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pH-induced kinetic co-operativity of a thylakoid-bound polyphenol oxidase

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A study of the catecholase activity of a latent plant polyphenol oxidase, extracted and purified from the chloroplast membranes of grapes (*Vitis vinifera* cv. Airen), revealed for the first time a lag phase above pH 5.0, whereas a steady-state rate was reached immediately when pH values were lower, thus suggesting the hysteretic nature of the enzyme. During steady state, the enzyme showed negative co-operativity concomitant with the presence of the lag period, and followed classical Michaelis–Menten kinetics under more acid pH conditions. Statistical analysis of these data showed a minimal value for the extreme Hill coefficient of 0.54 at pH 6.0. This kinetic behaviour of polyphenol oxidase has been interpreted in terms of the pH-induced 'slow' transition mechanism reported by Ricard, Noat & Nari [(1984) Eur. J. Biochem. **145**, 311–317] in which the conformational change does not affect the active site of the enzyme.

INTRODUCTION

It has been shown that many enzyme systems show a time-dependent behaviour that is related to 'slow' conformational changes occurring under non-equilibrium conditions, and which may play a role in the regulation of competing metabolic pathways. These enzymes, which were termed 'hysteretic' (Frieden, 1970), may display kinetic cooperativity in the steady state with appropriate values of the rate constants (Ainslie *et al.*, 1972).

An important example of this type of kinetic co-operativity occurs when the hysteresis is induced by pH changes in the medium. A theoretical kinetic model was developed by Ricard *et al.* (1984) in order to account for this phenomenon and was useful in explaining the pH response of enzymes bound to cell envelopes. Thus the model was applicable to the kinetic study of the behaviour of a plant-cell-wall β-glycosyltransferase (Nari *et al.*, 1984) and of a fructose 1,6-bisphosphatase obtained from spinach (*Spinacia oleracea*) chloroplasts (Gontero *et al.*, 1984). The main assumption of the model is that the 'slow' transition does not affect the active site of the enzyme, but rather another region of the protein that has an ionizable group, suggesting that co-operativity must be suppressed at 'extreme' pH values.

Polyphenol oxidase (EC 1.14.18.1) is widely distributed in Nature and is mainly involved in the biosynthesis of melanins in animals and in the browning phenomenon in plants. It catalyses both the *o*-hydroxylation of monophenols (cresolase activity) and the oxidation of *o*-diphenols to *o*-quinones (catecholase activity). That polyphenol oxidase exists in a latent state has been reported in both animals (Ashida, 1971; Galindo *et al.*, 1983) and plants (Mayer & Harel, 1979), and its activation is thought to play a potentially important role in the regulation of pigment formation.

The latent enzyme has been shown to be activated *in vitro* by exposure to acid or basic pH, the process being accompanied by a change in the electrophoretic mobility of the enzyme, as well as by a change in the Stokes' radius of the protein, which suggests the involvement of conformational changes during activation (Lerner *et al.*, 1972; Lerner & Mayer, 1975). Such changes may be part of a mechanism controlling polyphenol oxidase activity.

The aim of the present paper was to determine the kinetics as a function of pH of a polyphenol oxidase, extracted and purified in a latent state from chloroplast thylakoid membranes of grapes (*Vitis vinifera*, cv. Airen). The results obtained show that the activation of the enzyme by pH changes in the medium may be classified as a hysteretic response.

MATERIALS AND METHODS

Materials

4-t-Butylcatechol (4tBC) was from Aldrich and used without further purification. Triton X-114 was obtained from Fluka AG (Buchs, Switzerland) and condensed as described by Bordier (1981) by using 100 mM-sodium phosphate buffer (pH 7.3). The detergent phase of the third condensation had a Triton X-114 concentration of 25% (w/v) and was used as the stock solution of detergent for all the experiments.

Methods

Polyphenol oxidase was extracted in its latent state from chloroplast membranes of grapes. Chloroplasts were isolated as previously described (Lerner *et al.*, 1972). A 50 g portion of grapes in 25 ml of 100 mM-phosphate buffer, pH 7.3, containing 10 mM-sodium ascorbate, was homogenized in a blender for 15 s, filtered through eight layers of gauze, and centrifuged at 4000 *g* for 15 min.

The precipitate thus obtained was extracted with 20 ml of 1.5% (w/v) Triton X-114 in 100 mM-sodium phosphate buffer, pH 7.3, for 30 min at 4 °C. After high-speed centrifugation (60000 *g* for 15 min), this dark-green extract yielded a slight green supernatant with polyphenol oxidase activity. This was subjected to temperature phase partitioning by adding Triton X-114 at 4 °C to give a final concentration of 4% (w/v). The mixture was kept at 4 °C for 15 min and then warmed to 35 °C. After 10 min, the solution became spontaneously turbid owing to the formation, aggregation and precipitation of large mixed micelles of detergent, which contained hydrophobic proteins and the remaining chlorophylls. This solution was centrifuged at 5000 *g* for 10 min at room temperature (Sánchez-Ferrer *et al.*, 1989). The clear supernatant thus obtained, after dialysis against

Abbreviation used: 4tBC, 4-t-butylcatechol.

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1 mM-sodium phosphate buffer, pH 7.3, was used as latent enzyme source. Leupeptin and *N*-ethylmaleimide were added before and after dialysis to give final concentrations of 0.04 and 5 mM respectively, in order to avoid any possible activation of the enzyme by endogenous proteinases.

The catecholase activity of the enzyme was determined at 25 °C by spectrophotometrically monitoring, at 400 nm, the appearance of the *o*-benzoquinone product of the reaction (ϵ 1150 $M^{-1} \cdot cm^{-1}$) (Waite, 1976). The steady-state rate (V_s) was defined as the slope of the linear zone of the product accumulation curve. The lag period (L) was estimated by extrapolation of the linear portion of the product accumulation curve to the abscissa. Unless otherwise stated, the reaction media contained 4.5 mM-4tBC at the indicated pH in 50 mM-sodium acetate (pH 3.5–5.5) or phosphate (pH 5.5–7.0) buffers and 0.026 unit of enzymic activity, in a final volume of 1 ml. The reaction was started by the addition of the enzyme.

One unit of enzyme activity was taken as the amount that produces 1 μmol of 4-t-butyl-*o*-benzoquinone/min as measured at pH 3.5 under the above experimental conditions.

Oxygen consumption throughout the time course of the reaction was monitored with an oxygen electrode (Rank Brothers Co.).

SDS/PAGE was performed as described by Angleton and Flurkey (1984), using the method of Laemmli (1970). The SDS concentration was 0.1 %, conditions under which the enzyme remains active, allowing the detection of different enzyme forms of polyphenol oxidase (Angleton & Flurkey, 1984; Sánchez-Ferrer *et al.*, 1989). Samples were mixed with glycerol and Bromophenol Blue before being applied to 7.5% (w/v) polyacrylamide gels. Electrophoresis was carried out for 6 h at room temperature. Gels were stained for polyphenol oxidase activity in 100 ml of sodium acetate buffer, pH 4.5, containing 5 mM-L-dopa (3,4-dihydroxyphenylalanine) as substrate.

The rate data at steady state versus substrate concentration were initially analysed graphically by double-reciprocal plots (Neet, 1980). Those showing linearity were fitted to the Michaelis–Menten equation. In the case of double-reciprocal plots with downwards-concave lines, the steady-state rates were fitted to eqn. (1) from the Results and Discussion section. Statistical analyses were carried out by fitting the data by non-linear regression using the program 3R from the BMD (Los Angeles, CA, U.S.A.) statistical package. Initial estimations of the parameters for data fitting were calculated graphically from the double-reciprocal plots as described by Neet (1980). Residual S.D. values thus obtained were within the range 7.79×10^{-4}–1.16×10^{-2} μM/s.

RESULTS AND DISCUSSION

The measurement of the catecholase activity of latent grape polyphenol oxidase as a function of pH indicated an optimum pH of 4.7 (Fig. 1*a*), above which the activity sharply decreased, these results being in agreement with those obtained with the active enzyme purified at the same pH by $(NH_4)_2SO_4$ fractionation (Valero *et al.*, 1988). Furthermore, above pH 5.0, the progress curve of the reaction showed a lag phase and then became a straight line, whereas under more acid pH conditions the rate of the reaction reached its steady-state value immediately (Fig. 1*b*, curves a and b). The length of the lag period showed a bell-shaped curve as a function of pH, displaced with respect to that of the activity (Fig. 1*a*), the maximum being observed at pH 5.5.

The appearance of a lag period in the expression of the catecholase activity of polyphenol oxidase is a phenomenon that has never been previously described. It was not an artifact of the enzyme assay, as was demonstrated by measuring enzymic activity with other *o*-diphenolic substrates and also by monitoring oxygen consumption during catalysis (results not shown).

This response of the enzyme to pH changes in the medium is a characteristic property of a hysteretic enzyme undergoing slow transition to another kinetically different form during catalysis (Frieden, 1970; Neet & Ainslie, 1980). The lag observed in a hysteretic enzyme can be abolished by preincubation with ligand (protons in this case), which causes the slow transition (Neet & Ainslie, 1980). The results presented in Fig. 1(*b*) (curve c) show that the lag observed in the expression of the catecholase activity of latent polyphenol oxidase was indeed abolished on preincubating the enzyme with a sufficient amount of acetic acid. Thus this lag period may be caused by slow pH-induced conformational changes in the enzyme to a catalytically more active form. This suggestion is in agreement with the above-mentioned one of Lerner *et al.* (1972) and Lerner & Mayer (1975).

Computer simulation has shown that, in those cases where a slow transition between enzyme forms occurs, co-operativity can be expected in the steady-state kinetics (Ainslie *et al.*, 1972). Figs. 2(*a*) and 2(*b*) slow Lineweaver–Burk double-reciprocal plots of

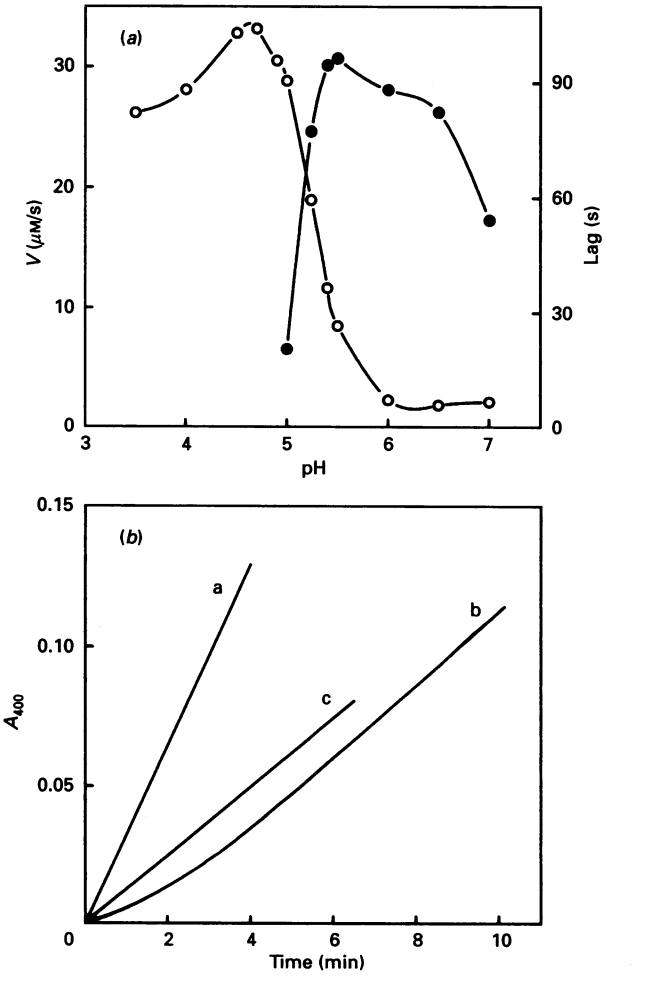

Fig. 1. (*a*) Effect of pH on the catecholase activity of latent grape polyphenol oxidase and (*b*) time course of catecholase activity of latent grape polyphenol oxidase at pH 4.0 (curve a) and 5.4 (curves b and c)

In (*a*) the open circles represent the steady-state rates and the closed ones represent the length of the lag period. In (b), curve c, the enzyme was previously preincubated at pH 3.3 at 25 °C with 50 mM-acetic acid for 10 min.

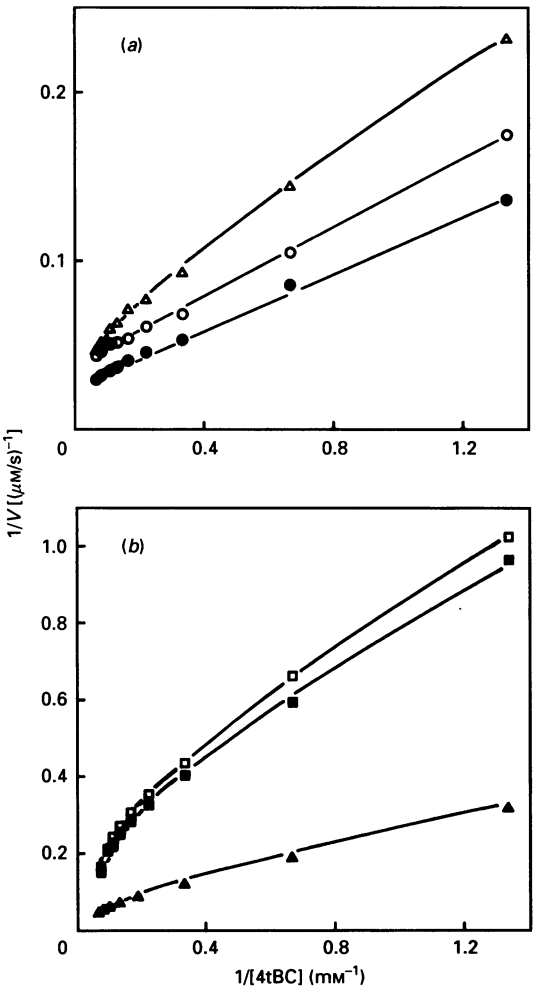

Fig. 2. Double-reciprocal plots of steady-state rates versus 4tBC concentration at several pH values

The following pH values are plotted, with the indicated enzyme concentrations: ○, pH 3.5, 0.016 unit of enzyme activity; ●, pH 4.0, 0.016 unit; △, pH 5.0, 0.008 unit; ▲, pH 5.4, 0.016 unit; □, pH 6.0, 0.032 unit; ■, pH 6.5, 0.048 unit.

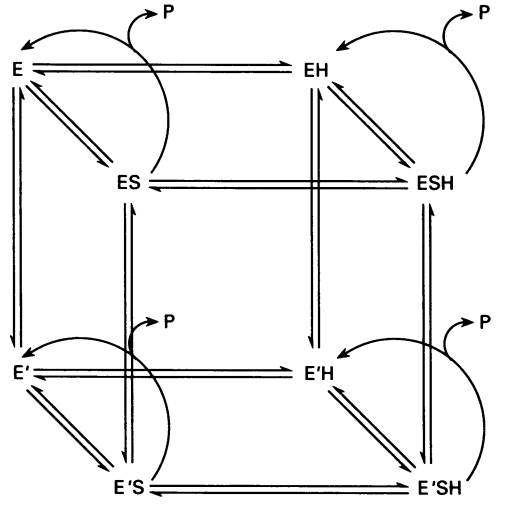

Fig. 3. A hysteretic model which shows how pH may influence co-operative effects

E, enzyme in one conformation; E', enzyme in another conformation; S, substrate; P, product. The isomerization steps (E → E', with or without substrate and with or without protons) are 'slow' in comparison with the other steps in the mechanism.

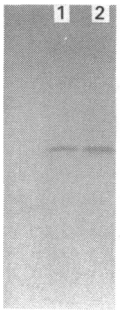

Fig. 4. Electrophoresis and enzyme staining of Airen-grape polyphenol oxidase

Lane 1, latent enzyme; lane 2, activated enzyme (the latent enzyme) was passed through a trypsin (an activator of polyphenol oxidase)–CNBr-activated Sepharose 4B column.

steady-state rates against 4tBC concentration at several pH values. It can be seen that, under acid pH conditions, where the activity did not display a lag period, polyphenol oxidase exhibited classical Michaelis–Menten kinetics. However, as the pH was raised, the enzyme displayed negative co-operativity (see also Fig. 5 below).

This kinetic behaviour of polyphenol oxidase, where the appearance and disappearance of the lag period in an intermediate pH range and at 'extreme' pH values are concomitant with the appearance and disappearance respectively of co-operativity, is consistent with the kinetic model of pH-induced co-operativity for hysteretic enzymes reported by Ricard *et al.* (1984). The simplest formulation of this model for a one-substrate one-product enzyme is shown in Fig. 3. The basis of the model is that co-operativity does not arise from the existence of two different conformations of the active site, but rather from the ionization, or the protonation, of a group located outside the active site, in a different domain of the protein, which undergoes the conformational transition.

The equation that describes the dependence of the steady-state rate on the rate constants and substrate concentration pertaining to this model is of the form (Ainslie *et al.*, 1972; Ricard *et al.*, 1984):

$$V = \frac{c[S]^2 + d[S]}{1 + a[S]^2 + b[S]} \quad (1)$$

where V is the steady-state rate and the parameters a, b, c and d are combinations of the rate constants from the model. This equation is also obtained by combining two Michaelis–Menten terms. Therefore it remained to be established that only one isoenzyme of polyphenol oxidase was present in the extract. To do this, electrophoretic analysis was performed using the method of Angleton & Flurkey (1984) to detect polyphenol oxidase isoenzymes, and the presence of only one isoenzyme was revealed in the extract (Fig. 4), both in its latent state and activated by trypsin. Thus co-operativity expressed by the enzyme does not seem to be correlated with the presence of two or more isoenzymes. Co-operativity and hysteresis may therefore be explained as a reflection of the model of slow isomerization mediated by pH shown in Fig. 3, in accordance with Ainslie *et al.* (1972). Steady-state rates obtained at the different pH values by varying the substrate concentration in the reaction medium were

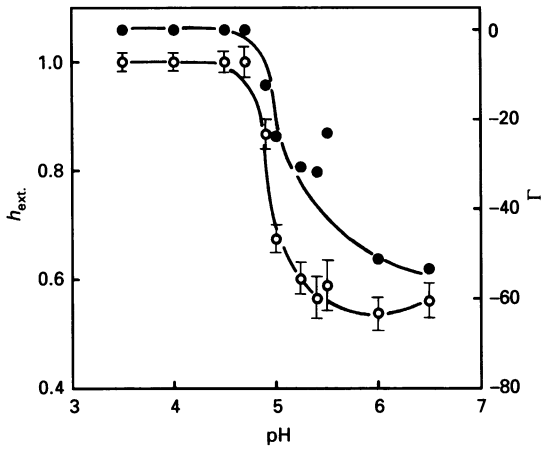

Fig. 5. Variation of the extreme Hill coefficient ($h_{ext.}$) (○) and of the Γ parameter (●) as a function of pH

Experimental conditions are as in Fig. 2.

fitted by non-linear regression to eqn. (1), and good agreement was obtained.

The most widely used ways to express the extent of kinetic co-operativity of an enzyme system that follows a rate equation of this type involve the use of the maximum or minimum Hill coefficient ($h_{ext.}$) and the so-called Γ parameter:

$$\Gamma = \lim_{[S]\to\infty} d^2(V^{-1})/d([S]^{-1})^2$$

(Neet, 1980; Ricard *et al.*, 1984; Ricard & Cornish-Bowden, 1987)

whose expressions as a function of the kinetic parameters from eqn. (1) are the following:

$$h_{ext.} = \frac{2\{cd + c^2[d/(bc-ad)]^{\frac{1}{2}}\}}{2cd + [c^2 + d(bc-ad)][d/(bc-ad)]^{\frac{1}{2}}} \quad (2)$$

$$\Gamma = \frac{2[c^2 - d(bc-ad)]}{c^3} \quad (3)$$

Fig. 5 shows the values of $h_{ext.}$ and those of the Γ parameter obtained when the sets of parameters *a*, *b*, *c* and *d* at each pH, estimated by non-linear least-squares fitting of the rate data from Fig. 2 to eqn. (1), were substituted in these expressions (eqns. 2 and 3). It can be seen by the $h_{ext.}$ index that the apparent kinetic co-operativity was minimal for pH 5.5 and 6.0. The Γ parameter showed a similar dependence, although the minimum was displaced towards higher pH values, since it expressed the degree of curvature of the double-reciprocal plot at infinite substrate concentration, which is not necessarily the substrate concentration associated with the greatest co-operativity. At pH values above 6.5 it is difficult to monitor the 4-t-butyl-*o*-benzoquinone spectrophotometrically because of its instability, and therefore kinetics for these pH values could not be determined.

These results are in good agreement with the existence of a slow conformational transition of polyphenol oxidase during its catalytic action, induced by a pH change in the medium, and which does not affect the active site of the enzyme (Ricard *et al.*, 1984), but which is responsible for the kinetic co-operativity observed in the steady state.

The kinetic behaviour as a function of pH reported here for polyphenol oxidase has probably not been previously described, since most kinetic studies have been performed with the enzyme extracted from acetone-dried powders or purified by means of $(NH_4)_2SO_4$ fractionation, and these two agents have been shown to be capable of activating or modifying the enzyme (Mayer & Harel, 1979; Golbeck & Cammarata, 1981). The latency of polyphenol oxidase is a well-known phenomenon (Mayer & Harel, 1979; Vaughn & Duke, 1984), and the enzyme has been activated *in vitro* by many different treatments, although the mechanism by which polyphenol oxidase activity is regulated *in vivo* remains unknown. The kinetic behaviour presented here may help in clarifying the activation mechanism of the enzyme within the cell.

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