) and Nari *et al.* (1974). Both fitting and simulations were effected with a Wang 2200 S minicomputer provided with an extended memory.

Theory

(1) The basic model

Let us consider a monomeric one-sited enzyme homogeneously distributed within a charged and insoluble matrix. If the substrate of the enzyme bears one or several charges, its concentration will be different within the charged matrix and in bulk solution. The same will indeed apply to any charged species. This situation is illustrated in Fig. 1. For a reaction mixture containing different anions of different valency, B^- , B^{2-} ... B^{z-} , and different univalent cations A^+ , an electrostatic partition coefficient $\mathcal{P}$ may be defined:

$$\mathcal{P} = \frac{\Sigma[B_o^-]}{\Sigma[B_i^-]} = \frac{\Sigma[B_o^{2-}]^{1/2}}{\Sigma[B_i^{2-}]^{1/2}} = \dots = \frac{\Sigma[B_o^{z-}]^{1/z}}{\Sigma[B_i^{z-}]^{1/z}} = \frac{\Sigma[A_i^+]}{\Sigma[A_o^+]} = \exp\left(-\frac{\mathcal{F}\Delta\psi}{RT}\right) \quad (1)$$

In this equation, *o* and *i* refer to bulk and local (inside the matrix) concentrations, $\Delta\psi$ is the Donnan potential, $\mathcal{F}$ is the Faraday, and R and T have their usual significance.

Moreover, electroneutrality conditions must apply outside and inside the matrix, and the local electroneutrality equation assumes the form:

$$\frac{\Sigma[B_o^-]}{\mathcal{P}} + \frac{2\Sigma[B_o^{2-}]}{\mathcal{P}^2} + \dots + \frac{z\Sigma[B_o^{z-}]}{\mathcal{P}^z} = \Sigma[B_o^-] + 2\Sigma[B_o^{2-}] + \dots + z\Sigma[B_o^{z-}] \pm \Delta^\pm \quad (2)$$

where $\Delta^\pm$ is the effective concentration of fixed charges within the matrix. Eqn. (2) may be rearranged to:

$$\mathcal{P}^{z+1} \pm \frac{\Delta^\pm}{\Sigma[B_o^-] + \dots + z\Sigma[B_o^{z-}]} \mathcal{P}^z - \frac{\Sigma[B_o^-]}{\Sigma[B_o^-] + \dots + z\Sigma[B_o^{z-}]} \mathcal{P}^{z-1} - \frac{2\Sigma[B_o^{2-}]}{\Sigma[B_o^-] + \dots + z\Sigma[B_o^{z-}]} \mathcal{P}^{z-2} - \dots - \frac{z\Sigma[B_o^{z-}]}{\Sigma[B_o^-] + \dots + z\Sigma[B_o^{z-}]} = 0 \quad (3)$$

Fig. 1. Schematic representation of the ionic partition between a polyelectrolyte matrix bearing negative charges and the medium

E stands for the enzyme and S^- for the charged substrate. The medium is supposed to contain different mono-anions (B^-), one di-anion (B^{2-}) and several cations (A^+).

which is a generalization of an equation given by Engasser & Horvath (1976) for one univalent anion and one univalent cation.

It is noteworthy that the coefficients of $\mathcal{P}$ in this local electroneutrality equation involves the concentration of anions but not that of cations. This is because only univalent cations are supposed to be

present in the reaction mixture. If (z^-)-valent cations were also present, the local electroneutrality equation would have been exceedingly complex and

would have been expressed in terms of the concentrations of both anions and cations.

The rate of equation of the enzyme bound within the charged matrix is:

$$v = \frac{V_{\max} [S_i^{z-}]}{K_m + [S_i^{z-}]} \quad (4)$$

where S^{z-} is a z -valent substrate. Making use of eqn. (1), one finds:

$$v = \frac{V_{\max} [S_o^{z-}]}{K_m \mathcal{P}^z + [S_o^{z-}]} \quad (5)$$

In eqns. (4) and (5) the subscripts i and o still refer to local and bulk concentrations respectively. As already outlined by Engasser & Horvath (1976), when $[S_o^{z-}]$ is varied, $\mathcal{P}$ varies, and the response of the bound enzyme cannot be hyperbolic. If the matrix is a polyanion, the kinetic behaviour of the enzyme will seem co-operative, and if the matrix is a polycation, the enzyme will display an apparent anti-co-operative behaviour (Engasser & Horvath, 1976). However, if the contribution of $[S_o^{z-}]$ to the total ionic strength is very small, the variation of $\mathcal{P}$ when $[S_o^{z-}]$ is varied will be very small too, and the bound enzyme will display an apparent hyperbolic behaviour. The apparent K_m will be different from the true K_m by a value equal to $\mathcal{P}^z$.

Maximal positive or negative apparent co-operativity may be expected to occur when the local 'concentration' of charges of the polyelectrolyte in the vicinity of the active site is very high, and when most of the total ionic strength is due to the substrate anion. Under these conditions, and if the valence of the substrate anion is z , the local electroneutrality equation is:

$$\frac{z[S_o^{z-}]}{\mathcal{P}^z} + \Delta^- - z\mathcal{P}[S_o^{z-}] = 0 \quad (6)$$

for a polyanion, and

$$\frac{z[S_o^{z-}]}{\mathcal{P}^z} - \Delta^+ - z\mathcal{P}[S_o^{z-}] = 0 \quad (7)$$

for a polycation.

At low ligand concentration, and for apparent positive co-operativity, the maximal value of the partition coefficient, $\mathcal{P}_{\max}$, is much greater than 1, and the first term of eqn. (6) is negligible. Therefore:

$$\mathcal{P}_{\max} = \frac{\Delta^-}{z[S_o^{z-}]} \quad (8)$$

Similarly, for negative apparent electrostatic co-operativity, the minimal value of the partition coefficient, $\mathcal{P}_{\min}$, is much smaller than 1, and it is

the last term of eqn. (7) that can be dropped. The expression of $\mathcal{P}_{\min}$ is then:

$$\mathcal{P}_{\min} = \left(\frac{z[S_o^{z-}]}{\Delta^+} \right)^{1/z} \quad (9)$$

Eqns. (8) and (9) may be inserted into the steady-state rate equation of the bound enzyme and a Hill equation may be derived. A particularly interesting case is obtained if the substrate is a univalent anion. For apparent positive co-operativity, the maximal Hill coefficient (h) is equal to 2 and is independent of the ionic composition of the medium. If apparent co-operativity is negative, the minimal Hill coefficient is equal to zero.

When an enzyme exhibits a positive co-operativity explainable, for instance, by a two-site Adair equation (Levitzki, 1978), the maximum value of h is obtained when the enzyme is half-saturated by the substrate. For both very low and very high substrate concentrations, the slope of the Hill plot is equal to unity (Fig. 2). If the one-sided polyelectrolyte-bound enzyme exhibits an apparent positive co-operativity due to Donnan effects, the maximum value of h is equal to 2 at low substrate concentration and continuously declines to unity when substrate concentration is increased. This decline with respect to the logarithm of the substrate concentration is sigmoidal (Fig. 2). This continuous decline of apparent positive co-operativity when concentration is increased is probably the most specific diagnostic test for the existence of sigmoidal kinetics generated by Donnan effects.

A similar reasoning may be applied to negative co-operativity. 'Classical' negative co-operativity explainable by a two-site Adair equation (Levitzki, 1978) results in a value of h that is minimum at half-saturation of the enzyme by the substrate and is equal to one at both low and high substrate concentrations (Fig. 2). If non-hyperbolic kinetics are explainable by electrostatic attraction of the substrate, h increases from a value equal to zero at low substrate concentration to a value of unity when the substrate concentration is increased (Fig. 2). The increase in h with substrate concentration can be taken as a good test for apparent negative co-operativity generated by Donnan effects.

(2) Effect of a variation of the effective concentration of fixed charges in the Donnan phase on non-hyperbolic kinetics

When the substrate concentration in the bulk phase is varied, the local pH in the Donnan phase varies as well. This change of pH may produce a protonation of fixed charged groups in the matrix if the pK of these groups is precisely located within the domain of variation of the local pH. Then the effective concentration of fixed charges in the

Fig. 2. Comparative variation of the Hill coefficient (h) predicted by the Adair equation and by electrostatic Donnan effects (computer simulations)

(a) Positive co-operativity. Curve 1 corresponds to the Adair equation for a dimer. h becomes maximum at half-saturation of the enzyme by the substrate (marked by an arrow). The values of the two apparent binding constants $\bar{K}_1$ and $\bar{K}_2$ are 1 and 30 respectively. Curve 2 describes what should happen for apparent positive co-operativity resulting from the Donnan effect. The apparent binding constant ($1/K_m$) equals 30 and the partition coefficient ($\mathcal{P}$) varies from 20 ($\log[S_0^-] = 3.5$) to 1 ($\log[S_0^-] = 2.5$). (b) Negative co-operativity. Curve 1 corresponds to the Adair equation for a dimer. h becomes minimum at half-saturation of the enzyme (marked by an arrow). The values of the two apparent binding constants $\bar{K}_1$ and $\bar{K}_2$ are set equal to 30 and 0.5 respectively. Curve 2 describes what should happen for apparent negative co-operativity resulting from the Donnan effect. The apparent binding constant ($1/K_m$) is 30 and the partition coefficient ($\mathcal{P}$) varies from 0 to 1. In either positive or negative co-operativity the substrate is univalent. Note the maximum value of h (2) for apparent positive electrostatic co-operativity and the minimum value of h (0) for apparent negative electrostatic co-operativity. Half-saturation is indicated by an arrow. In all cases the rate constant for product appearance is set equal to 1.

Fig. 3. Modulation of the electrostatic effect by protonation of the fixed charges of the matrix (computer simulations) (a) Variation of the partition coefficient; (b) variation of the local (internal) pH. In either case the total concentration of fixed ionizable groups in the matrix was set at 0.1 M, the external pH was 6.0 and the medium does not contain any salt apart from the univalent substrate. For curves 1 (a and b) the concentration of fixed charges in the matrix is assumed constant, whereas for curves 2 (a and b) the fixed ionizable groups are assumed to ionize with a pK of 5.0.

Donnan phase decreases, as well as the values of the Donnan potential, $\Delta\psi$.

If this situation occurs, the variation of both the local pH and the electrostatic partition coefficient

becomes buffered when the concentration of the charged substrate in the bulk phase is varied. The computer simulation of this effect is shown in Fig. 3.

If Δ_T is the total concentration of fixed groups

that may ionize in the Donnan phase, Δ^- the effective concentration of fixed charged groups, pH_i the local pH, and pK_A the pK of fixed charged groups, one has:

$$\Delta^- = \frac{\Delta_T \times 10^{(\text{pH}_i - \text{pK}_A)}}{1 + 10^{(\text{pH}_i - \text{pK}_A)}} \quad (10)$$

(3) Non-hyperbolic kinetics for a Michaelian enzyme heterogeneously distributed within the Donnan phase

If a population of the enzyme molecules is not submitted, or poorly submitted, to electrostatic effects, whereas another population is strongly submitted to these effects, the overall reaction rate for a univalent substrate is expressed by the equation:

$$v = \frac{V_{\max,1}[\text{S}_0^-]}{K_m + [\text{S}_0^-]} + \frac{V_{\max,2}[\text{S}_0^-]}{K_m \mathcal{P} + [\text{S}_0^-]} \quad (11)$$

where $V_{\max,1}$ and $V_{\max,2}$ still represent the maximum reaction rates of the two enzyme populations. Obviously, the ratio $V_{\max,1}/V_{\max,2}$ corresponds to the ratio of enzyme 'concentrations' associated with these two populations. This situation, described by eqn. (11), may occur if, for instance, a population of enzyme molecules is located at the interface between the matrix and the bulk phase, whereas another population of the same enzyme is buried in the Donnan phase. This situation is pictured in Fig. 4. Although it is not compulsory, one may expect that the enzyme molecules of the first population are poorly submitted to electrostatic effects, whereas molecules belonging to the second population are strongly submitted to these effects. If the variation of substrate concentration does not produce a significant variation of the partition coefficient, the situation expressed by eqn. (11) is formally equivalent to a reaction conditioned by two different enzymes acting on the same substrate. This situation is classically known (Dixon & Webb, 1958) to generate reciprocal plots that are concave-downwards. Eqn. (11) may be cast in its reciprocal form, and one finds:

$$\frac{1}{v} = \frac{1 + K_m(1 + \mathcal{P})\frac{1}{[\text{S}]} + K_m^2 \mathcal{P} \frac{1}{[\text{S}]^2}}{V_{\max,1} + V_{\max,2} + K_m(V_{\max,1} \mathcal{P} + V_{\max,2})\frac{1}{[\text{S}]}} \quad (12)$$

where the subscript o and the minus sign have been omitted for sake of clarity. One can show that the first derivative with respect to $1/[\text{S}]$ is always positive and that the second derivative (for $1/[\text{S}] \rightarrow 0$)

Fig. 4. A possible schematic representation of a heterogeneous distribution of an enzyme within a polyelectrolyte matrix

The outer enzyme (E) molecules are supposed not to be submitted to electrostatic effects. The univalent substrate is represented by S^- . For the sake of clarity, only univalent ions (B^- and A^+) are assumed to be present.

Fig. 5. Non-hyperbolic kinetics (at constant partition coefficient) for a Michaelian enzyme heterogeneously distributed in the polyelectrolyte matrix (computer simulations)

The curves (reciprocal co-ordinates) were obtained by using eqn. (11) and with the following numerical values: $V_{\max,1} = V_{\max,2} = 0.5$, $K_m = 10^{-5} \text{ M}$, $\mathcal{P} = 1$ (curve 1), 5 (curve 2), 10 (curve 3), 100 (curve 4).

is always negative, except for $\mathcal{P} = 1$. The extent of the downward curvature of the plots is increased for high or low values of $\mathcal{P}$ (Fig. 5 and eqn. 13). This situation is indeed similar to negative co-operativity. The dependence of non-hyperbolic kinetics on the $\mathcal{P}$ values, which are assumed not to vary when $[S]$ is varied, means that the departure from classical hyperbolic kinetics is controlled by the local concentration of fixed charges in the Donnan phase. As a matter of fact, the expression of a Hill coefficient can be exactly derived from eqn. (11). Its expression

$$\frac{1}{v} = \frac{1 + K_m \frac{1}{[S]} + K_m \Delta^- \frac{1}{[S]^2} + K_m^2 \Delta^- \frac{1}{[S]^3}}{V_{\max.,1} + V_{\max.,2} + K_m V_{\max.,2} \frac{1}{[S]} + K_m V_{\max.,1} \Delta^- \frac{1}{[S]^2}} \quad (14)$$

is particularly simple if $V_{\max.,1} = V_{\max.,2}$ and is found to be (see the Appendix):

$$h = 4\sqrt{\mathcal{P}} \frac{(1 + \sqrt{\mathcal{P}})^2}{(1 + \mathcal{P} + 2\sqrt{\mathcal{P}})^2} \quad (13)$$

The above results hold if the electrostatic partition coefficient does not vary when the substrate concentration is varied. If this is not the case, the variation of initial rate with respect to substrate

concentration may become quite complex and depends on the ionic composition of the reaction medium. The analytical expression of the reaction rate, however, can be easily derived if the effective concentration of fixed charges in the Donnan phase is much greater than the concentration of mobile ions in the bulk phase. If so, eqns. (8) and (9) apply. For electrostatic repulsion of a negatively charged substrate by the negative charges of a polyanionic matrix, eqn. (13) may be written in its reciprocal form as:

Its first derivative with respect to $1/[S]$ is always positive. Therefore the steady-state rate cannot exhibit inhibition by excess substrate. The second derivative when $1/[S] \rightarrow 0$ has one real positive root, therefore the graph of $1/v$ versus $1/[S]$ has one inflection point. The abscissa of this inflection point markedly depends on the effective concentration, Δ^- , of fixed charges in the Donnan phase. If Δ^- is large, the inflection point may be so close to the ordinate axis that it may escape detection. The

Fig. 6. Non-hyperbolic kinetics (at variable partition coefficient) for a Michaelian enzyme heterogeneously distributed in the polyelectrolyte matrix (computer simulations)

The curves (reciprocal co-ordinates) were obtained according to eqn. (14), with the following numerical values: $V_{\max.,1} = V_{\max.,2} = 0.5$, $K_m = 10^{-5}$ M, matrix charge (Δ) equal to 2×10^{-3} M (curve 1), 10^{-2} M (curve 2), 0.1 M (curve 3), 1 M (curve 4).

Fig. 7. Effect of relative concentrations of enzyme, not submitted and submitted to electrostatic effects, on the reciprocal plots (computer simulations)

The curves (reciprocal co-ordinates) are obtained according to eqn. (14), with the following numerical values: $K_m = 10^{-5}$ M, $\Delta = 0.1$ M, $V_{\max.,1}/V_{\max.,2} = 0.1$ (curve 1), 0.2 (curve 2), 1 (curve 3), 10 (curve 4).

abscissa of the inflection point increases when Δ^- declines (Fig. 6).

The shape of the reciprocal plots also markedly depends on the ratio $V_{\max,1}/V_{\max,2}$, that is, on the ration of enzyme concentrations not submitted and submitted to electrostatic effects respectively (Fig. 7). Indeed if most of the bound enzyme is not submitted to electrostatic effects, the reciprocal plots will be linear (Fig. 7).

Results and interpretation

Purified acid phosphatase exhibits Michaelian steady-state kinetics in *p*-nitrophenyl phosphate hydrolysis (Crasnier *et al.*, 1980). For $I = 0.1$, both the purified enzyme in free solution and the enzyme tightly bound to small cell-wall fragments or to unbroken cells exhibit exactly the same behaviour (Crasnier *et al.*, 1980) with the same K_m of about 1.2×10^{-4} M.

However, if the enzyme activity of the bound enzyme is measured at lower ionic strengths, the reaction exhibits an apparent negative co-operativity that can be suppressed by increasing the ionic strength (Fig. 8a). Although an ionic-strength increase does not activate the purified enzyme in free solution, it does when phosphatase is bound to either cell-wall fragments or intact cells. The apparent co-operativity of the bound enzyme is not obviously due to pure diffusional effects, for it is suppressed by increasing the ionic strength of the medium. Moreover, the K_m of the bound enzyme, at 'high' ionic

strength ($I 0.1$) is the same, within experimental error, as that of the purified enzyme in free solution. Last, the maximum velocity of the bound enzyme, which is attained at high substrate concentration, is the same whether NaCl has been added to the reaction mixture or not. All these results suggest that departure from Michaelian behaviour is due to electrostatic effects.

As already found (Noat *et al.*, 1980), the negative charges of polygalacturonates must generate, in cell-wall fragments, a Donnan potential that tends to increase the local concentration of cations and to decrease that of anions with respect to the corresponding concentrations in bulk solution. If fixed negative charges were uniformly spread out within the matrix around the enzyme molecules, one would have expected the apparent co-operativity to be positive. Since it is precisely the converse that is obtained, it is logical to assume that the fixed charges are not uniformly distributed around the enzyme molecules, that some of these enzyme molecules are not submitted to electrostatic effects, whereas others are. As shown above, this situation may be obtained if a population of enzyme molecules is located at the interface between the insoluble polyanion and the free solution and another one is buried in the polyanion. The assumption that some of the phosphatase molecules are not influenced by cell-wall electrostatic potential is the simplest way to reconcile the existence of electrostatic repulsion of the substrate with apparent negative co-operativity.

Although eqn. (13) may predict downward

Fig. 8. Non-hyperbolic kinetics of bound acid phosphatase at 'low' ionic strength

(a) Lineweaver-Burk plots. Curves 1-4 were respectively obtained in presence of the following NaCl concentrations: 0 (curve 1), 1.66 mM (curve 2), 16.6 mM (curve 3), 0.1 M (curve 4). Symbols correspond to averages of rate measurements. Dotted lines are non-linear least-square fits obtained from eqn. (15). $[S]_0$ is the total substrate concentration in the bulk phase. (b) Curve fitting of experimental results with two or three 'classes' of enzyme. Open symbols (O) are the data pertaining to curve 1 (in a). Curve 1 corresponds to the best fit obtained with eqn. (15). Curve 2 is the best fit obtained with eqn. (11). Similar results were obtained with rate data pertaining to curves 2, 3 and 4. They are not shown here for sake of clarity.

curvature of the reciprocal plots, experimental results of Fig. 8(a) cannot be fitted quantitatively to this equation for any value of the ionic strength. An example of poor fit is shown in Fig. 8(b). This is so because the ionic composition of the reaction mixture defines the value of the partition coefficient as well as its variation when the substrate concentration is varied. A good fit is obtained if three classes of enzyme at least are assumed to exist within the cell wall, each with a particular ionic environment. If, as already outlined, one of these classes does not exhibit any electrostatic effect, the resultant steady-state equation is:

$$v = \frac{V_{\max,1}[S_0^-]}{K_m + [S_0^-]} + \frac{V_{\max,2}[S_0^-]}{K_m \mathcal{P} + [S_0^-]} + \frac{V_{\max,3}[S_0^-]}{K_m \mathcal{P}' + [S_0^-]} \quad (15)$$

The phosphate concentration that appears in this equation is that of the mono-anionic form, since it is known (Jorgensen, 1959; Alvarez, 1962; Hsu *et al.*, 1966) that this ionic form is the true substrate of acid phosphatase. The concentration of this form may be easily computed from the total concentration of *p*-nitrophenylphosphate introduced into the reaction mixture.

From eqn. (15) a minimum of two partition coefficients ($\mathcal{P}$ and $\mathcal{P}'$) is necessary to express quantitatively electrostatic interaction between substrate and cell-wall fragments.

The theory allows an estimation of the proportion of the enzyme belonging to any of three categories E_1 , E_2 and E_3 , as well as of the apparent local concentrations of Δ^- groups in the vicinity of E_2 and E_3 . If $[E]_T$ is the total enzyme concentration, the

results shown in Fig. 8 allow one to estimate the values of the following proportions: $[E_1]/[E]_T = 0.30$, $[E_2]/[E]_T = 0.32$ and $[E_3]/[E]_T = 0.38$. The local concentrations of Δ^- groups in the vicinity of enzyme forms E_2 and E_3 , namely Δ_2^- and Δ_3^- , are 4.6 mM and 0.22 M respectively. It is indeed quite possible, although not compulsory, that an enzyme molecule that has in its vicinity a large concentration of negative charges is deeply buried in the polyanionic matrix. If so, the enzyme form E_3 would be deeply buried in the matrix and strongly submitted to electrostatic effects. Moreover, the same theory allows one to study the variation in both the local pH values and the partition coefficients, $\mathcal{P}$ and $\mathcal{P}'$, associated with classes E_2 and E_3 as a function of substrate concentration (Fig. 9). When the concentration of *p*-nitrophenyl phosphate is increased, the local pH values increase and the two partition coefficients decline. Indeed, the good agreement between theory and experiment does not prove the existence of three well-defined compartments in the cell wall, but rather suggests that at least three classes of enzymes with different environments are needed in order to explain the experimental results. Most probably there is a continuous gradation of the effect, and this might also be indicated by the nearly equal distribution found between the three enzyme classes.

For sake of simplicity the model above does not take account of a possible ionization of the enzyme. This is justified by the recent observation (Van Etten & McTigue, 1977) that no *pK* is detectable under pH 7.8. Moreover, simulation studies show that the main conclusions of the model are nearly independent of the number of ionization states of the enzyme.

Fig. 9. Calculated values of local pH values (pH_i) and partition coefficients ($\mathcal{P}$) as a function of substrate concentration. The results shown here correspond to the data of curve 1 of Fig. 8. Curves 1 of the present Figure (a and b) correspond to local pH values and partition coefficients, $\mathcal{P}$, in vicinity of enzyme molecules E_2 (see the text). Curves 2 of this Figure (a and b) correspond to local pH values and partition coefficient, $\mathcal{P}'$, in the vicinity of E_3 (see the text) enzyme molecules. (a) Variation of local pH values; (b) variation of partition coefficients $\mathcal{P}$ and $\mathcal{P}'$.

Discussion

It is usually believed that a departure from hyperbolic kinetics (generated by Donnan effects) of necessity implies that the electrostatic partition coefficient ($\mathcal{P}$) varies when the substrate concentration is varied. This is so only when the enzyme is homogeneously spread out in the insoluble matrix. If one part of the enzyme population is located at the surface of the Donnan phase, and therefore not submitted to electrostatic effects, but another one is buried in the matrix, the whole system may display an apparent negative co-operativity. This effect is observed even if the electrostatic partition coefficient does not significantly vary when the substrate concentration is varied. A variation of the partition coefficient may generate a sequence of positive and negative apparent co-operativities. These effects, which are controlled by the ionic concentration in the Donnan phase, do not seem to have been studied or predicted so far.

For an enzyme homogeneously distributed within the matrix, the departure from hyperbolic behaviour may be distinguished from true co-operativity by an analysis of the Hill plots.

Attraction of positively charged mobile species by fixed negative charges in the Donnan phase may result in a marked decrease of the local pH in the polyanionic matrix. This effect is limited if the pK of fixed charged groups of the matrix is located in the domain of variation of the local pH. If so, when the local pH decreases the negatively charged groups of the matrix become protonated and the value of the Donnan potential decreases. Therefore the local pH cannot become much lower than the pK of the fixed charged groups in the Donnan phase. In a certain sense this process can be considered as a regulatory device of the local pH.

The above theoretical treatment has been applied to the kinetic study of acid phosphatase bound to plant cell walls. The results obtained show the existence of an apparent negative co-operativity that can be explained by assuming that the distribution of acid phosphatase within the cell wall fragments is heterogeneous. Depending on the nature of their ionic environment, several classes of enzyme may be distinguished. A proportion of phosphatase molecules is not submitted to electrostatic effects and responds in a pure Michaelian way. The rest of the enzyme molecules are buried in the the polyanionic matrix of the cell wall and displays a positive electrostatic co-operativity. Owing to their ionic environment, at least two classes of enzyme that display positive electrostatic co-operativity may be distinguished. Superimposition of these effects generates apparent negative co-operativity. Indeed, this departure from hyperbolic kinetics is highly controlled by the ionic strength. Although the enzyme is by itself not

activated by a slight increase of ionic strength, it does become activated when bound to cell-wall fragments. This apparently surprising effect is understandable in the light of the Donnan theory.

Diffusion of the substrate within cell-wall fragments is not the limiting step of the overall kinetic process. At 'high' ionic strength (10.1 or above) the kinetic behaviour of the bound enzyme becomes identical with that of the pure enzyme in free solution. Therefore electrostatic effects are the only source of the observed non-hyperbolic behaviour of the bound acid phosphatase.

The decrease of the local pH values within cell walls, generated by an increase of the substrate concentration in the bulk phase, can be calculated from the above theoretical treatment and has been found to be quite significant.

Finally, the important idea that can be drawn from the above results is that on association with a cell wall or any other biological polyelectrolyte, an enzyme may acquire both quantitative and qualitative novel properties, namely apparent co-operativity and a strict control.

References

- Alvarez, E. F. (1962) *Biochim. Biophys. Acta* **59**, 663–672
- Cleland, W. W. (1967) *Adv. Enzymol. Relat. Areas Mol. Biol.* **29**, 1–32
- Cornish-Bowden, A. & Koshland, D. E., Jr. (1970) *Biochemistry* **9**, 3325–3336
- Crasnier, M., Noat, G. & Ricard, J. (1980) *Plant Cell Environ.* **3**, 217–224
- Dixon, M. & Webb, E. C. (1958) *Enzymes*, Longmans, Green and Co., London, New York and Toronto
- Douzou, P. & Maurel, P. (1977a) *Trends Biochem. Sci.* **2**, 14–17
- Douzou, P. & Maurel, P. (1977b) *Proc. Natl. Acad. Sci. U.S.A.* **3**, 1013–1015
- Engasser, J. M. & Horvath, C. (1975) *Biochem. J.* **145**, 431–435
- Engasser, J. M. & Horvath, C. (1976) *Appl. Biochem. Bioeng.* **1**, 194–204
- Goldstein, L. (1976) *Methods Enzymol.* **44**, 397–526
- Goldstein, L., Levin, Y. & Kalchalsky, E. (1964) *Biochemistry* **3**, 1913–1919
- Hsu, R. Y., Cleland, W. W. & Anderson, L. (1966) *Biochemistry* **5**, 799–807
- Jorgensen, O. B. (1959) *Acta Chem. Scand.* **13**, 900–909
- Levitzki, A. (1978) *Biochem. Pharmacol.* **27**, 2083–2088
- Maurel, P. & Douzou, P. (1976) *J. Mol. Biol.* **102**, 253–264
- Morvan, C., Demarty, M. & Thellier, M. (1979) *Plant Physiol.* **63**, 1117–1122
- Nari, J., Mouttet, C., Fouchier, F. & Ricard, J. (1974) *Eur. J. Biochem.* **41**, 499–515
- Noat, G., Crasnier, M. & Ricard, J. (1980) *Plant Cell Environ.* **3**, 225–229

Pettersson, G. & Pettersson, I. (1970) *Acta Chem. Scand.* **24**, 1275–1286

Van Etten, R. L. & McTigue, J. J. (1977) *Biochim. Biophys. Acta* **484**, 386–397

Wharton, C. W., Crook, E. M. & Brocklehurst, K. (1968a) *Eur. J. Biochem.* **6**, 565–571

Wharton, C. W., Crook, E. M. & Brocklehurst, K. (1968b) *Eur. J. Biochem.* **6**, 572–578

APPENDIX

Eqn. (14) in the main paper may be rewritten as:

$$\frac{v}{V_{\max}^*} = \frac{K_m \frac{\mathcal{P} + \alpha}{1 + \alpha} [S_o^-] + [S_o^-]^2}{K_m^2 \mathcal{P} + K_m (\mathcal{P} + 1) [S_o^-] + [S_o^-]^2} \quad (1')$$

The expression of the Hill coefficient (h) at half of the maximum rate is:

$$h = 4 \frac{d\left(\frac{v}{V_{\max}^*}\right)}{d[S_o^-]} [S_o^-] \quad (5')$$

that is:

$$h = 4 [S_o^-]_{0.5} \frac{K_m \frac{\mathcal{P} \alpha + 1}{\alpha + 1} [S_o^-]_{0.5}^2 + 2 K_m^2 \mathcal{P} [S_o^-]_{0.5} + K_m^3 \mathcal{P} \frac{\mathcal{P} + \alpha}{1 + \alpha}}{\{[S_o^-]_{0.5}^2 + K_m (\mathcal{P} + 1) [S_o^-]_{0.5} + K_m^2 \mathcal{P}\}^2} \quad (6')$$

where:

$$V_{\max}^* = V_{\max.,1} + V_{\max.,2} \quad (2')$$

$$\alpha = \frac{V_{\max.,2}}{V_{\max.,1}}$$

In order to find the substrate concentration that gives a value of $v/V_{\max}^* = 0.5$, one has to solve the quadratic equation:

$$[S_o^-]_{0.5}^2 + K_m \frac{1 - \alpha}{1 + \alpha} (\mathcal{P} - 1) [S_o^-]_{0.5} - K_m^2 \mathcal{P} = 0 \quad (3')$$

whose solution is:

$$[S_o^-]_{0.5} = \frac{-K_m \frac{1 - \alpha}{1 + \alpha} (\mathcal{P} - 1) + K_m \sqrt{\left(\frac{1 - \alpha}{1 + \alpha}\right)^2 (\mathcal{P} - 1)^2 + 4 \mathcal{P}}}{2} \quad (4')$$

This expression is indeed extremely complex when $[S_o^-]_{0.5}$ is substituted by its expression (eqn. 4'). However, if $\alpha = 1$, that is, if $V_{\max.,1} = V_{\max.,2}$, eqn. (4') becomes beautifully simple, namely:

$$[S_o^-]_{0.5} = K_m \sqrt{\mathcal{P}} \quad (7')$$

Substituting this expression in eqn. (6') yields:

$$h = 4 \sqrt{\mathcal{P}} \frac{(1 + \sqrt{\mathcal{P}})^2}{(1 + \mathcal{P} + 2\sqrt{\mathcal{P}})^2}$$