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Hysteresis of plant cell-wall β-glucosidase

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A transient-kinetic study of β-glucosidase from soyabean cell walls was performed with the use of a stopped-flow apparatus. The progress curve of the reaction exhibits a 'slow' burst of about 1 s before the steady state is reached. In the time scale investigated this burst may be accounted for by only one exponential, whose time constant varies with the substrate concentration. As this concentration is increased the value of the time constant increases at first, then decreases. Premixing the enzyme with glucose, the last product of the reaction sequence, reverses the 'slow' burst into a 'slow' lag. Taken together, these results are only compatible with a model that involves the existence of a 'slow' conformational transition of the enzyme.

INTRODUCTION

Apparent positive or negative kinetic co-operativity can be generated by subunit interactions but also through 'slow' conformational changes of an enzyme upon substrate or product binding. The enzymes that display this type of behaviour have been termed hysteretic (Frieden, 1970; Neet & Ainslie, 1980; Cornish-Bowden & Cardenas, 1987). In addition to apparent kinetic co-operativity occurring under steady-state (but not pseudo-equilibrium) conditions, these enzymes may display 'slow' bursts or lags during the pre-steady-state phase (Hatfield *et al.*, 1970; Bearer & Neet, 1978; Frieden, 1979). These lags and bursts must depend on the pre-equilibrium between two free enzyme forms. As this equilibrium depends on both substrate concentration and product concentration, the transient phase should be dependent upon the concentrations of the substrate and of the product.

A β-glucosidase is present in plant cell walls. It is a monomeric enzyme of 70 kDa molecular mass. This enzyme can hydrolyse *p*-nitrophenyl β-D-glucopyranoside. Under acidic pH conditions this hydrolysis process follows Michaelis–Menten kinetics and the reaction is competitively inhibited by glucose, the last product of the reaction sequence. Although the enzyme remains monomeric under all the experimental conditions tested and is able to bind only one molecule of *p*-nitrophenyl β-D-glucopyranoside, the enzyme displays a negative kinetic co-operativity when the pH approaches neutrality (Nari *et al.*, 1984). Since the second substrate of the enzyme reaction is a water molecule, one cannot argue that the deviation observed from the Michaelis–Menten behaviour could be due to the random binding of substrates occurring under steady-state conditions. Therefore this apparent negative co-operativity has been considered as indicative of the existence of a 'slow' conformational transition of the enzyme. As outlined by Neet & Ainslie (1980), 'slow' means 'not fast' with respect to the other reaction steps.

It is thus of interest to obtain new experimental evidence for the existence of a 'slow' conformational transition whose equilibrium is shifted by the substrate or the product of the reaction. This may be done through a kinetic study of the transient phase of the reaction.

MATERIALS AND METHODS

Enzyme purification

p-Nitrophenyl β-D-glucopyranoside was obtained from Sigma Chemical Co. Soyabean cells (*Glycine maximalis* L.) were cultured *in vitro* under sterile conditions and harvested during the exponential growth phase for cell-wall preparation. The β-glucosidase was isolated from these cell walls and purified as described previously (Nari *et al.*, 1982). With respect to the previously published procedure, an additional CM-Trisacryl chromatography proved to be necessary in order to remove a slight contamination of the preparation by invertase. In this step, the gel was equilibrated in 0.01 M-succinate buffer, pH 4.5, and the elution was carried out with a linear gradient of 0.15–0.25 M-succinate. The β-glucosidase was eluted at a concentration of 0.18 M-succinate. The active fractions were concentrated on Diaflo membranes. The enzyme specific activity was about 3300 units (μmol/min)/mg.

Stopped-flow procedure

The enzyme activity was assayed by monitoring the hydrolysis of *p*-nitrophenyl β-D-glucopyranoside and the appearance of *p*-nitrophenol at 400 nm. Stopped-flow kinetic experiments were performed with a Durrum–Gibson device (model D110) connected to a data-storage system and to a Plessey 6622 (PDP 11/23) mini computer. The apparatus is basically as described by Gibson (1969) except for the drive, which is pneumatic (Durrum), and the 20 mm-path-length observation tube. The photomultiplier output is linked through a programmable real-time clock triggered by the stopping syringe, and an analogue-into-digital converter (10 bits, 2049 points full scale) coupled to the PDP 11/23. The reaction was carried out in potassium phosphate buffer, pH 7 and *I* 0.3, at 10 °C. The absorbance change at 400 nm was proportional to the concentration of *p*-nitrophenol produced. Premixing experiments were affected by mixing the enzyme and glucose for 15 min in one of the drive syringes of the stopped-flow apparatus while the substrate was in the other syringe.

Data analysis

In order to eliminate some noise of the progress curve, the data were filtered by using an algorithm based on a convolution

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principle (Savitzky & Golay, 1964). Fitting the experimental results to a theoretical equation was effected through the Newton–Gauss method (Wilkinson, 1961) or the Simplex method (Nedler & Mead, 1965). Computer stimulations were performed with an adapted version, made by M. Bidaud (personal communication), of the KINSIM program of Frieden (Barshop *et al.*, 1983).

THEORY

The experimental results may be interpreted by assuming that the free enzyme exists under two slowly interconvertible forms that are able to perform catalysis at different rates (Scheme 1). This model is a typical specific case of the so-called hysteretic model proposed by Frieden (1979) and later formalized by Neet & Ainslie (1980).

If it is assumed, as proposed by Ainslie *et al.* (1972), that the two catalytic cycles reach a steady state long before the overall steady state is attained, the equation of the progress curve of product appearance assumes the form:

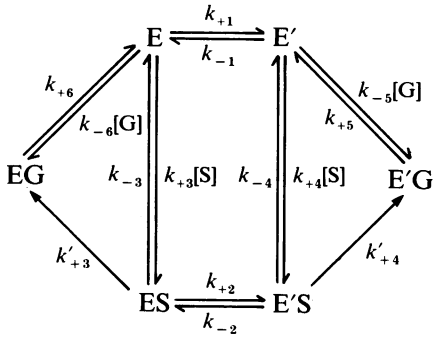
$$\frac{[P]_t}{[E]_T} = \frac{V_s}{[E]_T}t + \psi(1-e^{\lambda t}) \qquad (1)$$

where V_s is the steady-state rate, $[E]_T$ is the total enzyme concentration, $[P]_t$ is the product concentration at time t, ψ is the amplitude of the transient and, as already outlined, λ is its time constant. ψ may take positive or negative values depending on whether the reaction displays a burst or a lag. Under these conditions the approach to the steady state occurs in one exponential only. Moreover, the time constant of this exponential as a function of substrate concentration assumes the form:

$$\lambda = \frac{\alpha_0 + \alpha_1[S] + \alpha_2[S]^2}{\beta_0 + \beta_1[S] + [S]^2} \qquad (2)$$

where α_0, α_1, α_2, β_0 and β_1 are groupings of rate constants (Table 1) and [S] is the substrate concentration. The hysteretic model and the above equation may thus predict the existence of a maximum value of the time constant, λ, for a particular substrate concentration.

It is important to stress that monitoring the formation of *p*-nitrophenol may result in the appearance of a burst if *p*-nitrophenol is released first and glucose afterwards. This may indeed occur in the absence of any enzyme hysteresis. In that case, however, the time constant of the 'slow' exponential should reach a plateau as the substrate concentration is increased.



Scheme 1. Hysteretic model for the β-glucosidase

The enzyme exists under two conformational states E and E′ connected through a 'slow' transition. S and G stand respectively for *p*-nitrophenyl β-D-glucopyranoside and glucose.

Table 1. Significance and values obtained of the kinetic parameters of eqn. (2)

k and k' correspond to $(k'_{+3}+k_{-3})/k_{+3}$ and $(k'_{+4}+k_{-4})/k_{+4}$ respectively. The parameter values are given as means ± S.D.

	Kinetic parameters	Parameters values
α_0	$kk'(k_{+1}+k_{-1})$	0.034 ± 0.002
α_1	$k(k_{-2}+k_{+1})+k'(k-1+k_{+2})$	11.6 ± 2.2
α_2	$k_{+2}+k_{-2}$	(3.44 ± 0.55) × 10^{-3}
β_0	kk'	0.376 ± 0.034
β_1	$k+k'$	1.23 ± 0.41

RESULTS

Kinetics and analysis of the progress curves

Progress curves of *p*-nitrophenyl β-D-glucopyranoside hydrolysis monitored at pH 7 are shown in Fig. 1. Whatever the substrate concentration, the reaction displays a 'slow' burst before the steady state is reached. The duration of this burst is about 1 s.

Within the time scale of these experiments, the burst phase may be fitted to one exponential only (Fig. 2). This does not mean, of course, that a 'fast' exponential term does not exist in addition to the 'slow' one.

The time constant, λ, of this 'slow' exponential varies as the substrate concentration is varied, as shown in Fig. 3(*a*), but is independent of the enzyme concentration (Fig. 3*b*).

Premixing experiments

Probably one of the most efficient ways to test the reality of a hysteretic conformational transition of an enzyme is to perform premixing experiments in which this enzyme is premixed with the last product of the reaction (Ricard *et al.*, 1977; Meunier *et al.*, 1979). If the burst observed at the beginning of the reaction were

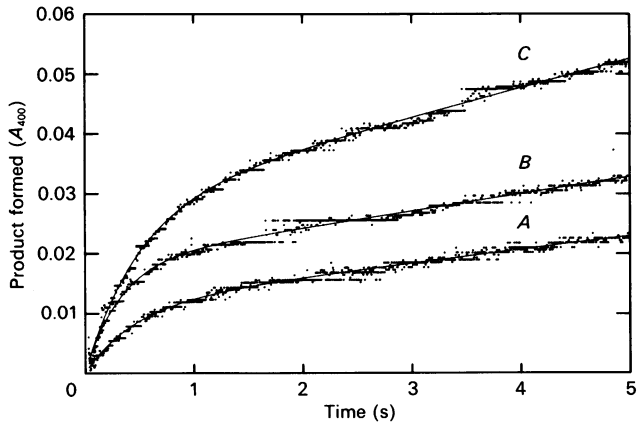


Fig. 1. Pre-steady-state kinetics of β-glucosidase

The progress curves were obtained by mixing the enzyme and the substrate in the stopped-flow device (phosphate buffer, pH 7 and I 0.3, at 10 °C). The enzyme concentration was 4.94 μM. The substrate concentrations (after mixing) were 0.1 mM (curve *A*), 2 mM (curve *B*) and 5 mM (curve *C*). The points are experimental, and the continuous lines are theoretical and correspond to eqn. (1) (see the text).

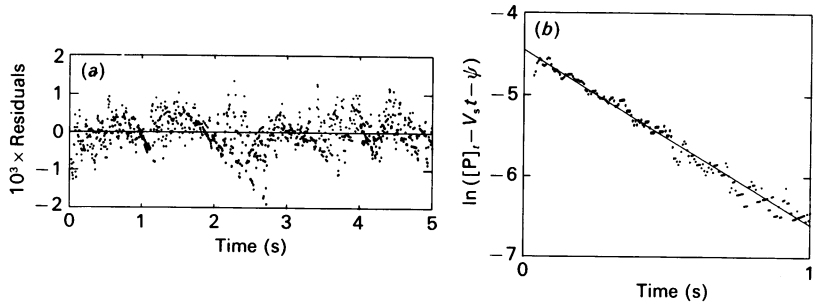


Fig. 2. Fit of the results to a single-exponential function

(*a*) Distribution of residuals. The data of the progress curve of Fig. 1 (curve *B*) were filtered by using a convolution method (see the Materials and methods section). Then the curve was adjusted to a single exponential. The difference between the theoretical and the experimental values (residuals) is plotted as a function of time. This fit to a single exponential leads to a random distribution of the residuals. (*b*) Semi-logarithmic plot of the progress curve. This shows a semi-logarithmic plot of $([P]_t - V_s t - \psi)$ versus time for curve *B* of Fig. 1. The plot is linear over 1 s. The slope of this plot is equal to $-\lambda$ (the value of λ is 2.148 s^{-1}) and the extrapolated value at $t = 0$ corresponds to $\ln\psi$.

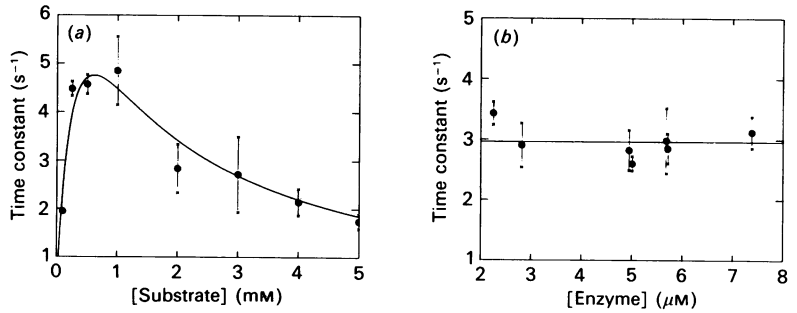


Fig. 3. Analysis of the transient

(*a*) Variation of the time constant as a function of the substrate concentration. The experimental conditions were identical with those described in Fig. 1 legend. The points are the means of several experimental results and the error bars are S.E.M. values ($n = 8$). The line shows the best non-linear least-squares fit of results to eqn. (2) (see the text). The values of the fitted parameters are given in Table 1. The parameters obtained allow us to calculate some grouping rate constants: $k_{+2}+k_{-2} = 3.44 \times 10^{-3}\,s^{-1}$, $k_{+1}+k_{-1} = 9.05 \times 10^{-2}\,s^{-1}$, $k = 0.65\ mM^{-1}$ and $k' = 0.578\ mM^{-1}$ (see Table 1). (*b*) Variation of the time constant versus the enzyme concentration. The substrate concentration was 2 mM, the pH of the reaction was 7 (phosphate buffer) and the ionic strength was 0.3. The temperature was 10 °C. The error bars are S.E.M. values ($n = 3$). In these conditions the half-time of the transition, calculated by the expression $t_{\frac{1}{2}} = 0.69/\lambda$, had a mean value of 234 ± 18 ms ($n = 18$) and was independent of the enzyme concentration.

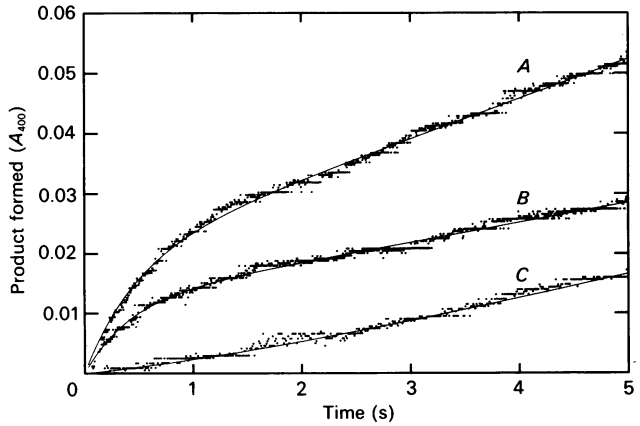


Fig. 4. Glucose effects on the transient kinetics

In all experiments the β-glucosidase concentration was equal to 2.84 μM after mixing in syringe 1. Curve *A*: the enzyme (syringe 1) was mixed with 5 mM substrate (syringe 2); this curve is the same as curve *C* shown in Fig. 1. Curve *B*: mixing of the enzyme (syringe 1) with substrate (5 mM) and glucose (2.5 mM) already premixed in syringe 2. Curve *C*: premixing of the enzyme with glucose; glucose (2.5 mM) was incubated with the enzyme (syringe 1) for 15 min; the syringe 2 contained only the substrate (5 mM). The final concentrations after mixing of all the reactants are the same for curves *B* and *C*, and the steady-state progress curve is then the same for these two experiments.

due to the existence of the hysteretic transition, this would imply that the free enzyme normally exists mostly in its more active form. If the less active form is stabilized by the product, premixing of the enzyme with that product should reverse the burst into a lag. If the β-glucosidase is mixed with both its substrate and glucose (the last product), the reaction displays a burst. If it is premixed with glucose and then mixed with its substrate, the time course of the reaction exhibits a lag. If, as shown in Fig. 4, one manages to obtain identical reactant (substrate and glucose) concentrations after mixing, regardless of the order of mixing of these reactants, the steady-state rate of product appearance is the same in both cases, but premixing the β-glucosidase with glucose results in the reversal of the burst into a lag.

DISCUSSION

Most experimental studies devoted to hysteretic enzymes have been performed through the analysis of the deviations displayed from steady-state Michaelis–Menten kinetics. In fact the quantitative study of the transient phase and its dependence upon the substrate concentration, as well as premixing experiments with the last product of the reaction sequence, offer a very efficient tool for the demonstration of the occurrence of 'slow' conformation changes that occur in the time course of the enzyme reaction.

The aim of this paper was thus to study the transient phase

of a cell-wall β-glucosidase. This enzyme was chosen because, although monomeric and apparently having one site for its substrate, it displays a negative kinetic co-operativity, which appears to be due to enzyme hysteresis (Nari *et al.*, 1984). As expected, the reaction exhibits a 'burst' of *p*-nitrophenol of about 1 s when the enzyme is rapidly mixed with its substrate. In the time scale investigated, this burst may be resolved in only one exponential, whose time constant reaches a maximum and then decreases as the substrate concentration is increased. This type of behaviour is compatible with the hysteretic model, but does not match the view that the burst is solely due to a 'slow' hydrolysis of a glycosyl-enzyme complex.

Premixing the β-glucosidase with glucose, i.e. the last product of the reaction sequence, should shift the equilibrium between the two free enzyme forms and should therefore alter the transient phase. This is what has been observed experimentally. Premixing the enzyme with glucose results in a reversal of a burst into a lag.

The results that have been presented in this paper are thus compatible with the hysteretic model of Scheme 1. Admittedly this does not prove that other models of a similar type could not fit these results equally well, but in any model the presence of a 'slow' transconformation step appears to be necessary in order to take account of these experimental results.

The β-glucosidase that was studied is specifically bound to plant cell walls and probably plays a role in the extension of the external envelope of the cell. One may therefore speculate whether the 'slow' conformational transition of the enzyme is altered thanks to its association with the wall, and whether this 'slow' transition has any physiological role. It is impossible to answer these questions at the moment.

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