A kinetic study of irreversible enzyme inhibition by an inhibitor that is rendered unstable by enzymic catalysis

The inhibition of polyphenol oxidase by L-cysteine

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A kinetic study of the irreversible inhibition of an enzyme by an inhibitor that is depleted in the medium by its reaction with the product of enzymic analysis was made. The model is illustrated by the study of the inhibition of catecholase activity of polyphenol oxidase by L-cysteine. The inhibition is characterized by an initial lag period followed by a concomitant decrease in enzymic activity expressed when the steady state is reached, both kinetic parameters being modulated by enzyme, substrate and inhibitor concentrations. There is no analytical solution to the non-linear differential-equation system that describes the kinetics of the reaction, and so computer simulations of this dynamic behaviour are presented. The results obtained show that the system here studied presents kinetic co-operativity for a target enzyme that follows the simple Michaelis–Menten mechanism in its action on the substrate.

INTRODUCTION

Polyphenol oxidase (monophenol mono-oxygenase; monophenol, dihydroxy-L-phenylalanine: oxygen oxidoreductase, EC 1.14.18.1.) is a widely distributed copper-containing protein responsible for melanization in animals and browning in plants. The enzyme catalyses two different reactions, both using molecular oxygen: cresolase activity, or *o*-hydroxylation of monophenols to *o*-diphenols, and catecholase activity, or oxidation of *o*-diphenols to their corresponding *o*-quinones. The quinones thus formed are very reactive with nucleophilic agents (Mason & Peterson, 1965; Agrup *et al.*, 1982; García-Carmona *et al.*, 1988; Valero *et al.*, 1988a) and have been shown to react with thiols by a fast non-enzymic reaction to form more stable adducts (Bouchilloux & Kodja, 1960; Sanada *et al.*, 1972). Thus cysteine is known to be involved in pheomelanin synthesis via cysteinyl-dopas (Prota, 1972, 1980; Ito & Prota, 1977; Ito *et al.*, 1988), the stoichiometry of the cysteinyl-dopa formation process having been well established (Jiménez *et al.*, 1986). However, the direct effect of these thiol compounds on polyphenol oxidase has always been the subject of controversy. In previous studies on polyphenol oxidase, several researchers (Lerner & Fitzpatrick, 1950; Lerner *et al.*, 1950; Dawson & Magee, 1965) reported that thiols could inhibit the enzyme by combining with the copper in the active site. However, it was generally assumed that their major effect was due to the formation of these thiol-conjugated reaction products rather than to the inhibition of enzymic activity (Seiji *et al.*, 1969; Prota, 1980).

The aim of the present paper was to make kinetic analysis of systems where the irreversible enzyme inhibitor is consumed by its reaction with the product of catalytic activity, illustrated by the kinetic study of the inhibition of catecholase activity of polyphenol oxidase by L-cysteine. According to the results obtained we have developed a kinetic model for this system based on the mechanism proposed for enzymic catalysis by polyphenol oxidase (Mason, 1957; Galindo *et al.*, 1983; Cabanes *et al.*, 1987).

KINETIC ANALYSIS

Notation and definitions are basically the same as detailed in the Appendix for a simple Michaelis–Menten mechanism.

The experimental data obtained here are qualitatively consistent with the mechanism shown in Scheme 1:

Scheme 1

where the inhibitor (I) binds to an intermediate form of the catalytic cycle of catecholase activity of polyphenol oxidase (Mason, 1957; Galindo *et al.*, 1983; Cabanes *et al.*, 1987), as will be discussed below. This mechanism postulates the successive use of two *o*-diphenolic substrate molecules (D) for completion of the catalytic cycle with the occurrence of an oxidized form of the enzyme, 2Cu^{2+} (E_{ox}), which, in comparison with the reduced form, 2Cu^{1+} (E_{met}), has greater affinity with the substrate (Galindo *et al.*, 1983).

The differential-equation system corresponding to this kinetic scheme was simplified by keeping in mind similar assumptions to those proposed for a general case in the Appendix ($[\text{E}_s] = [\text{E}_{\text{met}}] + [\text{E}_{\text{met}}\text{D}] + [\text{E}_{\text{ox}}] + [\text{E}_{\text{ox}}\text{D}] + [\text{E}_{\text{ox}}\text{I}]$) and the following equation set was obtained:

$$\frac{d[\text{E}_s]}{dt} = -[\text{I}][\text{E}_s] \frac{k_{+6}K_m/K_I}{[\text{D}] + K_m \left(1 + \frac{[\text{I}]}{K_I}\right)} \quad (1)$$

Abbreviation used: dopa, 3,4-dihydroxyphenylalanine.

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$$\frac{d[P]}{dt} = \frac{k_{cat}[D][E_s]}{[D] + K_m \left(1 + \frac{[I]}{K_I}\right)} - 2k_q[I][P] \quad (2)$$

$$\frac{d[I]}{dt} = -2k_q[I][P] \quad (3)$$

where K_m and K_I are the apparent Michaelis-Menten constant for the *o*-diphenolic substrate and the apparent dissociation constant of the $E_{oxy}I$ complex respectively:

$$K_m = \frac{k_{+1}k_{+2}(k_{-3} + k_{+4}) + k_{+3}k_{+4}(k_{-1} + k_{+2})}{k_{+1}k_{+3}(k_{+2}k_{+4})} \quad (4)$$

$$K_I = \frac{k_{-5}[k_{+1}k_{+2}(k_{-3} + k_{+4}) + k_{+3}k_{+4}(k_{-1} + k_{+2})]}{k_{+1}k_{+2}k_{+5}(k_{-3} + k_{+4})} \quad (5)$$

The catalytic component of the catalytic process is given by:

$$k_{cat} = \frac{2k_{+2}k_{+4}}{k_{+2} + k_{+4}} \quad (6)$$

the initial conditions being: $[E_s] = E_0$, $[I] = I_0$, $[P] = 0$.

MATERIALS AND METHODS

L-Cysteine, ascorbic acid and 4-methylcatechol were purchased from Sigma, and fresh solutions were prepared every day.

Polyphenol oxidase was extracted from grapes as previously described (Valero *et al.*, 1988b).

Catecholase activity of polyphenol oxidase was monitored by the appearance of 4-methyl-*o*-benzoquinone at 400 nm, since it has recently been established that the *o*-quinone has a half-life of 1400 s (Valero *et al.*, 1988a). A unit of enzyme activity was defined as the amount of enzyme that produces 1 μ mol of 4-methyl-*o*-benzoquinone/min. Unless otherwise stated, the reaction medium at 25 °C contained 30 mM-methylcatechol, 10 mM-sodium acetate buffer (pH 4.75), L-cysteine at the indicated concentration and 0.15 unit of enzymic activity. Protein concentration was determined by the method of Bradford (1976). The steady-state rate was defined as the slope of the linear zone of the product-accumulation curve. The lag period was evaluated by extrapolation of the linear portion of the product-accumulation curve to the abscissa.

Spectra of products obtained during 4-methylcatechol oxidation by polyphenol oxidase in the presence of L-cysteine were measured with a Beckman DU-7 HS spectrophotometer, interfaced on-line to a IBM PC-XT computer, with scanning speed of 1200 nm/min at 48 s intervals. Difference spectra were obtained by subtracting each tracing from the reference recording corresponding to 4-methylcatechol. The difference spectra thus obtained were submitted to a rank matrix analysis, by using a graphic method (Coleman *et al.*, 1970).

Computer simulations were performed by the numerical integration method of Euler, introduced in a compiled BASIC program using a IBM PC-XT microcomputer equipped with an 8087 co-processor chip. The integration step was varied automatically in the program by means of a 1–10% flow tolerance method (Barshop *et al.*, 1983). The kinetic parameters V_{max} and K_m corresponding to 4-methylcatechol were determined by fitting the experimental data obtained in the absence of inhibitor to the Michaelis-Menten equation by using non-linear regression (Marquardt, 1963).

RESULTS AND DISCUSSION

Fig. 1(a) shows the time course of the oxidation of 4-methylcatechol by polyphenol oxidase in the presence and

Fig. 1. (a) Progress curves for catecholase activity of polyphenol oxidase in the absence (i) or presence (ii) of 150 μ M-L-cysteine, and (b) computer simulations of progress curves corresponding to Appendix Scheme A1

(a) The points represent the experimental data obtained from a standard reaction mixture. The lines are computer simulations of Scheme 1 using the following kinetic constants: $K_m = 1.25 \times 10^{-2}$ M; $k_{cat} = 7.2 \times 10^4$ min $^{-1}$; $K_I = 2 \times 10^{-2}$ M; $k_{+6} = 24$ min $^{-1}$; $k_q = 2.4 \times 10^6$ M $^{-1}$ min $^{-1}$. The initial conditions were: i, S_0 (S_0 etc. are defined in the Appendix) = 3×10^{-3} M, $E_0 = 10^{-9}$ M, $I_0 = 0$; ii, the same as i, except $I_0 = 1.5 \times 10^{-4}$ M. P is the product. (b) The solid lines represent product (P) accumulation; the broken line represents inhibitor decay for ii conditions. The kinetic constants used were: $K_m = 5 \times 10^{-3}$ M; $k_{+2} = 1.2 \times 10^4$ min $^{-1}$; $K_I = 5 \times 10^{-6}$ M; $k_{+4} = 3 \times 10^{-1}$ min $^{-1}$; $k_q = 1.2 \times 10^6$ M $^{-1}$ min $^{-1}$. The initial conditions were: i, $S_0 = 10^{-3}$ M, $E_0 = 10^{-9}$ M, $I_0 = 5 \times 10^{-6}$ M; ii, the same as i, except $I_0 = 6 \times 10^{-6}$ M; iii, the same as i, except $S_0 = 7 \times 10^{-4}$ M; iv, the same as i, except $E_0 = 6.5 \times 10^{-10}$ M.

absence of L-cysteine and the corresponding theoretical curves obtained by simulation of eqns. (1)–(3). A selection of theoretical progress curves obtained by means of computer simulation of the Michaelis-Menten mechanism described by Appendix Scheme A1 (see the Appendix, eqns. A6–A8) is shown in Fig. 1(b). A lag period appears in the presence of the inhibitor as well as a reduction in the enzymic activity expressed when the steady state is reached. The dependence of these kinetic parameters on inhibitor concentrations found experimentally and theoretically by simulation of the mechanism described by Scheme 1 is shown in Fig. 2, an agreement between both of them being observed. The discrepancies observed between experimental data and those obtained by computer simulation may be due to the simplification introduced in the action mechanism of polyphenol oxidase, when considering that oxygen concentration is saturating so that the levels of the 'deoxy' form of polyphenol oxidase tend to zero, therefore the following equilibrium in the mechanism of polyphenol oxidase (Dietler & Lerch, 1982): ${}^{k_{+2}}E_{deoxy} + O_2 \rightleftharpoons E_{oxy}$ is

Fig. 2. Effect of L-cysteine concentration ([I]) on lag period (○, ●) and on enzyme reaction velocity at steady state (v_s) (△, ▲)

The open symbols represent the data obtained experimentally and the closed those obtained theoretically.

equivalent to: $\frac{k_{+2}}{k_{-2}} E_{oxy}$. It can be seen that the duration of the lag period is not linear with L-cysteine concentration. In order to check that it was a direct effect of L-cysteine on the enzyme, we measured the 4-methyl-*o*-benzoquinone accumulation rate under the same experimental conditions previously used, in the presence of ascorbic acid instead of L-cysteine, since the former acts by an analogous mechanism and it has been shown that it has no direct effect on polyphenol oxidase (Varoquaux & Sarris, 1979). It was found that the steady-state rates obtained after lag periods were not affected by the presence of ascorbic acid concentrations that produced lag periods similar to those obtained with L-cysteine (results not shown). By this means we were able to discard the existence of irreversible inactivation by enzymic catalysis (suicide inactivation), as has been shown with mushroom (Varoquaux & Sarris, 1979; Golan-Goldhirsh & Whitaker, 1984; Tudela *et al.*, 1987) and frog epidermis polyphenol oxidase (Escribano *et al.*, 1989), as well as the existence of substrate depletion.

When the enzyme concentration was varied in the reaction medium, an inverse dependence of the duration of the lag period and an irreversible-type inhibition were found (Fig. 3). The straight lines did not pass through the co-ordinate origin and were extrapolated back to give positive intercepts on the abscissa (Fig. 3b). This may be explained by taking into account that there is always a determined amount of enzyme which is completely inactivated by the inhibitor. Therefore the position where the lines intersect the horizontal axis is equivalent to this enzyme concentration that is irreversibly inactivated, and it will be higher as the inhibitor concentration increases. This inactivation effect of L-cysteine on polyphenol oxidase has, as was pointed out above, been the subject of controversy. Thus, in order to study it in depth, similar levels of enzymic activity were exposed to increased levels of the inhibitor and incubated under the same pH and temperature conditions as those used previously. Aliquots of these preparations were assayed for catecholase activity at various times. Data obtained revealed that L-cysteine

Fig. 3. Effect of polyphenol oxidase concentration ([E]) on lag period (a) and v_s (b) at different L-cysteine concentrations

Inhibitor (cysteine) concentrations used were: ○, 0; ●, 100 μM; △, 150 μM; ▲, 200 μM; □, 300 μM.

does not bind to the free (met) form of the enzyme, since, even using L-cysteine concentrations (5 mM) that would not allow the spectrophotometric measurement of enzymic activity, there was no loss of enzymic activity in any one case (results not shown). These results can only be interpreted by taking into account the internal mechanism of catecholase activity of polyphenol oxidase (Scheme 1), which indicates that L-cysteine must bind irreversibly to the oxy form of the enzyme. This form is an obligatory intermediate in the catalytic turnover, and so the presence of the substrate, and therefore of the catalytic activity, are necessary to obtain it in significant levels to enable L-cysteine to act.

The effect of 4-methylcatechol concentration on the lag period and steady-state rate is illustrated in Fig. 4, which shows that there is an inverse dependence of the length of the lag period on substrate concentration and that V_{max} is not affected by the presence of L-cysteine. Furthermore, a positive kinetic co-operativity effect with respect to catalytic activity can be seen. Then, these results were fitted to the Hill equation:

$$v = V_{max} [S]^h / (K_H^h + [S]^h)$$

by using non-linear regression (Marquardt, 1963). Hill coefficients and constants of the Hill equation thus obtained are shown in the inset to Fig. 4, an increase in both kinetic parameters being observed as L-cysteine concentration increases. This response of the system to substrate concentration, and the existence of the lag period in the product accumulation curves (Fig. 1), is similar to that of an enzyme with hysteretic behaviour due to isomerization or displacement of a ligand which causes a shift to a more active form of the enzyme, as was reported by Ainslie *et al.* (1972). Thus this co-operativity effect must be included within the so-called 'kinetic co-operativity'. All these dependences of the lag period and steady-state rate as well as the co-operativity effect are also obtained by computer simulation of the Michaelis-Menten mechanism shown in Appendix Scheme A1 (results not shown).

Fig. 4. Effect of 4-methylcatechol (4MC) on lag period (a) and ν_s (b) at different L-cysteine concentrations

Conditions are as described in Fig. 3. The inset shows corresponding values of Hill coefficient (h) (○) and the constant of the Hill equation (K_H) (●). L-Cysteine concentrations were as in Fig. 3.

The appearance of the lag period in the product-accumulation curve (Fig. 1) must be interpreted as the rapid removal of 4-methyl-*o*-benzoquinone due to its reaction with L-cysteine present, according to data in the literature. In order to confirm this, we made an analysis of the reaction media using spectroscopic scanning techniques. Figs. 5(a) and 5(b) show the results obtained in the form of difference spectra. A disappearance of the maximum at 280 nm corresponding to 4-methylcatechol and the appearance of a new maximum at 300 nm, attributed to the product of the reaction between L-cysteine and 4-methyl-*o*-benzoquinone (Q), can be seen. Furthermore, in the spectral set shown in Fig. 5(a), two isosbestic points were seen at 269 and 285 nm, indicating a constant ratio between the disappearance of 4-methylcatechol and the formation of Q, although with time they disappeared (Fig. 5b), just when 4-methyl-*o*-benzoquinone started to appear ($\lambda_{\text{max.}} = 400$ nm). This experiment was performed again under the same conditions, the time course of the reaction at 300 and 400 nm being monitored as shown in Fig. 6. There is a linear increase in the absorbance at 300 nm, whereas the absorbance at 400 nm remains unchanged, and that the increase at 300 nm slows when the increase in the absorbance at 400 nm starts.

Graphical analysis of the recordings of Fig. 5 by the matrix method of Coleman *et al.* (1970) (results not shown) gave a good fit for two, kinetically related, absorbing species in solution with respect to the first spectra set (Fig. 5a), the two species being 4-methylcatechol and the adduct resulting from the reaction between 4-methyl-*o*-benzoquinone and L-cysteine (Q). The lines

Fig. 5. Difference spectra for 4-methylcatechol oxidation ($350 \mu\text{M}$) by polyphenol oxidase (0.15 unit) in the presence of L-cysteine ($12.5 \mu\text{M}$)

The spectra set shown in (b) are a time continuation of that shown in (a). The scan speed was up to 1200 nm/min, at intervals of 48 s.

Fig. 6. Time course of 4-methylcatechol oxidation by polyphenol oxidase in the presence of L-cysteine monitored at 300 and 400 nm

Experimental conditions were as indicated in Fig. 5.

deviated as a result of graphical analysis of the second spectra set (Fig. 5b), indicating, in accordance with Coleman *et al.* (1970), a shift in the number of absorbing species in the reaction medium, due to the appearance of 4-methyl-*o*-benzoquinone when all the inhibitor had been exhausted. From these data it can be concluded that the lag period shown by catecholase activity of polyphenol oxidase in the presence of L-cysteine is the result of the chemical reactions of nucleophilic addition of L-cysteine to the *o*-quinone formed by polyphenol oxidase action, leading to the formation of more stable colourless products.

The results presented here indicate that L-cysteine is an irreversible inhibitor of polyphenol oxidase, rendered unstable in the reaction medium by the enzymic catalysis; it binds to the oxy form of the enzyme, an intermediate in the catalytic turnover, and it reacts also with the *o*-quinone product of enzymic catalysis, thus preventing its appearance.

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APPENDIX

In this section, the kinetic analysis of systems where the irreversible enzyme inhibitor is consumed by its reaction with the product of catalytic activity is made, assuming a Michaelis-Menten mechanism for the enzyme under study.

NOTATION AND DEFINITIONS

Species and concentrations

- E_0 and E_i Initial concentration of enzyme under study and inactive enzyme respectively
- E_s Set of enzyme species involved in a restricted steady state
- S_0 Initial concentration of substrate
- I_0 Initial concentration of irreversible inhibitor
- P Product formed from S
- Q Product formed from the reaction between the inhibitor and the product of enzymic catalysis (P)
- f_x Concentration factor corresponding to enzyme species X with regard to E_s ; $f_x = [X]/[E_s]$.

Kinetic parameters

- v_s Enzyme reaction velocity at steady-state
- L Lag period
- h Hill coefficient

Kinetic constants

- k_{cat} Catalytic constant of the catalytic route
- K_i Dissociation constant of the EI complex
- k_q Rate constant of the chemical reaction between the inhibitor and the product of enzyme reaction (P)
- K_H Constant of the Hill equation

BASIC MODEL AND ASSUMPTIONS

Assuming a Michaelis-Menten mechanism for the enzyme under study, the kinetic analysis of this type of process, where the irreversible enzyme inhibitor is made unstable by its reaction with the product of enzymic catalysis, may be described by Scheme A1 if the irreversible inhibition is considered to be competitive:

Scheme A1

The simple bimolecular reaction $E + I \rightarrow E_i$ is not considered, because it is a particular case of the above Scheme when the condition $k_{-3} \ll k_{+3} \ll k_{+4}$ is satisfied (Varón *et al.*, 1990).

The differential-equation system corresponding to this kinetic scheme is not linear, and particular solutions were obtained by means of numerical integration as described in the Materials and methods section of the main paper. The following assumptions were made.

- (1) Steady-state conditions are reached instantaneously between E, ES and EI.
- (2) Substrate concentration is much greater than enzyme concentration, so that depletion of free substrate by binding to the enzyme is negligible.
- (3) Inhibitor concentration is much greater than enzyme concentration, so that depletion of free inhibitor is solely governed by binding to the product of enzyme reaction.
- (4) Observations are made only while the effects of substrate depletion (by conversion into the product) and of product inhibition (P and Q) on the reaction velocity are negligible.
- (5) The reaction is started by addition of enzyme.

According to these assumptions and following the method of Cha (1968), the differential equation system may be simplified to the following:

$$\frac{d[E_s]}{dt} = -k_{+4}f_{EI}[E_s] \quad (A1)$$

$$\frac{d[P]}{dt} = k_{+2}f_{ES}[E_s] - k_q[I][P] \quad (A2)$$

$$\frac{d[I]}{dt} = -k_q[I][P] \quad (A3)$$

where f_{EI} and f_{ES} have the following meaning:

$$f_{EI} = \frac{[I]K_m/K_i}{[S] + K_m \left(1 + \frac{[I]}{K_i}\right)} \quad (A4)$$

$$f_{ES} = \frac{[S]}{[S] + K_m \left(1 + \frac{[I]}{K_i}\right)} \quad (A5)$$

Inserting the expressions (A4) and (A5) into eqns. (A1) and (A2) respectively, we obtain the following differential-equation system:

$$\frac{d[E_s]}{dt} = -[I][E_s] \frac{k_{+4}K_m/K_i}{[S] + K_m \left(1 + \frac{[I]}{K_i}\right)} \quad (A6)$$

$$\frac{d[P]}{dt} = \frac{k_{+2}[S][E_s]}{[S] + K_m \left(1 + \frac{[I]}{K_i}\right)} - k_q[I][P] \quad (A7)$$

$$\frac{d[I]}{dt} = -k_q[I][P] \quad (A8)$$

the initial conditions being: $[E_s] = E_0$, $[I] = I_0$, $[P] = 0$.

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