Effect of pH on the oxidation pathway of α -methyldopa catalysed by tyrosinase

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This paper deals with the quantitative description of the effect of pH on the oxidation pathway of α -methyldopa. Tyrosinase catalyses the oxidation by molecular oxygen of α -methyldopa to *o*- α -methyldopaquinone, which evolves non-enzymically through a branched pathway with cyclization or hydroxylation reactions. The intermediates of the hydroxylation branch have been identified, and the corresponding rate constants have been determined. These compounds, which have been detected in melanosomes and in tumour cells, have great cytotoxic power and could have physiological significance in acidic media.

INTRODUCTION

Melanins are polymeric compounds responsible for enzymic browning in fruits and vegetables, as well as for the pigmentation of eyes, hair and skin in mammals. Melanogenesis begins by the oxidation by molecular oxygen of monophenols and/or *o*-diphenols to yield the respective *o*-quinones. This step can be catalysed or not by tyrosinase, depending on the physiological compartmentation of the enzyme. In both cases, however, the *o*-quinones evolve through coupling of non-enzymic reactions towards the formation of melanins (Mason, 1957; Robb, 1984).

There are a number of monophenols and *o*-diphenols which give rise to melanins in plants and animals (Mason, 1957; Robb, 1984), and neuromelanins resulting from the catabolism of dopamine or noradrenaline have been reported (Langston, 1988). L- α -Methyldopa, DL-isoprenaline (isoproterenol) and about forty compounds related to L-tyrosine and L-dopa have been detected in the hair melanins of humans and guinea pigs (Yu & Scott, 1973; Harrison *et al.*, 1974). Thus the existence of a secondary metabolic pathway for adrenergic drugs could be considered (Harrison *et al.*, 1974; Jiménez *et al.*, 1986).

In rat brain, α -methyldopa is metabolized to catecholamines such as α -methyldopamine and/or α -methylnoradrenaline (Wick, 1978). Other studies (Langston, 1988) have considered the possible role of neuromelanins in neuron damage, or in the promotion of the selective vulnerability of neurons in Parkinson's disease.

Furthermore, α -methyldopa, together with some catecholamines and other adrenergic drugs, has also been used as a new class of anti-tumour agents against malignant melanoma. This anti-tumoural effect *in vitro* as well as *in vivo* has been ascribed to the reactivity of quinones produced on oxidation of *o*-diphenol by tyrosinase (Wick, 1979, 1980); this enzyme is detected in large amounts in malignant melanoma cells (Burnett, 1971; Nishioka, 1978).

On the other hand, α -methyldopa is known pharmacologically for its hypotensive properties. It is generally considered that α -methyldopa interferes with blood pressure regulation by modifying noradrenergic neuronal activity, and its efficiency is related to the stimulation of α -adrenergic receptors (Wand-Routledge & Marsten, 1988).

The oxidation of α -methyldopa (Jiménez *et al.*, 1986) by molecular oxygen yields *o*- α -methyldopaquinone; this process is catalysed by tyrosinase. This quinone is then metabolized non-enzymically through a branched pathway consisting of cyclization or hydroxylation reactions (see Fig. 1). The pathway has been partially characterized, but only at pH values where the intermediates of the cyclization branch can be identified, and the corresponding rate constants have been determined.

The aim of these studies was to characterize the oxidation pathway of α -methyldopa, catalysed by tyrosinase. The experimental study attempts to identify the intermediates of the hydroxylation branch, and to determine the corresponding rate constants. This allows the quantitative description of the regulatory effect of pH on this pathway. The intermediates of the hydroxylation branch, such as those detected in melanosomes (Sacki & Oikawa, 1983, 1985; Moelkman *et al.* 1988) and in tumour cells (Wike-Hooley *et al.*, 1984; Hedley & Jorgensen, 1989), have great cytotoxic power (Liang *et al.*, 1977), and could have physiological significance in acidic media.

MATERIALS AND METHODS

Materials

Mushroom tyrosinase (monophenol/*o*-diphenol: oxygen oxidoreductase; EC 1.14.18.1; 3300 units/mg) and α -methyldopa were purchased from Sigma (Germany). All other chemicals were of analytical grade, and were supplied by Merck (Germany).

Rapid-scan assays

Spectrophotometric measurements were carried out with an Aminco DW-2 spectrophotometer equipped with a Hewlett-Packard recorder with kinetic response, allowing the recording dead time to be minimized. The scan speed was 20 nm·s⁻¹, and the first recording was started 20 s after the beginning of the reaction. The assay medium contained 25 mM-acetate buffer, pH 4.1. Matrix analysis of the iterative spectra was carried out by application of the test for two or three absorbing species in solution, with stoichiometric restrictions (Coleman *et al.*, 1970).

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Kinetic assays

Product accumulation was monitored spectrophotometrically at 475 nm using a Perkin-Elmer Lambda 3 spectrophotometer interfaced on-line with a Perkin-Elmer DS-3600 computer. The reaction medium was 25 mM-acetate buffer containing 100 mM-KNO₃ in order to obtain a constant ionic strength at all pH values. Saturation conditions for α -methyldopa were obtained at 10 mM. Other reagents and conditions are detailed in the legends to the Figures.

Oxygen determination

Oxygen consumption was measured using a Hansatech DW oxymeter, based on the Clark electrode. Temperature was controlled at 20 °C using a Hetofrig circulating water bath with a heater/cooler and checked using a Cole-Parmer digital thermometer with a precision of ± 0.1 °C.

RESULTS AND DISCUSSION

Rapid-scan assays

Oxidation reactions of α -methyldopa at pH 3.7–4.3 in the presence of tyrosinase or sodium periodate have been monitored by means of rapid-scan spectroscopy. The iterative spectra have been analysed by the rank matrix method to determine the minimum number of absorbing species in solution.

An isosbestic point was obtained at 385 nm with sodium periodate in excess, whereas this isosbestic point disappeared on oxidation with sodium periodate at limiting levels or in the presence of high enzyme concentrations (results not shown). These results suggest the accumulation of hydroxy- α -methyldopa (Fig. 1), an intermediate not detected in similar rapid-scan assays carried out at pH ≥ 4.5 (Jiménez *et al.*, 1986).

Fig. 1. Pathway proposed for the oxidation of α -methyldopa catalysed by tyrosinase, taking into consideration the effect of pH

E, tyrosinase; D, α -methyldopa; QH, *o*- α -methyldopaquinone-H⁺; Q, *o*- α -methyldopaquinone; L, leuco- α -methyldopachrome; T, hydroxy- α -methyldopa; PQ, hydroxy-*p*- α -methyldopaquinone; DC, α -methyldopachrome.

Thus these results with tyrosinase and periodate, as well as data obtained with cyclic voltammetry assays (García Cánovas *et al.*, 1982; Jiménez *et al.*, 1986), confirm the oxidation pathway of α -methyldopa proposed here (Fig. 1).

Kinetic assays

The course of the oxidation pathway of α -methyldopa was monitored by measuring the appearance of products (A_{475}) during the entire assay time. At pH values lower than 4.5 (Figs. 2a and 2b) the lag period decreased as the enzyme concentration rose, whereas no such relationship was obtained at higher pH values (Jiménez *et al.*, 1986). This kinetic behaviour has not yet

Fig. 2. Kinetic assays of product accumulation for the oxidation of α -methyldopa catalysed by tyrosinase

Reagents were 10 mM- α -methyldopa and 0.26 mM-O₂, at pH 4.1. Other conditions were as detailed in the Materials and methods section. (a) Spectrophotometric recordings. Tyrosinase concentrations were (μ g/ml): 1.7, 3.3, 6.7 and 10.0 (b) Corresponding values of the lag period (τ) versus enzyme concentration (E_0) (●), and plot of product accumulation rate in steady state conditions against $[E_0]$ (▲).

Fig. 3. Kinetic assays of oxygen consumption for the oxidation of α -methyldopa catalysed by tyrosinase

(a) Reagents were 10 mM- α -methyldopa, 0.26 mM-O₂ and tyrosinase (μ g/ml): 1.7, 3.3, 6.7 and 10.0 at pH 4.1. (b) Corresponding values of V_0 versus $[E_0]$.

been analysed and could be useful for the quantitative characterization of the pathway.

Furthermore, the slopes of the spectrophotometric recordings are not suitable for estimating the steady-state rate of the pathway. This is due to the presence of two absorbing products, α -methyl dopachrome ($\lambda_{\max}$, 475 nm) and hydroxy- p - α -methyl dopaquinone ($\lambda_{\max}$, 485 nm; Graham & Jeffs, 1977), whose relative proportions are not known. The absolute values of the steady-state rate of the enzymic step have been determined by measuring oxygen consumption (Fig. 3). These rates are one-half of that of o - α -methyl dopaquinone- H^+ formation (Fig. 1), since one molecule of oxygen generates two molecules of quinone in each turnover of the reaction (Galindo *et al.*, 1983).

Kinetic analysis

The oxidation pathway of α -methyl dopa catalysed by tyrosinase at pH values lower than 4.5 is depicted in Fig. 1. This scheme involves the steady-state rate of the enzymic step (V_0), and first-order (k_1 and k_c) and second-order (k_{-1} , k_2 , k_3 and k_4) rate constants.

The experimental results show the accumulation of hydroxy- α -methyl dopa (Fig. 1). This implies a significant contribution of the second-order step controlled by k_4 to the kinetic behaviour of the pathway. Thus there are no analytical solutions with which to describe the evolution of the products during the transient phase of the kinetic assays (Fig. 2a). At the steady state of the pathway, however, it is possible to derive explicit equations relating the product concentrations to the assay time.

Kinetic analysis of this pathway requires α -methyl dopa consumption as well as the breakdown of the products to be negligible during the reaction time. Both conditions have been verified in the experimental assays. The mass balance between the reagents (Fig. 1) states that the consumed o -diphenol ($V_0 t$) yields intermediates ($\Sigma[I]$), products ($[DC]$, $[PQ]$) and regenerated o -diphenol through the cyclization ($[D]_c$) and hydroxylation ($[D]_h$) branches of the pathway (see the legend to Fig. 1 for definition of abbreviations):

$$V_0 t - ([D]_c + [D]_h) = \Sigma[I] + [DC] + [PQ] \quad (1)$$

According to Fig. 1,

$$[D]_c = [DC] \text{ and } [D]_h = [PQ] \quad (2)$$

$$\Sigma[I] = [QH] + [Q] + [T] \quad (3)$$

since leuko- α -methyl dopachrome (L) is not accumulated in the assay medium (Jiménez *et al.*, 1986).

During the steady state of the pathway, the concentration of the intermediates remains constant:

$$[QH] = V_0 + k_{-1}[H^+][Q] - k_{-1}[QH] - k_c[Q] - k_2[QH] - k_4[QH][T] = 0 \quad (4)$$

$$[Q] = k_1[QH] - (k_{-1}[H^+] + k_c)[Q] = 0 \quad (5)$$

$$[T] = k_2[QH] - k_4[QH][T] = 0 \quad (6)$$

where $k'_2 = k_2[H_2O]$. Note that the term $k_3[QH][L]$ has been replaced by $k_c[Q]$ in eqn. (4), since this step is rate determining for α -methyl dopachrome production, as indicated by the non-accumulation of leuko- α -methyl dopachrome. From the above equations, the following expressions for the concentration of the intermediates at the steady state (ss) are obtained:

$$[QH]_{ss} = \frac{(k_{-1}[H^+] + k_c)V_0}{2k_1k_c + 2k'_2(k_{-1}[H^+] + k_c)} \quad (7)$$

$$[Q]_{ss} = \frac{k_1V_0}{2k_1k_c + 2k'_2(k_{-1}[H^+] + k_c)} \quad (8)$$

$$[T]_{ss} = k'_2/k_1 \quad (9)$$

Consideration of eqns. (1)–(3) and (7)–(9) leads to the expression:

$$[DC]_{ss} + [PQ]_{ss} = \frac{V_0}{2} \left\{ t - \left[\frac{k_1 + k_{-1}[H^+] + k_c}{2k_1k_c + 2k'_2(k_{-1}[H^+] + k_c)} + \frac{k'_2}{k_4V_0} \right] \right\} \quad (10)$$

where t = time. Thus the accumulation of the products of the pathway during the steady state follows a straight line (eqn. 10), whose intercept with the time axis defines the lag period (τ):

$$\tau = \frac{k_1 + k_{-1}[H^+] + k_c}{2k_1k_c + 2k'_2(k_{-1}[H^+] + k_c)} + \frac{k'_2}{k_4V_0} \quad (11)$$

Since $k_c \gg k_1$ (Jiménez *et al.*, 1986), the eqn. (11) becomes:

$$\tau = \frac{k_{-1}[H^+] + k_c}{2k_1k_c + 2k'_2(k_{-1}[H^+] + k_c)} + \frac{k'_2}{k_4V_0} \quad (12)$$

This equation predicts that τ can decrease when the enzyme (tyrosinase) concentration ($[E_0]$, involved in V_0) is raised, according to experimental data at pH values lower than 4.5 (Fig. 2). At higher pH values, however, τ is not dependent on the enzyme concentration (Jiménez *et al.*, 1986). Thus, when $[H^+]$ decreases, $2k'_2(k_{-1}[H^+] + k_c) \ll k_1k_c$, ($k'_2 \ll k_1$) and eqn. (12) is transformed into:

$$\tau = \frac{k_{-1}[H^+] + k_c}{2k_1k_c} + \frac{k'_2}{k_4V_0} \quad (13)$$

Furthermore, since V_0 increases when the pH is raised, a negligible contribution of the second quotient compared with the first gives:

$$\tau = (k_{-1}[H^+] + k_c)/2k_1k_c \quad (14)$$

This expression is not dependent on the enzyme concentration.

On the other hand, eqn. (12) can be rearranged as:

$$\tau = 2\alpha + \beta(1/V_0) \quad (15)$$

where

$$2\alpha = \frac{\frac{1}{k'_2}[H^+] + \frac{k_cK_a}{k_1k'_2}}{[H^+] + \frac{k_cK_a}{k'_2}} \quad (16)$$

and

$$\beta = k'_2/k_4 \quad (17)$$

where $K_a = k_1/k_{-1}$. The above expressions can be useful in determining the rate constants of the pathway.

Kinetic data analysis

Two series of spectrophotometric (Fig. 2) and electrometric (Fig. 3) assays were carried out using the same set of enzyme concentrations. From A_{475} versus t recordings, data for the final portion were fitted by linear regression and the corresponding τ versus $[E_0]$ values were calculated. The initial portion of the $[O_2]$ versus t recordings were fitted by linear regression, yielding the respective values of $V_0/2$.

The kinetic assays provide, therefore, τ versus $(1/V_0)$ data at the same $[E_0]$ values. This procedure was applied for several pH values (Fig. 4a) and the data were fitted by linear regression according to eqn. (15). The great similarity between the slopes of the straight lines (Fig. 4a) confirms the non-dependence of β on pH (eqn. 17). Thus the mean value of the slopes enables k'_2/k_4 to be calculated (Fig. 4b). The intercepts on the ordinate axis, however, show hyperbolic behaviour for $[H^+]$ (Fig. 4b). The non-linear regression fitting of 2α versus $[H^+]$ data to eqn. (16) leads

Fig. 4. Kinetic assays of the oxidation of α -methyldopa catalysed by tyrosinase, at different pH values, for several enzyme concentrations

pH values were 3.7 ($\blacktriangle$), 3.9 ($\bullet$), 4.0 ($\blacksquare$), 4.2 ($\blacktriangledown$), 4.4 ($\blacklozenge$), 4.5 ($\triangle$), 4.7 ($\circ$), 4.9 ($\square$) and 5.4 (∇). Tyrosinase concentrations ($\mu\text{g}/\text{ml}$) tested at each pH were 3.3, 6.7, 10.0, 13.3 and 16.7. Other assay conditions were as described in the Materials and methods section. (a) Dependence of the lag period (τ) on $1/V_0$; kinetic parameters were obtained from spectrophotometric and oxymetric recordings respectively. Experimental data and linear regression fittings are shown. (b) Corresponding values of 2α ($\bullet$) and of β ($\blacktriangle$) versus $[\text{H}^+]$. The broken line shows data calculated by using eqn. (16), with the initial estimations of kinetic constants. $\bullet$ — $\bullet$, Data calculated by using eqn. (16), with final estimations of kinetic constants obtained from non-linear regression fitting; $\blacktriangle$ — $\blacktriangle$, mean values of β versus $[\text{H}^+]$, according to eqn. (17).

Table 1. Values of the rate constants for the oxidation pathway of α -methyldopa catalysed by tyrosinase (Fig. 1) at 20 °C

Constant (units)	Value
k_1 (s^{-1})	0.67 ± 0.12
k_2 ($\text{M}^{-1}\cdot\text{s}^{-1}$)	$(1.29 \pm 0.04) \times 10^{-4}$
k_c (s^{-1})	106.1 ± 22.6
k_4 ($\text{M}^{-1}\cdot\text{s}^{-1}$)	$(2.78 \pm 0.38) \times 10^3$

to the determination of all of the rate constants of the pathway, by considering $K_a = 1.91 \text{ nM}$ (Gray & Weitzman, 1968). Initial estimations of k_1 , k_2 and k_c arise respectively from the lower limit, higher limit and half-saturation value of the hyperbola (Fig. 4b). The values of the rate constants of the pathway (Fig. 1) are given in Table 1.

Effect of the pH on the pathway

The spectral and kinetic assays described above confirm the existence of and characterize the oxidation pathway of α -methyldopa proposed here (Fig. 1). The pH of the medium determines the differential contributions of the cyclization and the hydroxylation branches, through the protonation/deprotonation of the key intermediate, *o*- α -methyldopaquinone. Thus the experimental data show that both branches are only significant simultaneously at acid pH. Indeed, there is evidence concerning the acidic character of melanosomes (Sacki & Oikawa, 1983, 1985; Moelhman *et al.*, 1988) and tumour cells (Wike-Hooley *et al.*, 1984; Hedley & Jorgensen, 1989).

The cytotoxicity of α -methyldopa and other *o*-diphenols has been attributed to the further products of oxidation (Wick *et al.*, 1977; Graham *et al.*, 1978). Compounds similar to the intermediates of the hydroxylation branch also have cytotoxic power (Liang *et al.*, 1977), with greater selectivity than the intermediates of the cyclization branch (Wick *et al.*, 1979) against melanoma cells. The quinonic intermediates show high reactivity with amino and thiol groups of amino acids and proteins, inorganic anions and reductant agents, processes which could be related to their cytotoxic ability (Mason, 1957; Robb, 1984). Therefore reagents causing changes in the pH of the physiological microenvironment could determine the actual cytotoxicity of α -methyldopa and other catecholamines.

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