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pH MODULATION OF TRANSIENT STATE KINETICS OF ENZYMES

II. TRANSIENT STATE KINETICS OF PLANT CELL WALL ACID PHOSPHATASE

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The pre-steady-state kinetics of plant cell wall acid phosphatase has been investigated at different pH values. The approach of the steady state lasts about 1 or 2 s and may be fitted with two exponential terms. For certain pH values the approach to the steady state exhibits damped oscillations. Plotting the sum and the product of the two time constants of these exponentials as a function of substrate concentration yields two straight lines. From the slopes and intercepts of these lines one may determine the values of rate and ionization constants involved in the reaction scheme. The results obtained are consistent with the view that the binding of the substrate to the enzyme does not induce a 'slow' conformation change of the enzyme. The enzyme reacts with its substrate while being mostly in its ionized form. Release of *p*-nitrophenol is also favoured by this ionized form of the enzyme. However, the hydrolysis of the phosphoryl-enzyme complex mostly occurs from the protonated form of the enzyme. The ionization constants of the free enzyme and of the various enzyme-ligand complexes are very similar.

1. Introduction

Plant cell walls in sterile culture contain several hydrolases [1–4] that take part in the process of cell extension and in the transport or organic solutes within the cell. Clumps of sycamore (*Acer pseudoplatanus*) cells cultured in vitro are particularly rich in an acid phosphatase. This enzyme is an exocellular monomeric glycoprotein of M_r 100000 [5]. Although the purified enzyme does follow classical Michaelis-Menten kinetics, its pH profile is surprisingly bimodal. A plot of $\log(\tilde{k}_{cat}/\tilde{K}_m)$ vs. pH exhibits two maxima at pH 4 and 7.5, whereas the value of $\log(\tilde{k}_{cat}/\tilde{K}_m)$ is minimum at pH 5.5. It has been tentatively assumed that this very unusual type of pH profile represents a complex regulation imposed on the enzyme by the very peculiar structure of the plant cell wall which behaves as a polyanion. This complex pH behaviour tends to buffer the variation of phosphate transport and hydrolysis against possible variations of organic phosphate concentrations in the external bulk phase [6,7]. Therefore, it is extremely tempting to consider that complexity of the pH profile represents a remarkable adaptation to a given type of compartmentalization and function.

In view of this complexity, it is therefore of interest to study the pH modulation of the transient phase of enzyme reaction. This study may be easily performed in the light of the theoretical results presented in the companion paper [8].

The experimental study of the transient phase of this acid phosphatase as well as its pH modulation are precisely the goals of this paper.

2. Materials and methods

Clumps of sycamore (*A. pseudoplatanus*) cells were cultured in vitro as described previously [5].

Acid phosphatase was isolated from cell wall fragments and purified to homogeneity by methods already described [5]. Rate determinations were effected in a Durrum-Gibson (model D 110) stopped-flow apparatus by following *p*-nitrophenyl phosphate hydrolysis. The temperature was maintained at 30°C by circulating thermostatted water. The stopped-flow device was connected with a data analyzer and a micro-computer. The data analyzer was a Zoomax device (Société d'électronique Industrielle et Nucléaire, Paris, France) connected with a Novelec (Paris) data sampler and analogue digital computer. This device allowed sampling, storing and averaging of numerical data. The mathematical treatment of these data was effected with a Wang 2200 computer with extended memory.

p-Nitrophenyl phosphate and *p*-nitrophenol were obtained from Sigma.

3. Results

The pre-steady-state kinetics of hydrolysis of *p*-nitrophenyl phosphate by the acid phosphatase is rather complex. Under acidic pH conditions, the rate of *p*-nitrophenol appearance, during the transient phase, is smaller than the steady-state rate. This implies that the difference Δp (see companion paper [8]) between the actual and the steady-

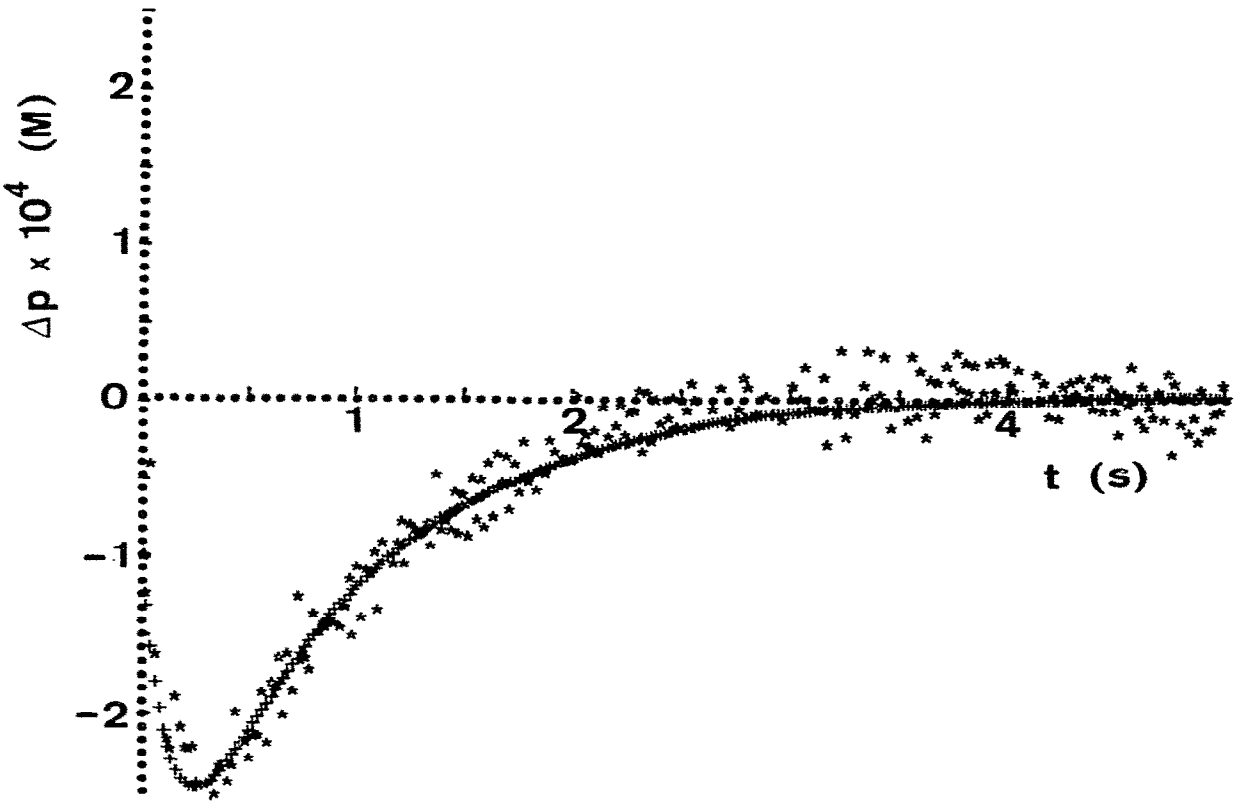

Fig. 1. Approach to the steady state of acid phosphatase reaction, under slightly acidic pH conditions. Conditions: pH, 6.6; substrate (*p*-nitrophenyl phosphate) concentration, 2×10^{-4} M. (•) Averages of experimental data. Curve is theoretical and has been fitted to the experimental data with the following values: $\psi_1 = 3.84$ M, $\psi_2 = -2.83$ M, $\lambda_1 = 1.17\ s^{-1}$, $\lambda_2 = 7.49\ s^{-1}$.

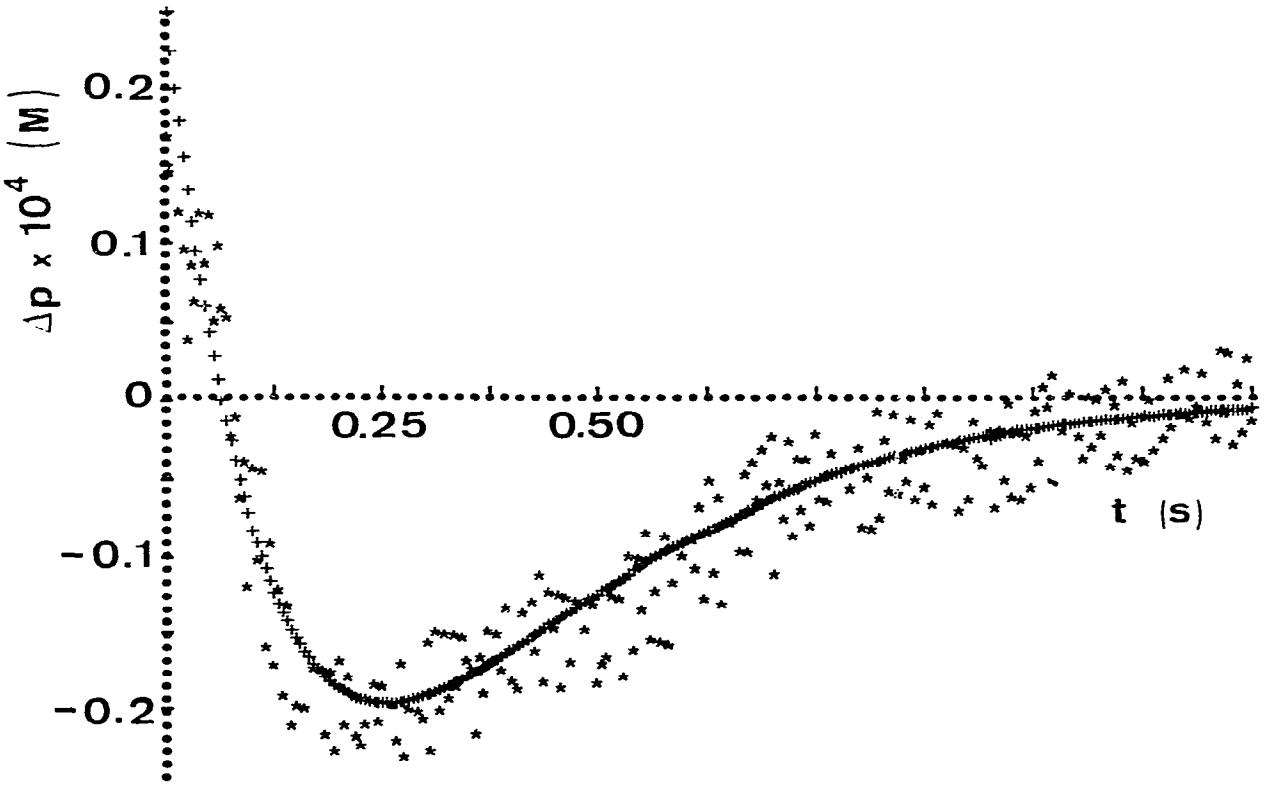

Fig. 2. Approach to the steady state of acid phosphatase reaction under slightly alkaline pH conditions. Conditions: pH 8; substrate (p-nitrophenyl phosphate) concentration, 10^{-3} M. (*) Averages of rate data. Curve is theoretical and has been fitted to the velocity data with the following values: $\psi_1 = -13$ M, $\psi_2 = 11.9$ M, $\lambda_1 = 6\ s^{-1}$, $\lambda_2 = 4.6\ s^{-1}$.

state progress curves is negative and decreases at first, then increases and approaches zero (fig. 1) as the steady state is reached. Alternately, for alkaline pH values, the value of Δp is positive, decreases and becomes negative, then approaches zero when the steady state is reached (fig. 2).

For intermediates pH values close to neutrality, the values of Δp exhibit damped oscillations as the steady state is approached (fig. 3). The frequency of these damped oscillations depends on substrate concentration (table 1).

From these and other data one may estimate the two time constants, λ_1 and λ_2, that govern the variation of Δp with time. Their sum, σ, and their product, ξ, are plotted against p-nitrophenyl phosphate concentration in (fig. 4).

In either case the experimental data fall, within experimental error, on straight lines. According to the theoretical results presented in the companion paper [8], the ordinates and slopes of these plots allow one to estimate the κ values which should be hyperbolic functions of proton concentration. Whereas κ_1 increases as proton concentration is increased, κ_{-1}, κ_2 and κ_3 decrease as a function of [H]. This is shown in figs. 5 and 6.

4. Interpretation and discussion

The above results are quite consistent with the simple reaction scheme shown in fig. 7 where it is assumed that the free enzyme and the various

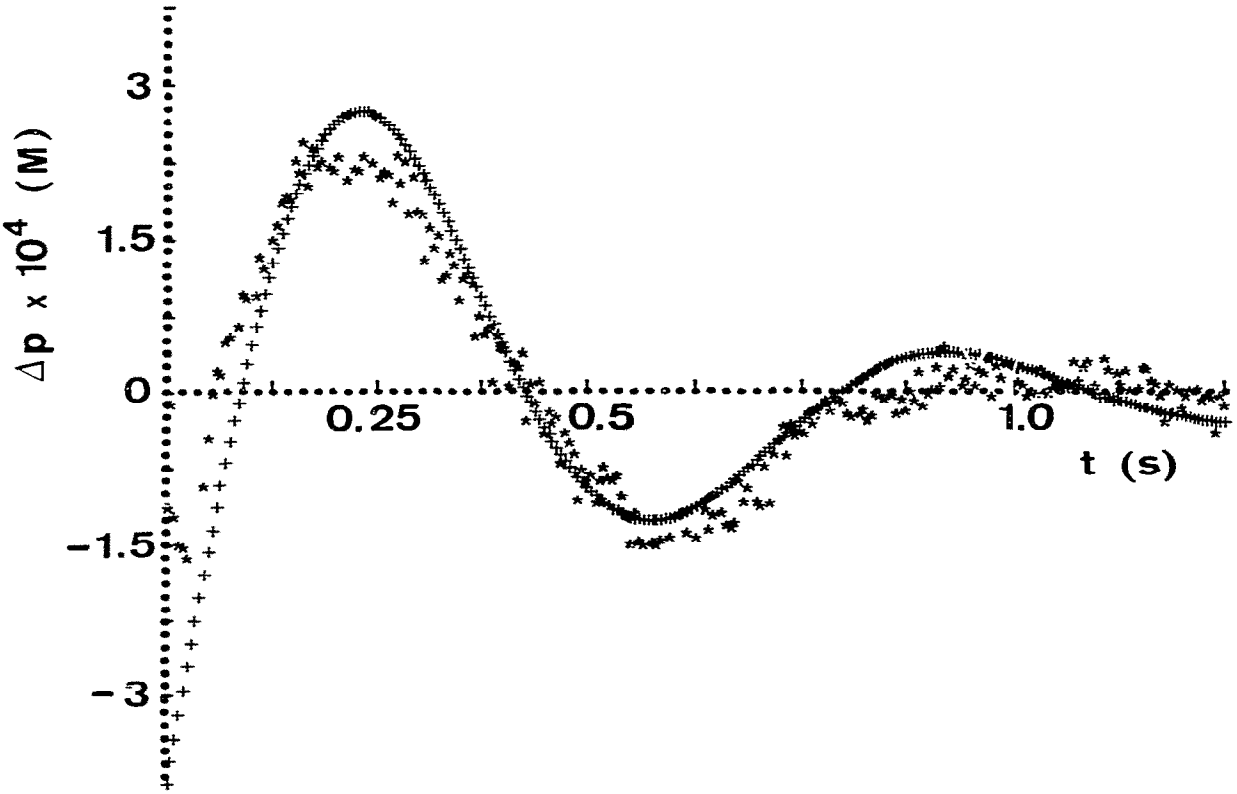

Fig. 3. Damped oscillations during the approach to the steady state. Conditions: pH of the reaction mixture, 7.25; substrate concentration, 10^{-4} M. The sum of the two λ parameters is $\sigma = 5.2\ s^{-1}$ and the ω value is $2.88\ s^{-1}$. (*) Averages of experimental values; the damped curve is theoretical.

enzyme-substrate and enzyme-product complexes exist in two different states, in rapid equilibrium. The finding that both the sum σ and the product ξ of the roots vary linearly with the substrate concentration strongly suggests that the substrate binding to the enzyme occurs in one detectable step and that no slow conformation change is induced upon substrate binding.

Fitting of the available data to this model allow estimation of the rate and the ionization constants involved in this reaction scheme. They are given in table 2. From these data one may derive several interesting conclusions. Firstly, the ionized state of the enzyme favours substrate binding and *p*-nitrophenol release, whereas protonation of this enzyme favours hydrolysis of the phosphoryl-enzyme complex. When the enzyme is protonated, the overall reaction rate is limited by *p*-nitrophenol release, whereas when the enzyme is ionized, the reaction is limited by the hydrolysis of the phosphoryl-enzyme complex. Secondly the transient phase of the enzyme reaction is a 'slow' process. One would have expected this slow process to be controlled by a slow induced conformation change of the enzyme [12]. In that case a plot of σ and ξ as a function of substrate concentration should have exhibited a plateau value. The present results show these plots to be linear. Therefore, the slowness of the overall reaction is mostly due to the slowness of product release. Lastly, one may note that the

Table 1

Theoretical and experimental variation of the natural frequency of oscillation ω as a function of substrate concentration during the approach to the steady state

The variation of ω has been calculated from the following values $\kappa_1 = 15\times10^3\ M^{-1}\ s^{-1}$, $\kappa_2 = 2\ s^{-1}$, $\kappa_3 = 1\ s^{-1}$, $\kappa_{-1} = 0.2\ s^{-1}$.

a_o (M)	ω_{theor} (s^{-1})	ω_{exp} (s^{-1})
2.0×10^{-5}	0.387	
2.5×10^{-5}	0.721	1.34 ± 0.99
3.0×10^{-5}	0.937	
3.5×10^{-5}	1.107	
4.0×10^{-5}	1.249	
4.5×10^{-5}	1.373	
5.0×10^{-5}	1.482	1.05 ± 0.50
5.5×10^{-5}	1.581	
6.0×10^{-5}	1.670	
6.5×10^{-5}	1.752	
7.0×10^{-5}	1.827	
7.5×10^{-5}	1.896	1.42 ± 1.20
8.0×10^{-5}	1.960	
8.5×10^{-5}	2.019	
9.0×10^{-5}	2.073	
9.5×10^{-5}	2.124	
10^{-4}	2.170	2.87 ± 1.00

binding of substrate and product does not dramatically change the pK of the enzyme.

One may observe that the on-constants for sub-

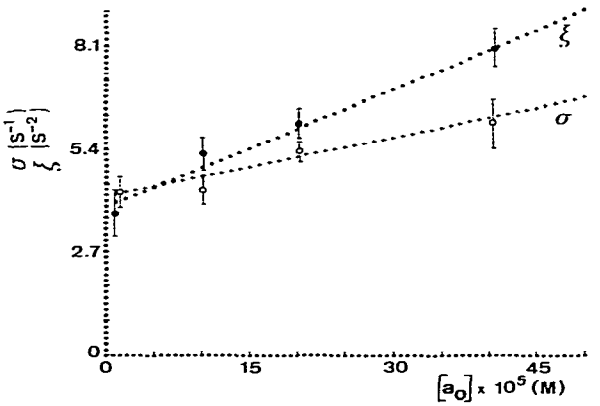

Fig. 4. Variation of the sum (σ) and the product (ξ) of the time constants (λ) as a function of substrate concentration. From the slope and intercept of these two straight lines, one may estimate the four κ values, namely: $\kappa_1 = 5\times10^3\ M^{-1}\ s^{-1}$, $\kappa_{-1} = 2.1\ s^{-1}$, $\kappa_2 = 0.58\ s^{-1}$, $\kappa_3 = 1.42\ s^{-1}$. pH of the reaction mixture, 6.25.

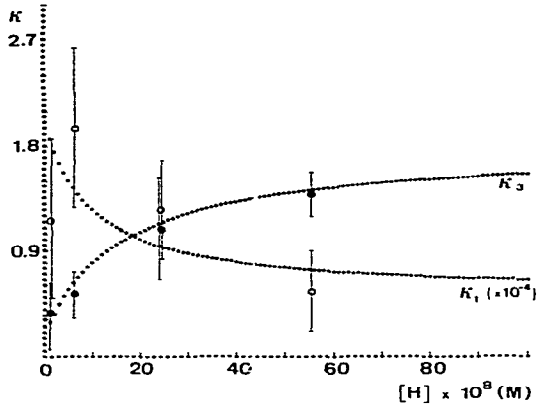

Fig. 5. Variation of κ_3 and κ_1 as a function of proton concentration. Points are experimental and curves theoretical. Fitting of the data was effected with the following values: $k_1 = 5\times10^3\ M^{-1}\ s^{-1}$, $k_1' = 2\times10^4\ M^{-1}\ s^{-1}$, $k_3 = 1.8\ s^{-1}$, $k_3' = 0.2\ s^{-1}$, $K_D = 10^{-7}$ M, $K_D'' = 1.6\times10^{-7}$ M.

strate binding are 5–6 orders of magnitude below the diffusion-controlled limit. Electrostatic repulsion between negative charges borne by the phosphate and the enzyme could represent a reasonable but speculative explanation of this difference.

Studies on pre-steady-state kinetics of enzymes have led to the view that the enzyme reaction may exhibit either a burst or a lag [9–11]. Strictly speaking this implies that the approach to the

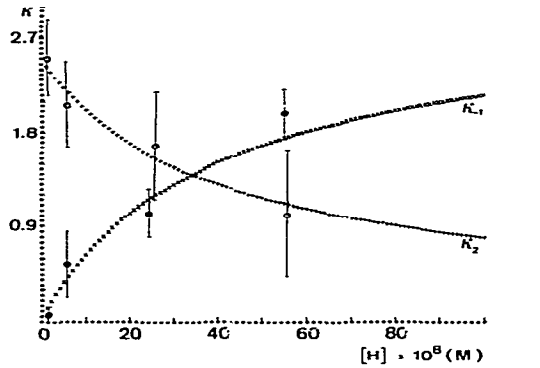

Fig. 6. Variation of κ_2 and κ_{-1} function of proton concentration. Points are experimental and curves theoretical. Fitting of the data was effected with the following values: $k_2 = 0.1\ s^{-1}$, $k_2' = 2.5\ s^{-1}$, $k_{-1} = 3\ s^{-1}$, $k_{-1}' = 5\times10^{-2}\ s^{-1}$, $K_D' = 4\times10^{-7}$ M.

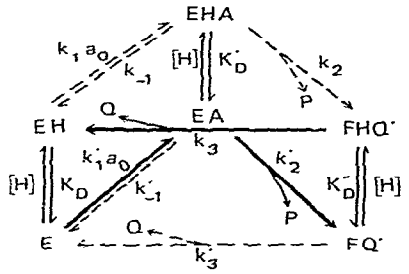

Fig. 7. Preferred reaction steps of the reaction scheme. The heavy arrows represent preferred reaction steps.

Table 2

Numerical values of rate and ionization constants involved in the reaction model

$K_D = 10^{-7}$ M	$k_1 = 5\times10^3$ M^{-1} s^{-1}
$K'_D = 4\times10^{-7}$ M	$k'_1 = 2\times10^4$ M^{-1} s^{-1}
$K''_D = 1.6\times10^{-7}$ M	$k_{-1} = 3$ s^{-1}
	$k'_{-1} = 5\times10^{-2}$ s^{-1}
	$k_2 = 0.1$ s^{-1}
	$k'_2 = 2.5$ s^{-1}
	$k_3 = 1.8$ s^{-1}
	$k'_3 = 0.2$ s^{-1}

steady state occurs in one exponential term only. this may be so, but only if the first step of substrate binding is sufficiently fast compared with the two steps of product release. The experimental results presented in this paper clearly show that the approach to the steady state occurs in two exponentials. This finding as well as the slowness of the transient phase is quite unexpected. Therefore, this concept of a burst or lag has little interest when applied to the experimental results of figs. 1 and 2 for the progress curves obtained may show both a lag and a burst.

Another striking result of the present study is the existence of damped oscillations of intermediates as the steady state is approached. This interesting behaviour occurs in a limited range of pH values. It is difficult at the moment to determine whether this kind of behaviour plays any role in the transfer of organic solutes in the cell. One may indeed speculate that the oscillatory behaviour of a chemical reaction may be associated with the transfer of a signal from one place to another. Whatever the case may be, there is no doubt that the pre-steady-state kinetics of cell wall acid phosphatase is much more complex than that of the corresponding transient phase of most of the enzymes studied thus far.

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