Effect of L-ascorbic acid on the monophenolase activity of tyrosinase

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The effect of ascorbic acid on the monophenolase activity of tyrosinase, using tyrosine as substrate, has been studied. Over the ranges of ascorbic acid concentration used, no direct effect on the enzyme is found. However, a shortening of the characteristic induction period of the hydroxylation reaction is observed. The evolution of the reaction is dependent on the concentration of ascorbic acid. Low concentrations permit the system to reach the steady state when all ascorbic acid is consumed, whereas high

concentrations do not. In the light of these results it is proposed that the influence of ascorbic acid on the reaction is due to its ability to reduce the enzymically generated *o*-quinones. A relationship between the ascorbic acid concentration, and the induction period generated by it, with the diphenolase activity of tyrosinase is established, which can be used as a basis for the determination of trace amounts of this reducing agent.

INTRODUCTION

Tyrosinase (monophenol,*o*-diphenol:oxygen oxidoreductase, EC 1.14.18.1) is a copper enzyme found in micro-organisms, plants and animals. This enzyme catalyses both the hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity). Some of these *o*-quinones are unstable in aqueous solutions and rapidly suffer a non-enzymic cyclization to leucoaminochrome, a compound which is oxidized non-enzymically by another molecule of *o*-quinone to yield aminochrome and one molecule of regenerated diphenol [1,2]. The monophenolase activity of tyrosinase shows an induction period [3–6] which depends on the enzyme and substrate concentrations [7] and is influenced by the presence of reductant agents. L-3,4-Dihydroxyphenylalanine (dopa), at very low concentration, is the most effective reductant agent in eliminating the induction period of tyrosinase acting on tyrosine. It produces an initial burst in the reaction when it is added at higher concentrations than that which the enzyme can maintain in the steady state [8].

Many studies on the effect of ascorbic acid on the action of tyrosinase on several substrates have been carried out with contradictory results. Some authors suggest that ascorbic acid affects specific structures of the enzyme in which copper is present [9,10] and shows an inhibitory action on the enzymic activity [11]. On another hand, ascorbic acid has been assigned the role of a reducing agent for the *o*-quinones produced by the enzymic oxidation of the substrates, showing no effect on the active site of the enzyme [12]. Ascorbic acid at high concentrations has been used as a reductant in measuring monophenolase activity by estimation of the diphenol formed from monophenol [13,14].

The present paper studies the effect of ascorbic acid on the monophenolase activity on tyrosine. Different ascorbic acid concentrations are investigated, and the disadvantages of using high concentrations to measure monophenolase activity are discussed. Finally, the effect of ascorbic acid on diphenolase activity is suggested as a basis for a possible method to determine trace amounts of this reducing agent.

MATERIALS AND METHODS

Reagents

Tyrosinase (3300 units/mg), tyrosine and dopa were purchased from Sigma (St. Louis, MO, U.S.A.). All other chemicals were of analytical grade and supplied by Merck (Darmstadt, Germany). Mushroom tyrosinase was purified by the procedure of Duckworth and Coleman [15]. Protein concentration was determined by a modified Lowry method [16]. The enzyme concentration was calculated by taking a value for the M_r of 120000. Tyrosine was purified from possible dopa contamination by passing it through a column, 1 cm in diameter, containing 2 g of aluminium oxide suspended in 0.5 M ammonium acetate, pH 6.1 [17]. This tyrosine solution was further purified by Chelex-100 chromatography (100–200 mesh; Na⁺ form; Bio-Rad) to remove traces of metal ions [18,19]. Ascorbic acid solution was freshly prepared for each experiment using orthophosphoric acid as solvent to prevent auto-oxidation. Milli-Q system (Millipore Corp., Bedford, MA, U.S.A.) ultrapure water was used throughout this research to avoid contamination by metal ions. In all the assays, 0.1 M phosphate buffer, pH 6.8, previously passed through a Chelex-100 column to ensure the stability of the ascorbic acid [20], was used.

Measurement of enzyme activity

The monophenolase activity of tyrosinase was monitored at 25 ± 0.1 °C by different methods.

Spectrophotometric assays

2-Carboxy-2,3-dihydroindole-5,6-quinone (dopachrome) formation was monitored at 475 nm (ϵ 3600 M⁻¹·cm⁻¹) using a Perkin-Elmer Lambda-2 spectrophotometer interfaced on-line with an Amstrad PC2086 computer. The induction period, τ , was estimated by extrapolation of the linear portion of the dopachrome accumulation curve to the abscissa in the plots of dopachrome accumulation versus time.

Abbreviations used: dopa, L-3,4-dihydroxyphenylalanine; dopachrome, 2-carboxy-2,3-dihydroindole-5,6-quinone; leukodopachrome, 2,3-dihydro-5,6-dihydroxyindole-2-carboxylate; tyrosine and ascorbic acid are their L isomers.

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Polarographic assays

Oxygen consumption was monitored with a Hansatech DW oxymeter, based on the Clark electrode, covered with a Teflon membrane, equipped with an Amel 863 digital *X/Y* recorder. Calibration was made by the 4-*t*-butylcatechol/tyrosinase method [21].

H.p.l.c. assays

The formation of dopa was monitored in a discontinuous way using a Beckman System Gold liquid-chromatograph system equipped with a model 110B pump for isocratic elution, a 168 programmable diode array detector (monitoring 250 nm; scanning 200–600 nm) and a System Gold IBM PS/2 model 5.1. Samples were introduced via a Rheodyne fixed-volume injector (20 μ l). The compounds were separated in an Ultrasphere-ODS reverse-phase column (4.6 nm $\times$ 250 nm; internal diameter 5 μ m) and eluted with a flow rate of 1 ml/min with 50 mM ammonium acetate and 5 mM Na₂-EDTA adjusted to pH 3.0 at 25 °C. The different peaks were characterized by their absorption spectra. The purity of the peaks was determined by the Real Time Purity Algorithm of the 168 diode array detector. The values of dopa concentration were quantified by interpolation of the peak areas on the linear calibration from their standards.

RESULTS

Addition of dopa as reducing agent in the tyrosine hydroxylation catalysed by tyrosinase

Figure 1 shows the effect of the addition of micromolar concentrations of dopa on the production of dopachrome from tyrosine catalysed by tyrosinase. This effect can also be followed discontinuously by estimating the dopa accumulated during the reaction (Figure 2). In the absence of dopa, an induction period is observed in the production of dopachrome (Figure 1, trace a) and a sigmoidal pattern of dopa concentration ([D]) versus time, whose final plateau corresponds to the level of dopa con-

Figure 1 Time course of dopachrome accumulation in the monophenolase activity with several initial dopa concentrations $\{[D]_0$ (μ M)}: a, 0; b, 5; c, 68; d, 100

The inset shows the dependence of the induction period, τ , on $[D]_0$. The data points of the inset have been obtained from plots similar to those of the main Figure, but using different $[D]_0$ values. In all cases, 1.2 mM tyrosine, 0.26 mM O₂ and 15 nM tyrosinase were used. Each point represents the mean for three separate experiments.

centration in the steady state ($[D]_{ss}$), is obtained (Figure 2, trace a). The $[D]_{ss}$ is not dependent on enzyme concentration, whereas it is proportional to the monophenol concentration ($[T]_0$), and so $[D]_{ss} = R[T]_0$ ($R = 0.057$) [8]. The induction period involves (see Scheme 1) the generation of dopa from *o*-dopaquinone-H⁺, in a non-enzymic reaction, as well as the removal of Emet from the dead-end complex EmetT to incorporate EmetD in the oxidase cycle [7,8]. This suggests that the induction

Figure 2 Pattern of dopa accumulation in the monophenolase reaction with several initial dopa concentrations $\{[D]_0$ (μ M)}: a, 0; b, 5; c, 68; d, 100

In all cases, 1.2 mM tyrosine, 0.26 mM O₂ and 5 nM tyrosinase were used. Each point represents the mean for three separate experiments.

Scheme 1 Reaction mechanism for the monophenolase activity of tyrosinase, coupled to non-enzymic reactions from *o*-dopaquinone-H⁺ up to dopachrome in the melanin-biosynthesis pathway

Abbreviations used: E, tyrosinase; Emet, met-tyrosinase or oxidized form of tyrosinase with Cu²⁺–Cu²⁺ in the active site; Ered, reduced form of tyrosinase with Cu²⁺–Cu⁺ in the active site; Eoxy, oxytyrosinase or oxidized form of tyrosinase with peroxide; T, L-tyrosine; D, L-dopa; QH, *o*-dopaquinone-H⁺; Q, *o*-dopaquinone; L, leukodopachrome; DC, dopachrome.

Figure 3 Time course of dopachrome accumulation in the monophenolase reaction with several values of [ascorbic acid]₀ (μM): a, 0; b, 5; c, 30; d, 80; e, 100

In all cases 1.2 mM tyrosine, 0.26 mM O₂ and 15 nM tyrosinase were used.

Figure 4 Time course of oxygen consumption in the monophenolase reaction with several values of [ascorbic acid]₀: a, 0; b, 5 μM; c, 30 μM; d, 3 mM

In all cases 1.2 mM tyrosine, 0.26 mM O₂ and 25 nM tyrosinase were used.

period can be shortened by the addition of dopa at the start of the reaction or by the formation of dopa in another reaction simultaneous with the enzymic reaction, because less time is required for the attainment of the corresponding [D]_{ss}. In fact there are three possible initial conditions concerning the initial concentration of dopa ([D]₀): (i) [D]₀ < [D]_{ss} leads to the induction period (Figure 1, trace b) and to the sigmoidal pattern of [D] versus time (Figure 2, trace b); (ii) [D]₀ ≈ [D]_{ss} does not originate either the induction period (Figure 1, trace c) or the sigmoidal pattern of [D] versus time (Figure 2, trace c); and (iii) [D]₀ > [D]_{ss} leads to an initial burst (Figure 1, trace d) due to the consumption of the excess dopa to reach the steady state (Figure 2, trace d). The dependence of the induction period on [D]₀ is shown in the inset of Figure 1. When the reactions are monitored by measuring oxygen consumption (results not shown), the results and dependences are similar to those obtained by measuring dopachrome production.

Addition of ascorbic acid in the tyrosine hydroxylation catalysed by tyrosinase

The effect of various concentrations of ascorbic acid on the monophenolase activity of tyrosinase has been studied. When dopachrome production is followed (Figure 3), the influence of ascorbic acid on the induction period depends on its concentration. When low concentrations are used (in the micromolar

Figure 5 Pattern of dopa accumulation in the monophenolase reaction with several values of [ascorbic acid]₀: a, 0; b, 5 μM; c, 3 mM

In all cases 1.2 mM tyrosine, 0.26 mM O₂ and 5 nM tyrosinase were used. Each point represents the mean for three separate experiments.

range), shortenings of the induction period are observed (Figure 3, traces b and c). Higher concentrations (near millimolar range) increase the induction period, which is followed by a burst that evolves towards the steady state (Figure 3, traces d and e) when all the ascorbic acid has been consumed. When the concentration of ascorbic acid is higher than that of any substrate of the enzyme, the activity cannot be monitored by dopachrome production. Monitoring the consumption of oxygen, shortenings of the induction period are observed for all the ascorbic acid concentrations studied (Figure 4, traces b and c), although when these are very high, the system never reaches the steady state (Figure 4, trace d). The pattern of dopa accumulation during the different reactions is also dependent on the concentration of ascorbic acid, as Figure 5 shows. When this is low, it results in a sigmoidal pattern of [D] versus time, reaching the value corresponding to [D]_{ss} (Figure 5, trace b) after all the ascorbic acid has been consumed. High concentrations result in an exponential pattern of [D] versus time (Figure 5, trace c), and therefore the steady state is never reached.

Relationship between the ascorbic acid concentration and the induction period generated by this agent in the diphenolase activity of tyrosinase

The diphenolase activity of tyrosinase under the experimental conditions used does not show an induction period [22].

When the diphenolase activity of tyrosinase acting on dopa, in the presence of ascorbic acid, is monitored by measuring dopachrome production, an induction period, the length of which depends on the ascorbic acid concentration, is observed. Analysing the material balance of the steady-state of the pathway:

$$2V_0t - [\text{ascorbic acid}] = [\text{DC}] + [\text{D}] \quad (1)$$

where $V_0 = d[\text{DC}]/dt$ is the rate of dopachrome production at time t , [DC] is the dopachrome concentration and [D] is the dopa concentration. Since one molecule of dopa produces one of dopachrome, eqn. (1) results in:

$$2V_0t - [\text{ascorbic acid}] = 2[\text{DC}] \quad (2)$$

When $t = \tau$ (induction period), [DC] = 0, and so:

$$2V_0\tau = [\text{ascorbic acid}] \quad (3)$$

Eqn. (3) establishes a direct relationship between the length of the induction period and the ascorbic acid concentration, as Figure 6 shows.

Figure 6 Relationship between [ascorbic acid] and length of the induction period, τ , induced in the diphenolase activity of tyrosinase

2 mM dopa, 0.26 mM O_2 and 0.5 nM tyrosinase were used. Each point represents the mean for three separate experiments.

DISCUSSION

In the light of the results obtained in our experiments using different methods to measure enzymic activity, the effect of ascorbic acid on the monophenolase activity of tyrosinase might be explained by its reducing action on the enzymically generated *o*-quinones during the reaction, though a direct interaction between the copper ions of the protein and ascorbic acid cannot be ruled out. The former hypothesis seems to be supported by the fact that some authors [13,14] use high concentrations of ascorbic acid to measure the diphenol formed by reduction of the enzymically produced quinones as a way of estimating monophenolase activity. However, this use has its disadvantages, which will be discussed below. The steady state of the monophenolase activity of tyrosinase acting on tyrosine is characterized by a constant $[D]_{ss}$ during the action of the enzyme on tyrosine [7,8]. The evolution of the system up to the steady state follows the steps previously described (see the Results section), which result in the characteristic induction period of this enzyme activity. One of these steps is the generation of dopa from *o*-dopaquinone- H^+ in a non-enzymic reaction. Thus either the addition of dopa at the start of the reaction or the formation of dopa in another reaction simultaneous with the enzymic reaction influences the evolution of the system until it reaches the steady state, resulting in variations of the induction period. The presence of ascorbic acid in the medium produces this last type of reaction. It generates dopa by the reduction of *o*-dopaquinone- H^+ at a rate which is higher than that which occurs in its absence, in which dopa is generated from the reaction between this quinone and 2,3-dihydro-5,6-dihydroxyindole-2-carboxylate (leukodopachrome) (see Scheme 1), resulting in a shortening of the induction period. When this effect is monitored by measuring dopachrome production, low concentrations of ascorbic acid have been found to shorten the induction period, whereas high concentrations result in increases followed by a burst. The explanation is that the reductant is rapidly consumed in the first case, permitting dopachrome formation, whereas high concentrations need longer times to be totally consumed, resulting in apparent increases in the induction period. When all the ascorbic acid is consumed, the concentration of accumulated dopa is higher than $[D]_{ss}$ and, therefore, the system evolves towards the steady state through a burst, in a similar way to that which occurs when an equivalent dopa concentration is added at the start of the reaction. This theory is confirmed by the oxygen-consumption method and by the estimation of the dopa formed during the reaction. The polarographic assays show shortenings of the induction period at all the concentrations investigated,

revealing that the system cannot reach the steady state when the ascorbic acid concentration is higher than the concentration of either substrate of the enzyme (tyrosine or oxygen), since these are consumed before the depletion of the reductant. This would explain the increase in the rate of oxygen uptake observed by Krueger [12] when he used a high concentration of ascorbic acid. The exponential pattern of dopa accumulation in the reaction confirms that the steady state cannot be reached under these conditions. Thus the addition of an excess of ascorbic acid (or other reducing agents) to measure the monophenolase activity of tyrosinase by estimating the diphenol formed [13,14] can only be used as a fixed-time method. It would not permit the study of this activity from a kinetic point of view, since the steady state is never reached.

As regards the effect of ascorbic acid on the diphenolase activity of tyrosinase, the relationship established between the concentration of the former and the induction period generated by it, could be used as a basis for the elaboration of an easy and simple kinetic method to determine trace amounts of this agent in solutions and crude biological extracts. Only the limitations of current spectrophotometers would limit the sensitivity of this method, which is capable of detecting ascorbic acid in micromolar concentrations. In biological samples, the possible interference of other reducing agents and enzymes must be taken into account.

In conclusion, ascorbic acid is an effective reducing agent which, at high concentrations, can momentarily retard the melanin-biosynthesis pathway, but never eliminate it. On the contrary, the resultant accumulation of diphenol produces an indirect activation on this pathway when the reductant is completely depleted. This should be taken into account in the elaboration of protective creams which include ascorbic acid or other reducing and depigmenting agents in their composition.

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