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Combined effects of diffusional hindrances, electrostatic repulsion and product inhibition on the kinetic properties of a bound acid phosphatase

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The kinetic properties of a soybean cell wall phosphatase were studied and compared under different environmental conditions. The native enzyme isolated from the wall exhibits classical Michaelis Menten kinetics. When buried into the cell wall, its natural environment, the enzyme mimics an apparent negative cooperativity. This deviation from hyperbolic dependence of the activity vs. the substrate concentration may result from an heterogeneous distribution of the enzyme molecules between the surface and the inside of the wall, where an electrostatic partition effect takes place. Cell wall fragments compacted on a Millipore-type membrane allow the study of the dual effects of diffusional hindrances and electrostatic interactions on the global kinetic behavior. By increasing the ionic strength, partition effects can be suppressed and therefore diffusional effects alone can be taken into account. On the one hand, diffusion and partition act individually and synergetically to decrease the apparent global affinity of phosphatase with respect to glucose 6-phosphate. On the other hand, product inhibition by inorganic phosphate is subjected to dual effects from diffusion through accumulation and partition through repulsion. A simple diffusion–partition reaction model accounts qualitatively and quantitatively for the experimental observations.

Soybean phosphatase; Plant cell wall; Semi-natural membrane; Diffusion–partition reaction; Kinetic model; Phosphomonoester

1. INTRODUCTION

The plant cell wall is a complex structure which is made-up of cellulose microfibrils, polysaccharides, structural proteins and enzymes [1,2]. Due to the presence of polygalacturonic acids, the plant cell wall behaves as a polyanion. The kinetic properties of cell wall-bound enzymes are then quite different from what they would be free in solution [3,4]. This charged structure may cause electrostatic attraction or repulsion between the fixed negative charges of the wall and the charges of the substrates and/or products. If the substrate is negatively charged, there is repulsion and the kinetics mimic negative cooperativity. This cooperativity may be explained by the presence of two populations of enzymes, one located at the surface of the wall and the other buried inside the matrix. Upon raising the ionic strengh, this apparent cooperativity can be suppressed [5–7]. The external diffusional resistances for the substrate can be coupled to this electrostatic repulsion effect by increasing its depletion in the vicinity of the cell wall-bound enzymes. A theoretical study related to this latter aspect was proposed by Shuler et al. [8].

When the electrostatic repulsion is suppressed, an accumulation of the negatively charged product of the reaction can occur in the vicinity of the bound enzyme, and a combined effect between diffusional resistances and product inhibition is then possible [9]. This coupling results in the alteration of the kinetic behavior of such enzymes.

The present paper deals with soybean (*Glycine max.*) cell wall phosphatase which is strongly inhibited by inorganic phosphate, its negatively charged product. Phosphatases are known to be ubiquitous in higher plants. A sycamore cell wall phosphatase has already been purified by Crasnier et al. [10]. However, its biochemical and/or physiological role is far from being well understood. One hypothesis might be its involvement in transport and trapping of phosphate esters from the extracellular environment into the cytoplasmic compartment [11]: several studies have demonstrated that a phosphate deficiency in the immediate environment can cause an increase in extracellular phosphatase activities [12–14].

Our aim is to study the combined effects of diffusional resistances, electrostatic repulsion and product inhibition on the kinetic behavior of the soybean cell wall phosphatase, both from a theoretical and experimental point of view.

2. MATERIALS AND METHODS

2.1. *Chemicals and plant material*

Biochemicals were purchased from Sigma and Boehringer.

Soybean cells (*Glycine max.*) were cultured in vitro under sterile conditions as described by Gamborg et al. [15]. Walls were isolated from cells taken during the exponential growth phase. The cell walls

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were obtained by following the procedure described by Nari et al. [16] and they were then filtered through 1.2 μm Millipore membranes over a Büchner in order to obtain 'compacted' wall fragments.

In order to solubilize the acid phosphatase enzyme and other proteins, the cell wall fragments were incubated for 90 min in a 20 mM HEPES buffer in the presence of 1 M NaCl, and then centrifuged for 25 min at 5,000 rpm. The supernatant was then dialysed for 24 h against water.

2.2. *Enzyme assays*

Acid phosphatase activity was followed by measuring the inorganic phosphate (P_i) released by the hydrolysis of glucose 6-phosphate. The method developed by Allen [17] was used for the detection. The reaction medium containing either the solubilized enzyme, the cell wall fragments in solution, or the 'compacted' wall fragments was made of HEPES buffer 5 mM, pH 7. The absorbance was read at 715 nm on a Milton Roy spectrophotometer. All measurements were carried out at 40°C.

3. RESULTS

3.1. *Kinetic properties of the solubilized acid phosphatase (reaction)*

Soybean acid phosphatase can hydrolyse numerous natural and artificial phosphoesters. In the present study, glucose 6-phosphate (Glu6P) was chosen as the substrate, yielding glucose (Glu) and inorganic phosphate (P_i):

$$\text{Glu6P} \rightarrow \text{Glu} + P_i$$

In solution, the enzyme activity follows classical Michaelis–Menten kinetics with a K_m of about 0.25 mM for glucose 6-phosphate. The pH for optimal activity lies between 6 and 7 which are higher values than those found for other acid phosphatases from plant cell walls [18–20]. The enzyme activity is stable for several hours at 40°C. Inorganic phosphate acts as a strong competitive inhibitor of the enzyme with an inhibition constant, K_p, of 50 μM.

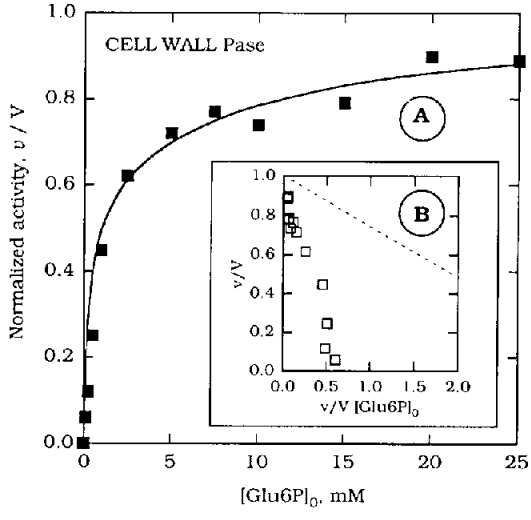


Fig. 1. Phosphatase bound to cell walls (reaction–partition). (A) Normalized activity (v/V) experimentally measured as a function of the (external) glucose 6-phosphate concentration ([Glu6P]$_0$]. The curve through data points was calculated from Eqn. 3 with parameter values as follows: V_1 = 35.2 μM·min^{-1}, V_2 = 27 μM·min^{-1}, Π = 34, K_m = 250 μM, K_p = 50 μM and α = 0.56. All measurements were carried out at pH 7 and 40°C. (B) (insert): Eadie–Hofstee transform (□) showing the deviation from the classical Michaelis–Menten kinetics (---).

3.2. *Kinetic properties of the enzyme bound to the cell walls (reaction–partition)*

Due to the presence of polygalacturonic acids, plant cell walls behave as polyanions with a pK_a around 3 [21]. Depending on the sign of the charge of the substrate, either attraction or repulsion may occur. Here, glucose 6-phosphate is negatively charged and is consequently repelled from the wall. Because of this electrostatic repulsion the enzyme activity as a function of Glu6P mimics a negative cooperativity as previously described by Ricard et al. [5] and resulting from the coexistence of two enzyme populations: one is located at the surface of the cell wall and is not subjected to electrostatic effects, whereas the other one is buried within the matrix and is subjected to these effects [6].

For each charged species, the electrostatic partition coefficient, Π, can be defined as follows:

$$\Pi = \frac{\Sigma [A_0]}{\Sigma [A_i^-]} = \frac{\Sigma [B_i^+]}{\Sigma [B_0^+]} = \exp\left(-\frac{F\Delta\psi}{RT}\right) \quad (1)$$

where subscripts 0 and i refer to the metabolite concentrations outside and inside the matrix, respectively. $\Delta\psi$ is the electrical or Donnan potential, F is the Faraday, and R and T have their usual significance.

If both glucose 6-phosphate and inorganic phosphate are considered as monovalent anions, then the expression of Π will be

$$\Pi = \frac{[\text{Glu6P}]_0}{[\text{Glu6P}]_i} = \frac{[P_i]_0}{[P_i]_i} \quad (2)$$

and under these conditions, the general form of the reaction rate, v, as a function of the external glucose 6-phosphate, [Glu6P]$_0$, and taking into account the competitive product inhibition by phosphate can be described by the following expression:

$$v = \frac{V_1 [\text{Glu6P}]_0}{K_m\left(1 + \frac{[P_i]}{K_p}\right) + [\text{Glu6P}]_0} + \frac{V_2 [\text{Glu6P}]_0}{K_m \Pi\left(1 + \frac{[P_i]}{\Pi K_p}\right) + [\text{Glu6P}]_0} \quad (3)$$

where Π is the electrostatic partition coefficient, K_m and K_p are the Michaelis and inhibition constants of glucose 6-phosphate and inorganic phosphate, respectively, for the solubilized enzyme, V_1 the maximal velocity of the enzyme molecules at the surface of the cell wall, and V_2 the maximal velocity of the enzyme molecules buried in

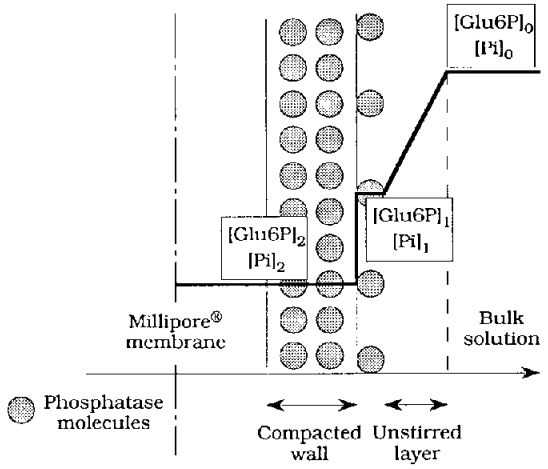


Fig. 2. Phosphatase bound to compacted cell walls (reaction–partition–diffusion). Schematic representation of the compacted cell wall fragments. The heavy line stands for the steady-state concentrations of glucose 6-phosphate, $[Glu6P]_i$, and inorganic phosphate, $[P_i]_i$, throughout the different layers of the structure, i.e. Millipore membrane, compacted wall, unstirred layer and bulk solution.

the polyelectronic matrix. The term $K_m\,\Pi$ (resp. $K_p\,\Pi$) represents the apparent Michaelis constant for the substrate, Glu6P (resp. the apparent inhibition constant for the product, P_i) with respect to the enzyme molecules buried inside the matrix and subjected to electrostatic repulsions.

The experimentally measured initial activities as a function of the Glu6P concentration are shown in Fig. 1A and can be fairly fitted by the rate expression (3), with parameter values as follows: $V_1 = 35.2\ \mu M \cdot min^{-1}$, $V_2 = 27\ \mu M \cdot min^{-1}$, $\Pi = 34$, $K_m = 250\ \mu M$, $K_p = 50\ \mu M$ and α [$= V_1/V$, where $V = (V_1 + V_2)$] = 0.56. The global apparent K_m ($K_{0.5}$), i.e. the $Glu6P_0$ concentration for which the observed global activity is half maximum, is about 1.62 mM, that is six times higher than the observed K_m for the free soluble enzyme. An Eadie–Hofstee representation clearly illustrates the deviation from the classical hyperbolic dependence of activity vs. the substrate concentration in terms of a concave-down curve (Fig. 1B).

3.3. *Kinetic behaviour of the compacted cell wall fragments (reaction–partition–diffusion)*

Compacting the cell wall fragments bearing phosphatase activity on Millipore filters, allows us to manufacture enzymatically active membrane-shaped supports. In that way, the effects of diffusional hindrances combined with the electrostatic interactions, on the kinetic behavior can be taken into account.

The new experimental device is depicted in Fig. 2. In such a configuration, the plant cell walls are no longer homogeneously distributed throughout the bulk of the solution. Let us stress that, due to its macroporous structure, the Millipore membrane just as the compacted wall fragments do not create diffusional constraints by themselves: the diffusional limitations take place significantly at the interface (unstirred layer) between the bulk of the solution and the membrane surface (compacted cell walls).

The bulk substrate molecules ($[Glu6P]_0$) diffuse through the unstirred layer (with thickness δ). Locally, these substrate molecules are transformed into product with concentration [P_i] by the population of enzymes located at the surface and not subjected to electrostatic interactions. The related steady-state concentrations are referred to as $[Glu6P]_1$ and $[P_i]_1$. The substrate molecules entering the compacted cell walls are subjected to an electrostatic partitioning (Π) and the steady-state concentrations for substrate and product, resulting from the coupling between catalysis and electrostatic repulsion, will be then $[Glu6P]_2$ and $[P_i]_2$.

Thus, the flux of substrate, J_{Glu6P} (resp. J_{P_i}, for the product) through the unstirred layer and towards the catalytic surface is given by the Fick's first law of diffusion:

$$J_{Glu6P} = h_{Glu6P}([Glu6P]_0 - [Glu6P]_1) \tag{5}$$

with

$$h_{Glu6P} = D_{Glu6P}/\delta \tag{6}$$

where h_{Glu6P} (resp. h_{P_i}) is the external mass transfer coefficient and D_{Glu6P} (resp. D_{P_i} is the diffusion coefficient of substrate (resp. product) through the layer.

The local $[Glu6P]_2$ (resp. $[P_i]_2$) concentration within the membrane is related to the $[Glu6P]_1$ (resp. $[P_i]_1$) concentration through the partition coefficient Π, that is $[Glu6P]_2 = [Glu6P]_1/\Pi$ (resp. $[P_i]_2 = [P_i]_1/\Pi$) and the time evolution of substrates and products is governed by the following pair of differential equations, set to zero at steady state:

$$\begin{cases} \dfrac{d([Glu6P]_1 + [Glu6P]_2)}{dt} = h_{Glu6P}\,([Glu6P]_0 - [Glu6P]_1) - v \\ \dfrac{d([P_i]_1 + [P_i]_2)}{dt} = h_{P_i}([P_i]_0 - [P_i]_1) + v \end{cases} \tag{7}$$

By using adimensional parameters, $s_i = [Glu6P]_i/K_m$ (resp. $p_i = [P_i]_i/K_P$), $\alpha = V_1/V$, $\mu = V/(h_{Glu6P}\,K_m)$, $\zeta = h_{Glu6P}\,K_m/h_{P_i}\,K_P$, where μ and ζ stand for the amplitude of diffusional limitations and the external accumulation factor, respectively, and by recalling that $s_2 = s_1/\Pi$ (resp. $p_2 = p_1/\Pi$), equation system (7) can be rewritten in the normalized form:

$$\frac{1}{\mu}(s_0 - s_1) = \frac{\alpha\, s_1}{1 + p_1 + s_1} + \frac{(1-\alpha)s_1}{\Pi + p_1 + s_1} = \frac{1}{\zeta\mu}(p_1 - p_0) \tag{8}$$

Under conditions where the product concentration in the bulk phase can be neglected ($[P_i]_0 = 0$), that is during

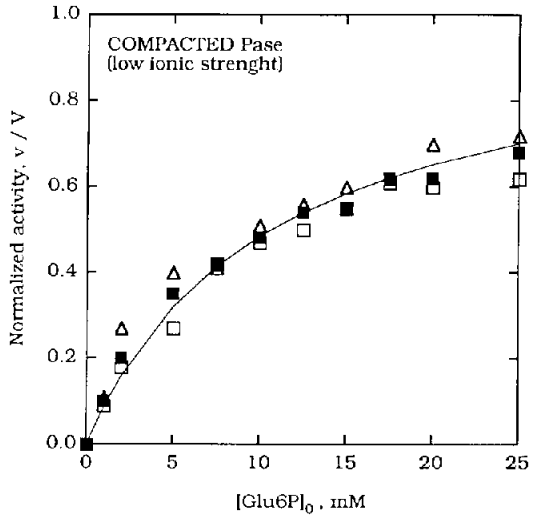


Fig. 3. Phosphatase bound to compacted cell walls (reaction–partition–diffusion). Normalized activity (v/V) experimentally measured as a function of the (external) glucose 6-phosphate concentration ([Glu6P]$_0$] for three independent series of measurements. The curve through data points was calculated from Eqn. 9 with parameter values as follows: $\mu = 3.5$, $\alpha = 0.2$, $\zeta = 2$ and $\Pi = 34$.

initial velocity measurements, the adimensional local product concentration, p_1, can be replaced by $[\zeta\,(s_0 - s_1)]$ and thus $[(p_1 - p_0)/\zeta\mu]$ will equal $[(s_0 - s_1)/\mu]$, and Eqn. 8 can be recast in the form:

$$\frac{1}{\mu}(s_0 - s_1) = \frac{\alpha s_1}{1 + \zeta(s_0 - s_1) + s_1} + \frac{(1-\alpha)s_1}{\Pi + \zeta(s_0 - s_1) + s_1} \qquad (9)$$

Given α, μ, ζ and Π, the s_1 steady-state concentrations as a function of s_0 can be calculated explicitly from Eqn. 9 by solving the following third-order polynomial in s_1

$$As_1^3 + Bs_1^2 + Cs_1 + D = 0 \qquad (10)$$

where

$$A = (1 - \zeta)^2$$

$$B = (1 - 4\zeta + 3\zeta^2)\, s_0 + \zeta + \Pi\,(\zeta - 1) - \mu\,(1 - \alpha - \zeta)$$

$$C = (-3\zeta^2 + 2\zeta)\, s_0^2 + (\Pi - 2\Pi\zeta - 2\zeta)\, s_0 - \Pi - \mu\,(\alpha\Pi + 1 + \zeta\, s_0 - \alpha)$$

$$D = \zeta^2 s_0^3 + (1 + \Pi)\,\zeta s_0^2 + \Pi s_0$$

The determination of the apparent activity, v, as a function of the external substrate concentration, s_0 ([Glu6P]$_0$), is then straightforward.

Fig. 3 shows the (normalized) phosphatase activity, (v/V), experimentally measured as a function of the external glucose 6-phosphate concentration for three independent series of measurements.

The quantification of μ and ζ requires independent measurements of the diffusion coefficients for Glu6P and P_i, along with an estimation of the thickness of the unstirred layer, δ. Combined diffusion experiments gave us the following numerical values, namely, 8×10^{-4} and 2×10^{-3} cm$^2 \cdot$min^{-1} for D_{Glu6P} and D_{P_i}, respectively, and 15×10^{-3} cm for δ.

The experimental data obtained can be fitted to Equation 9 with the following parameter values: $\Pi = 34$, $\mu = 3.5$ and $\zeta = 2$. Since we had no direct method determining the distribution of enzyme molecules between the surface and the inside of the membrane, α was taken as a free parameter. The best fit was obtained for a value of α equal to 0.2. From these experiments, the half-saturation substrate concentration, $K_{0.5}$, can be estimated to be 11 mM. This high value for $K_{0.5}$ mostly results from the cumulative (additional) effects of diffusional hindrances and electrostatic repulsion, both leading to a local depletion in the substrate concentration.

The slowing down of enzyme activity, the partitioning effect and the fast diffusivity of phosphate result in weak kinetic effects due to product inhibition.

This can be explained by the fact that when the cell wall fragments are compacted, the contact surface between the enzyme molecules and the bulk phase is decreased. Consequently V_{m1} (proportional to the enzyme molecules concentration) decreases, and α decreases as well.

3.4. *The effect of the ionic strength (reaction–diffusion)*

The electrostatic potential is under the control of the ionic strength, and therefore an increase of this ionic strength will tend to equalize the bulk and local ion concentrations, i.e. the electrostatic partition coefficient

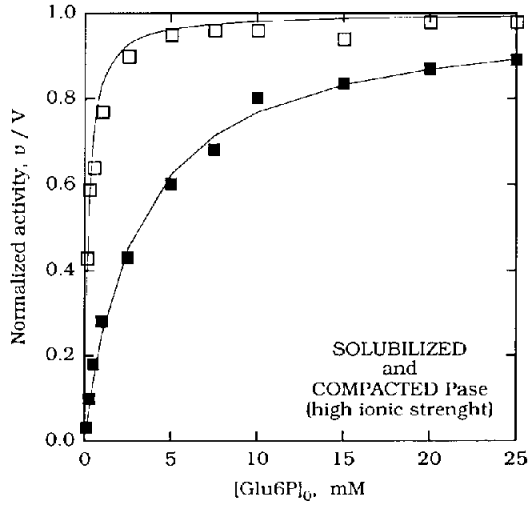


Fig. 4. Phosphatase bound to compacted cell walls under conditions of high ionic strength (reaction–diffusion). Normalized activity (v/V) experimentally measured as a function of the (external) glucose 6-phosphate concentration ([Glu6P]$_0$], in the presence of 0.2 M NaCl (■). The curve through data points was calculated from Eqn. 12 with parameter values as follows: $\mu = 3.5$ and $\zeta = 5$. For comparison, the activity of the solubilized enzyme (reaction) is also shown (□).

will tend to unity [21]. Clearly, the two enzyme populations will not be kinetically differentiated anymore, and S_1 will tend to equal S_2 ($\alpha \rightarrow 1$). It was shown with sycamore cell walls that, in the presence of a negatively charged substrate and at high ionic strength, the bound (cell-wall) acid phosphatase behaves as the enzyme in solution with an increase in the apparent affinity [4]. The same results were observed with soybean phosphatase (data not shown).

In the case of compacted cell walls, a high ionic strength also suppresses the electrical effects, but global diffusional hindrances are not altered. This leads to a building up of the inorganic phosphate concentration in the vicinity of the enzyme and which is no more repelled from the wall. As already discussed theoretically [9], a combined effect between diffusion and competitive product inhibition appears.

The overall reaction rate may thus be expressed as follows:

$$\frac{d[Glu6P]}{dt} = h_{Glu6P}([Glu6P]_0 - [Glu6P]) - \frac{V[Glu6P]}{K_m\left(1 + \frac{[P_i]}{K_p}\right) + [Glu6P]} \quad (11)$$

which, by using the adimensional parameters, s (and s_0), ζ and μ as previously defined, can be rewritten in the form

$$\frac{1}{\mu}(s_0 - s) = \frac{s}{1 + \zeta(s_0 - s) + s} \quad (12)$$

and the s steady-state solutions can be explicitly calculated by solving the second-order polynomial:

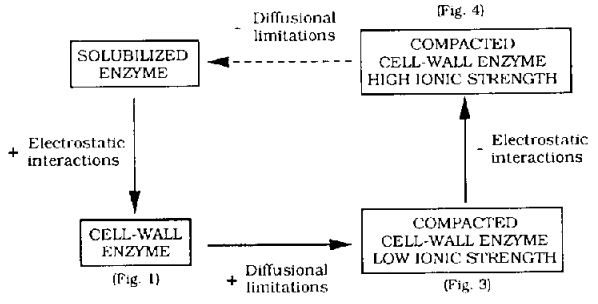
$$(\zeta - 1)s^2 + (s_0 - 2\zeta s_0 - \mu - 1)s + s_0 + \zeta s_0^2 = 0 \quad (13)$$

Fig. 4 shows the experimentally measured phosphatase activity as a function of the external Glu6P concentration and in the presence of NaCl 0.2 M. The curve through data points was calculated from Eqn. 12 with $\zeta = 5$, $\mu = 3.5$ and $p_0 = 0$. The higher value of ζ compared to the one obtained under the previous conditions ($\zeta = 2$) results from an increase in the product inhibition, i.e. the absence of electrostatic repulsion favors the local accumulation of phosphate at the surface of the compacted cell walls. The apparent global $K_{0.5}$ which takes into account both the product inhibition and the substrate and product diffusional limitations can be estimated to 3.3 mM.

4. DISCUSSION AND CONCLUSION

The compacted cell walls we used in the present work are an interesting and original material which can be viewed as a compromise between natural and artificial membranes. The natural presence of enzymes within the walls allows us to avoid the often tricky step of inclusion or immobilization. If one also takes advantage of the very long-lasting stability of the activities (numerous months at room temperature and in the open air), these cell walls, compacted or not, are a remarkable experimental tool to study the combined effects of diffusional limitations, electrostatic interactions, ionic strengh and product inhibition.

The experimental strategy we used in this work, is summarized in the following scheme:



Scheme 1

The solubilized enzyme taken as the reference, the independent or simultaneous contributions of electrostatic interactions and/or diffusional hindrances on the kinetic behavior of phosphatase can thus be analyzed and compared.

Provided that the matrix and the substrate have opposite net charges, partition and diffusional effects act synergetically to decrease the apparent global affinity of the enzyme with respect to its substrate. However, a comparison between the individual contribution of these effects in the alteration of apparent affinity, has to be analyzed cautiously. Indeed, (i) measurements carried out with compacted cell walls were made under gentle stirring conditions; more vigorous stirring would have led to a decrease in the unstirred layer thickness, that is, to a decrease in the contribution of diffusional hindrances, and (ii) the effective product inhibition results from a duel affect between accumulation (diffusion) and repulsion (electrostatic partiton).

One cardinal rule of the so-called heterogeneous enzymology is that the structure plays an amplificatory role with respect to endogeneously produced effectors. Under such conditions, inorganic phosphate will act as a more potent inhibitor compared to a situation where, due to electrostatic repulsion, it can be repelled from the structure. From a physiological point of view, the tuning of both diffusion and partition may act as a powerful controller on an enzyme activity through either alteration of the catalyst ionic microenvironment or by its translocation between the cytosol (no or weak diffusion) and the surface of a membrane (important mass transfers effects).

The ability to set up enzymatically active cell wall 'membranes' allows us to consider compartmentalized models where, for example, a kinase (soluble or immobilized) is separated from the external environment by a semi-permeable membrane bearing the phosphatase activity. Such an approach, which is presently in progress, should help to understand the role played by that very cell wall phosphatase, among others, in the transport of exogeneous phosphomonoesters.

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