Hysteresis and Cooperative Behavior of a Latent Plant Polyphenoloxidase

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ABSTRACT

Appearance of a lag period dependent on pH in the expression of the catecholase activity of a polyphenoloxidase extracted in a latent state from Airen grape (Vitis vinifera L.) berries, is revealed, suggesting the hysteretic nature of the enzyme. The lag time was independent of enzyme concentration, indicating that slow pH-induced conformational changes in the protein must occur during assay. Results obtained by varying substrate concentration show that the system presents hyperbolic or cooperative kinetics depending on the pH of the assay.

PPO2 (EC 1.14.18.1.) is a copper-containing monooxygenase widely distributed in nature that is responsible for melanization in animals and browning in plants. The enzyme catalyzes two distinct reactions involving molecular oxygen, namely (a) the o-hydroxylation of monophenols to o-diphenols, or cresolase activity, and (b) the subsequent oxidation of o-diphenols to o-quinones, or catecholase activity.

PPO is located in the chloroplast thylakoid membranes (10), and in some species it exists in a latent form; the latent enzyme was recently isolated and characterized from broad bean (11). A new method has been developed that permits the extraction of PPO in this latent state by using temperature-induced phase separation in Triton X-114 (14). The latent enzyme can be activated by different treatments such as trypsin (14, 17), fatty acids (4), aging (8), acid and base shock (5, 6), detergents (11, 15), and cations (16). As regards activation of the enzyme by pH changes in the medium, this phenomenon was first studied by Kenten (5) in Vicia faba, in which he ascribed the process to the removal of an inhibitory protein attached to the enzyme. More recently, Lerner et al. (6) found irreversible activation of the enzyme from grape berries following long exposure to acid pH, and Lerner and Mayer (7) further showed that the process was accompanied by a change in the Stokes' radius of the protein, indicating the involvement of a conformational change.

It has been shown that some enzymes located in cell envelopes display different kinetic behavior depending on the pH of the medium, which may represent a regulatory device operative in vivo (3). Furthermore, a kinetic model that explains pH-induced cooperative effects in hysteretic enzymes has been proposed to account for this behavior of enzymes attached to biological polyelectrolytes (13). The basis of this model is that, upon ionization or protonation of a strategic ionizable group, the protein undergoes a “slow” conformational transition.

In the present paper, the appearance of a lag period in the expression of catecholase activity of a PPO is reported for the first time. The enzyme shows a hysteretic nature and cooperative kinetics depending on the pH of the assay, these results being consistent with the above mentioned kinetic model (13).

MATERIALS AND METHODS

Materials

4-tert-Butylcatechol was purchased from Aldrich and used without further purification. Triton X-114 was obtained from Fluka AG (Bucks, Switzerland) and condensed as described by Bordier (2) by using 100 mm sodium phosphate buffer (pH 7.3). The detergent phase of the third condensation had a concentration of 25% Triton X-114 (w/v) and was used as the stock solution of detergent for all the experiments.

Methods

Airen grape berries (Vitis vinifera L.) used in this study were harvested and processed as previously described (18). PPO was extracted from grape berries in a latent form by using temperature-induced phase separation in Triton X-114 at pH 7.3 (14). The solution thus obtained, after dialysis against 1 mm sodium phosphate buffer (pH 7.3), was used as enzyme source. To avoid any possible activation of the enzyme by endogenous proteases, leupeptin and N-ethylmaleimide were added before and after dialysis to give a final concentration of 0.04 and 5 mm, respectively. This did not significantly affect polyphenoloxidase activity.

The catecholase activity of the enzyme was determined at 25°C by following spectrophotometrically at 400 nm the appearance of the o-benzoquinone product of the reaction.

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2 Abbreviation: PPO, polyphenoloxidase.
steady state was defined as the slope of the linear zone of the product accumulation curve. The lag period was estimated by extrapolation of the linear portion of the product accumulation curve to the abscissa axis. Unless otherwise stated, the reaction media contained 4.5 mM 4-tert-butylocatechol at the indicated pH in 50 mM sodium acetate (pH 3.5–5.3) or sodium phosphate (pH 7.0) buffers and 0.016 units of enzymatic activity, in a final volume of 1 mL. One unit of enzyme activity is the amount of enzyme that produces 1 μmol of 4-tert-butyl-o-benzoquinone/min as measured at pH 3.5 under the above experimental conditions.

SDS-PAGE was carried out as described by Angleton and Flurkey (1). Samples were mixed with glycerol and bromphenol blue before being applied to 7.5 or 12.5% polyacrylamide gels. Electrophoresis was carried out for 6 h at room temperature. Gels were stained for PPO activity in 100 mL of 10 mM sodium acetate buffer (pH 4.5) containing 5 mM L-dopa.

RESULTS AND DISCUSSION

When catecholase activity of latent grape PPO was assayed at pH 3.5, a steady-state rate was immediately attained (Fig. 1, curve a), in accordance with the results obtained with the active enzyme purified at the same pH by (NH₄)₂SO₄ fractionation (18). However, when the assay was performed at pH 5.3, the enzyme activity increased with time, reaching a steady-state after a discernible lag phase (Fig. 1, curve b). This lag period in the expression of catecholase activity of PPO has never been previously reported, and neither has any slow transition phenomenon affecting catecholase activity been described. This cannot be an artifact of the enzyme assay because the reaction is followed by measuring the appearance of the first product of catalytic activity, 4-tert-butylo-benzoquinone, which is very stable (19). The presence of only one catecholase enzyme was indicated by gel electrophoresis.

This response of the enzyme to pH changes in the medium is a characteristic property of a hysteretic enzyme undergoing slow transition to another form kinetically different during catalysis (12). The lag observed in a hysteretic enzyme can be abolished by preincubation with ligands that cause the slow transition (for example, protons in this case) (12). The results presented in Figure 2 (curve a) show that the lag observed in the expression of catecholase activity of latent PPO when the assay is performed at pH 5.3 was indeed abolished on preincubating the enzyme with a sufficient amount of acetic acid; in addition, a progress curve in the opposite direction, i.e., a burst in this case, is seen when, after preincubation with the ligand responsible for the slow transition, the enzyme is returned back to its previous experimental conditions (Fig. 2, curve b). The reaction rate decreases with time, reaching a steady-state rate lower than the one seen before (Fig. 2, curve a). This indicates slow pH-induced conformational changes in the enzyme to a catalytically less active form. We can also see that the process is reversible, because on adding new
Figure 4. Effect of substrate concentration on steady-state rates expressed at pH 3.5 (●) and 5.3 (○), and on the lag period that shows catecholase activity of the enzyme at pH 5.3 (△).

amounts of acetic acid to the medium after the steady-state has been reached, the activity recovered (Fig. 2, curve c). We then tested this behavior of latent PPO by measuring the lag time as a function of enzyme concentration. It was found that lag period remained constant and steady-state rates were linear with enzyme concentration (Fig. 3), indicating that the hysteresis observed is not due to oligomerization of the enzyme and must be due to pH-induced isomerization of the enzyme to another form that has different catalytic activity.

Another interesting aspect of study is the dependence of the lag period and steady-state rates upon substrate concentration. Figure 4 shows that lag period observed at pH 5.3 decreased when 4-tert-butylcatechol concentration was increased in the reaction medium. With respect to catalytic activity, it can be seen that, whereas at pH 3.5 the enzyme followed hyperbolic kinetics with an apparent K_m for the o-diphenolic substrate of 2.5 mM, at pH 5.3 it exhibited kinetic cooperativity. These results were fitted to the Hill equation  

\[ v = \frac{V_{\text{max}}[S]^n}{K_h + [S]^n} \]

by using nonlinear regression (9), a Hill coefficient of 0.74 being obtained. This result is consistent with the kinetic model described by Ricard et al. (13) to explain pH-induced cooperative effects of hysteretic enzymes, and which was believed to account for some pH responses of enzymes attached to biological membranes. The basis of this model is that cooperativity arises from the ionization or protonation of a group located outside the active site in a domain of the protein that undergoes the "slow" conformational transition.

Hysteretic enzymes are important in metabolic regulation. The pH response of latent PPO shown in this paper may represent a mechanism of regulation of its activity in vivo.

LITERATURE CITED