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pH-dependent interconversion of two forms of tyrosinase in human skin

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1. We have shown that the characteristic lag in cresolase activity of human skin tyrosinase at inhibitory concentration of tyrosine was absent at all pH values studied, i.e. pH 5.2, 5.7, 6.2 and 6.8, if the enzyme solubilized at low pH was used as the source of enzyme, but the same enzyme when dialysed against buffers of various pH values showed linear activity only at pH 5.2 and was not inhibited by excess tyrosine, whereas at higher pH values it exhibited a lag and inhibition by excess tyrosine. 2. However, the enzyme solubilized in buffer/detergent, pH 6.8, when dialysed against buffer of the same pH showed linear activity at pH 5.2 and non-linear activity at pH 6.8. 3. The water/detergent-solubilized enzyme from human skin melanosomes showed linear activity even at inhibitory concentrations of tyrosine at pH 5.2 and 6.8 up to 2 h, but acceleration of rate was observed after 2 h for the enzyme measured at pH 6.8. 4. After dialysis of the water/detergent-solubilized enzyme against double-glass-distilled water, it still exhibits linear activity at inhibitory concentration of tyrosines at pH 6.8 for the first 2 h, but the same enzyme when dialysed against 0.02 M-sodium phosphate buffer, pH 6.8, exhibits negligible activity up to $\frac{1}{2}$ h, in contrast with considerable activity before dialysis during the same interval of time, but without any loss of activity at later intervals of incubation time. 5. On the basis of these results, it is concluded that the enzyme exists in at least two interconvertible forms, one without lag and inhibition by excess tyrosine and the other with lag and inhibition by excess tyrosine. These two forms are interconvertible only by gradual change in pH over a period of hours.

INTRODUCTION

Tyrosinase (monophenol,dihydroxyphenylalanine:O_2 oxidoreductase, EC 1.14.18.1) catalyses the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (dopa) and oxidation of dopa to dopaquinone [1–5]. Tyrosinase from many sources was known to exhibit three characteristic properties: (*a*) a lag in cresolase activity, (*b*) a requirement for dopa as an essential cofactor, and (*c*) the inhibition of cresolase activity by excess tyrosine [1–11]. These properties, also observed for the enzyme from the human skin epidermis [12] and B-16 murine melanoma [13,14], were absent when these enzymes were partially purified by a single-step affinity-chromatographic procedure [12–14]. It was shown that the characteristic lag and inhibition by excess tyrosine of cresolase activity of tyrosinase from B-16 murine melanoma were absent at pH 5.0 [13,14].

In the present paper, it was demonstrated that both forms of the enzyme can be found in human skin epidermis if it was solubilized in water/detergent. These two forms are shown to be interconvertible by appropriate gradual change in pH by dialysis. The physiological implications of these results on regulation of melanin biosynthesis in human skin are discussed.

MATERIALS AND METHODS

L-tyrosine, L-ascorbic acid, L-dopa and bovine serum albumin were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Dialysis tubing was purchased from Thomas Scientific Co., Philadelphia, PA, U.S.A. The non-ionic detergent Igepal Co. 630 (GAF Corp., New York, NY, U.S.A.) was kindly given by Professor S. H. Pomerantz, Department of Biological Chemistry, University of Maryland, Baltimore, MD, U.S.A. All other chemicals were of analytical-reagent grade. Double-glass-distilled water with conductance corresponding to less than 0.5 p.p.m. as NaCl was used throughout the study.

Human cadaver skin was taken from accident cases which are kept cold, and material was removed within 48 h after the accident.

Preparation of pH 5.2 solution

The pH 5.2 solution at 0.005 M-NaH_2PO_4 was made by adjusting its pH to 5.2 by addition of 0.005 M-Na_2HPO_4.

Buffers

The 0.005 M-sodium phosphate buffers, pH 6.2 and 6.8, were made by mixing NaH_2PO_4 and Na_2HPO_4 to give a final concentration of 0.005 M. The required pH values were attained by addition of either 1 M-HCl or 1 M-NaOH. The 0.02 M-sodium phosphate buffer, pH 6.8, was made similarly, except that the final concentration of phosphate was 0.02 M.

The citrate/phosphate buffers, pH 5.2, 5.7, 6.2 and 6.8, were made by mixing citric acid and Na_2HPO_4 solutions adjusted to the respective pH values by addition of 1 M-NaOH. The final concentration of citrate and phosphate were 0.125 M and 0.25 M respectively.

Abbreviation used: dopa, 3,4-dihydroxyphenylalanine

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Table. 1. Specific activities and yield of tyrosinase solubilized by three different methods from melanosomal fraction of human cadaver skin

The conditions of assay for methods 1(a) and 1(b) were the same as described in Fig. 1, except that the activity was measured at 0.068 M-citrate/0.136 M-phosphate, pH 5.2. The conditions of assay for method 2(a) were the same as described in Fig. 1, except that activity was measured at 0.068 M-citrate/0.136 M-phosphate, pH 6.8. The activity for method 2(b), measured at 0.022 M-citrate/0.045 M-phosphate, pH 6.8, accelerates with time, as shown in Fig. 1. The conditions of assay for method 3(b) were the same as described in Fig. 5. Maximum rates were taken for calculations of specific activities.

Method of solubilization	Activity	
	Specific activity (units/mg of protein)	(units/g of epidermis)
1(a) pH 5.2 supernatant after sonication	200–295	69–155
(b) pH 5.2 pellet after sonication, treated with detergent at pH 5.2	17500	700
2(a) pH 6.8 supernatant after sonication	114	65.4
(b) pH 6.8 pellet treated with detergent	1584	776
3(a) Supernatant after sonication in double-glass-distilled water	Nil	Nil
(b) Water/detergent extract	1300–1536	130–264

Preparation of melanosomal fraction

This was done from human cadaver epidermal scrapings as described by Vijayan *et al.* [12], except that the pellet containing melanosomes was suspended in citrate (0.125 M) phosphate (0.25 M) buffer, pH 5.2 or 6.8, or double-glass-distilled water.

Solubilization of tyrosinase from melanosomal fraction

The pellet containing melanosomes was suspended in citrate/phosphate buffer, pH 5.2 or 6.8, or double-glass-distilled water and was sonicated with a Sonifier Cell Disruptor (B-30; Branson Sonic Power Co., Danbury, CT, U.S.A.) at 75 mV for 30 s; the tube was kept in ice. It was then centrifuged at 10000 ***g*** for 15 min (Varifuge-K centrifuge; Heraeus Christ Co., Germany). The supernatant solutions contained tyrosinase in the case of the melanosomal pellet suspended in citrate/phosphate buffers, pH 5.2 or 6.8, but much more tyrosinase was solubilized at pH 5.2 or 6.8 after the pellet thus obtained was kept overnight with Igepal at 0–4 °C. But the supernatant in the case of the melanosomal pellet suspended in double-glass-distilled water showed no tyrosinase activity. Therefore, the pellet obtained after sonication was further suspended in double-glass-distilled water containing 1 % (v/v) Igepal (0.4 ml/g of epidermis) and kept overnight at 0–4 °C, and was centrifuged at 10000 ***g*** for 15 min at 0–4 °C, and the supernatant, which contained most of the tyrosinase, was used for the studies in the case of the water/detergent-solubilized enzyme.

Dialysis

The supernatants containing tyrosinase were dialysed wherever mentioned as follows.

(*a*) The supernatant obtained at pH 5.2 was dialysed against 1 litre of either 0.005 M-NaH_2PO_4, pH 5.2, or 0.005 M-sodium phosphate buffers, pH 6.2 or 6.8, for 15–17 h, with one change of solution or buffers after 6 h at 0–4 °C.

(*b*) The supernatant obtained at pH 6.8 was dialysed against 1 litre of 0.005 M-NaH_2PO_4, pH 5.2. This in turn was dialysed against 0.005 M-sodium phosphate buffer, pH 6.8, at 0–4 °C for experiments on reversibility of the two forms of enzyme at pH 5.2 and 6.8 (Figs. 1 and 2).

(*c*) The water/detergent-solubilized supernatant was dialysed against 1 litre of double-glass-distilled water for 6 h, with one change of water after 3 h at 0–4 °C, since a longer time of dialysis results in inactivation of the enzyme.

(*d*) The water/detergent-solubilized supernatant was dialysed against 1 litre of 0.02 M-sodium phosphate buffer, pH 6.8, for 6 h, with one change of buffer after 3 h at 0–4 °C.

The precipitate that resulted during dialysis in the dialysis bag (in *a–d* above) was removed by centrifugation at 10000 ***g*** for 10 min at 0–4 °C, and the supernatants were used as the sources of enzyme.

Protein determination

Protein was measured by the micro-assay method of Peterson [15].

Measurement of tyrosinase activity

Tyrosinase activity was assayed by the measurement of dopa formed from tyrosine by a fluorimetric method [16] as described by Husain *et al.* [2,17], except that ascorbic acid was used as reductant at 0.004 M instead of at 0.75 mM in the reaction mixture. Ascorbic acid in this assay procedure could act as a reductant in the hydroxylation of tyrosine [13,14] and at the same time reduces any dopaquinone formed by tyrosinase back to dopa, so that measurement of dopa is a true estimate of cresolase activity of tyrosinase.

The reaction was conducted in air in rimless tubes (12 mm × 100 mm) in duplicate, kept in a water bath at 37 °C. The duplicate values agree within ±10 %. A unit of enzyme is defined as 1 pmol of dopa formed/min under the conditions described in the legends to Figures and Tables.

RESULTS

Solubilization of enzyme by three different procedures

The tyrosinase from the melanosomal fraction was solubilized in three different ways. The specific activities

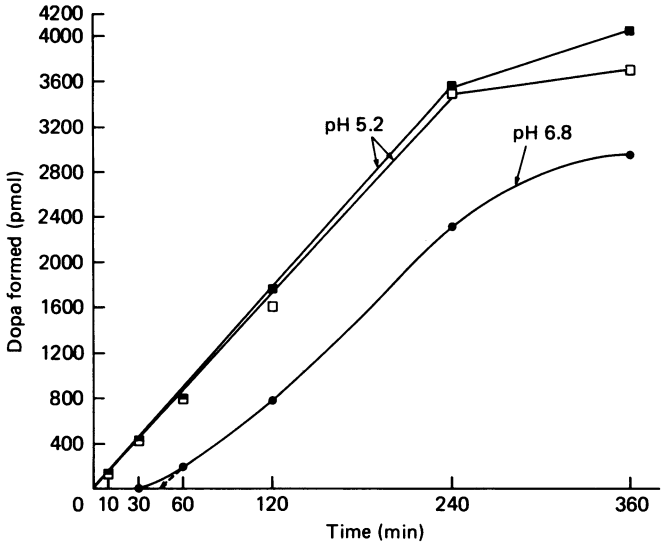

Fig. 1. Time course of cresolase activity of tyrosinase solubilized by buffer/detergent, pH 6.8

The reaction mixture contained, at 37 °C, the following components (final concns.) in a total volume of 55 μl: L-tyrosine, 0.0016 M: ascorbic acid, 0.004 M; citrate, 0.022 M; phosphate 0.045 M. (a) Curve (■), enzyme solubilized at pH 6.8 and adjusted to pH 5.2 by dialysis as described in the Materials and methods section. The enzyme activity was measured at pH 5.2 and protein concentration was 90 μg/ml of reaction mixture. (b) Curve (□), the enzyme used in (a) was returned to pH 6.8 as described in the Materials and method section, and its activity was measured at pH 5.2 and at pH 6.8 (●). Protein concentration was 52 μg/ml of reaction mixture.

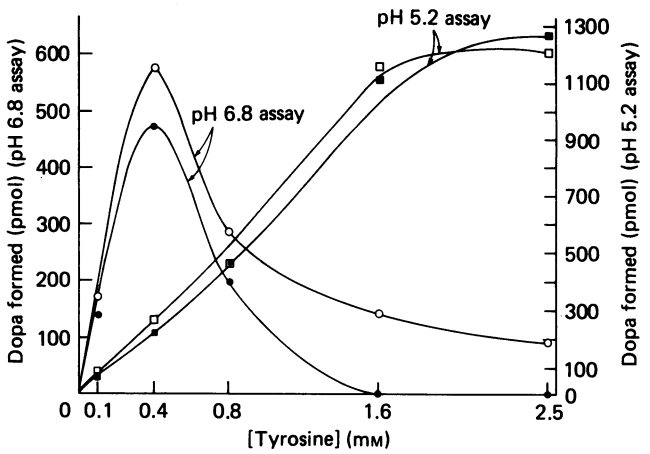

Fig. 2. Effect of tyrosine concentration on the cresolase activity of tyrosinase solubilized at pH 6.8

The enzyme solubilized by buffer/detergent, pH 6.8, was dialysed against sodium phosphate buffer, pH 6.8, and was used as source of enzyme. The conditions of assay were the same as described in the legend to Fig. 1, except that the time of incubation was fixed at $\frac{1}{2}$ h and the concentration of tyrosine was varied. (a) Curve (●), the enzyme activity was measured at pH 6.8. Protein concentration was 338.0 μg/ml of reaction mixture. (b) Curve (■), the enzyme solubilized at pH 6.8 was adjusted to pH 5.2 by dialysis as described in the Materials and methods section. The enzyme activity was measured at pH 5.2 and protein concentration was 290 μg/ml of reaction mixture. (c) The enzyme used in (b) at pH 5.2 was returned to pH 6.8 by dialysis as described in the Materials and method section. The enzyme activity was measured at pH 6.8 (curve ○) and at pH 5.2 (curve □). Protein concentration was 172.7 μg/ml of reaction mixture.

and yield per g of tissue obtained by these procedures are presented in Table 1. Some enzyme can be solubilized by simple sonication of melanosomes in citrate/phosphate buffers, pH 5.2 or 6.8, but not in double-glass-distilled water, where the enzyme is released by treatment with detergent only. Even in the case of melanosomes sonicated in citrate/phosphate buffer, much of the enzyme was released only after detergent treatment. Treatment of melanosomes with citrate/phosphate buffer, pH 5.2, and 1 % (v/v) Igepal results in solubilization of a larger amount of enzyme with high specific activity, and perhaps is the optimum condition for solubilization of the enzyme for its further purification.

Absence at pH 5.2 of the characteristic lag in cresolase activity of tyrosinase

The time course of cresolase activity of tyrosinase solubilized at pH 6.8 and dialysed against 0.005 M-sodium phosphate buffer, pH 6.8, was determined at pH 6.8 and 5.2, and the results are presented in Fig. 1. The characteristic lag in cresolase activity was seen at pH 6.8, but not at pH 5.2.

Absence of inhibition by excess tyrosine of cresolase activity of tyrosinase at pH 5.2

The cresolase activity of enzyme solubilized under conditions described in Fig. 1 was estimated at a fixed time of $\frac{1}{2}$ h at different concentrations of tyrosine at both pH 6.8 and pH 5.2, and the results are presented in Fig. 2. The human skin tyrosinase was not inhibited by excess tyrosine at pH 5.2, whereas, as was shown previously [12] and in Fig. 2, it is inhibited by excess tyrosine at pH 6.8.

These results indicate that loss of lag at pH 5.2 may be related to loss of inhibition by excess tyrosine. The characteristic lag that was observed in cresolase activity of tyrosinase at pH 6.8 (Fig. 1) was not due to hysteretic behaviour of the enzyme resulting either from slow dissociation of an oligomeric enzyme, dissociated enzyme being more active, or to slow conformational change of the enzyme, for the following reasons.

(1) If dissociation was involved, the lag should increase with increasing concentration of enzyme, and vice versa, but actually the lag decreases with increasing concentration of enzyme [37].

(2) If slow conformational change is involved, the lag should remain unaffected by enzyme concentration, but actually the lag decreases with increasing concentration of enzyme. The lag was also decreased when its activity was measured in the presence of dopa [2] and was completely absent when dopa was added in sufficient concentration to the enzyme preparation from other sources [6,7]. Therefore, the lag could be interpreted as due to inhibition by excess tyrosine and its reversal by dopa that accumulates during the course of the reaction. It could also be explained by association of the enzyme by dopa, associated enzyme being more active. But results presented in Fig. 3 discount such a possibility.

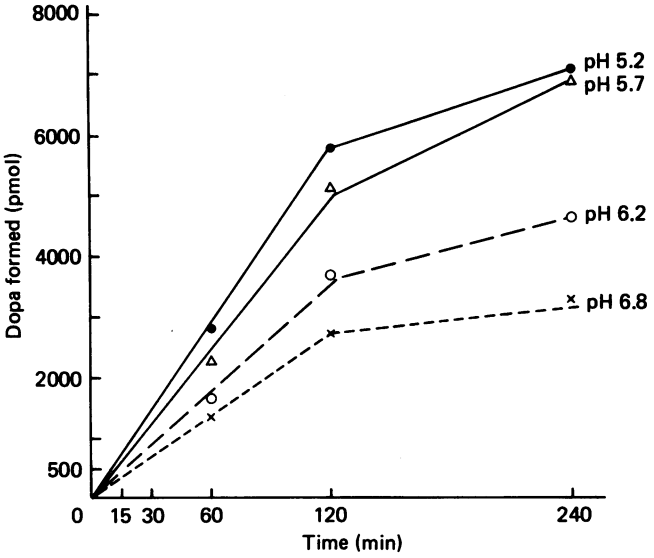

Fig. 3. Time course of cresolase activity at various pH values of tyrosinase solubilized by buffer/detergent, pH 5.2

The enzyme solubilized by buffer/detergent, pH 5.2, and dialysed against sodium phosphate solution, pH 5.2, as described in the Materials and methods section was the source of enzyme. The conditions of assay were the same as described in the legend to Fig. 1, and the enzyme activity was assayed at various pH values. Protein concentration was 185.4 μg/ml of reaction mixture at all pH values.

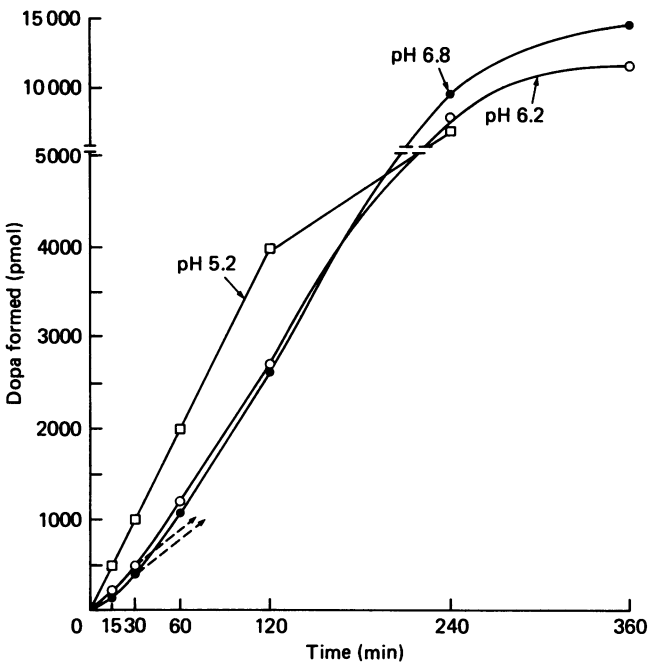

Fig. 4. Time course of cresolase activity of tyrosinase solubilized by buffer/detergent, pH 5.2, and adjusted to various pH values by dialysis and assayed at the corresponding pH values

The enzyme solubilized by buffer/detergent, pH 5.2, and dialysed against buffers of various pH values as described in the Materials and methods section was used as the source of enzyme at corresponding pH values. The conditions of assay were the same as described in the legend to Fig. 1, and the enzyme activity was assayed at different pH values. Protein concentration was 41.8, 134.5 and 123.6 μg/ml of reaction mixture at pH 5.2 (□), pH 6.2 (○) and pH 6.8 (●) respectively.

Interconversion of two forms of enzyme

To see whether the enzyme at pH 5.2, which did not exhibit lag and was not inhibited by excess tyrosine, could be converted by increase in pH into a form with lag and inhibition by excess tyrosine, the enzyme from the human skin melanosomal fraction was solubilized at pH 5.2 in the presence of detergent as described in the Materials and methods section. The enzyme was dialysed against 0.005 M-sodium phosphate solution, pH 5.2, and its cresolase activity was estimated at various pH values. These results are presented in Fig. 3; clearly, at all pH values, the enzyme activity was linear even at inhibitory concentrations of tyrosine for the first 2 h and declined at later intervals. These results rule out the possibility of association of enzyme at pH 6.8 by dopa that accumulates during the reaction, associated enzyme being more active, as an explanation for the lag that was observed in Fig. 1 at pH 6.8. These results also rule out the possibility of two or more non-interconvertible forms of enzyme being present in the impure preparation of enzyme used in these studies, such that they respond differentially to the changes in pH, since at all pH values there was neither lag nor inhibition by excess tyrosine. Moreover, partially purified tyrosinase from B-16 murine melanoma or partially purified mushroom tyrosinase were shown to be reversibly interconverted by appropriate change in pH ([13, 14; C. Chaya Devi, A. Ramaiah & R. K. Tripathi, unpublished work).

One form of enzyme could be converted into the other form only by gradual change in pH

To see whether conversion of the form not inhibitable by tyrosine and without lag into the tyrosine-inhibitable form with lag could be brought about by exposure of enzyme to higher pH values over an extended period, the enzyme was adjusted to pH 6.8 by adding a portion of enzyme to buffer at pH 6.8 and kept at 0–4 °C for 6 h, and then its activity was measured at pH 6.8. It was found to be in the tyrosine-uninhibitable form and without lag. Therefore it was thought possible that enzyme may require still more time for conversion into the tyrosine-inhibitable form, so the enzyme was kept at pH 6.8 in frozen state (−20 °C) overnight and again kept at 0–4 °C for 6 h, and then its activity was measured at pH 6.8, but the enzyme was still in the tyrosine-uninhibitable form and without lag. Even after storage of this enzyme at −20 °C for a further 24 h, the enzyme still exhibited properties characteristic of the enzyme assayed at pH 5.2, i.e. no lag and no inhibition by excess tyrosine (results not shown). Whether the conversion of this enzyme into the tyrosine-inhibitable form with lag can be accomplished by a gradual increase in pH was therefore investigated. The enzyme at pH 5.2 was dialysed against buffers of pH 5.2, 6.2 and 6.8 over a period of hours, and then activity of the enzyme was measured at various fixed pH values at various intervals of time. These results are presented in Fig. 4; under these conditions tyrosinase shows a characteristic lag in its cresolase activity at pH 6.2 or 6.8. This enzyme is also inhibited by excess tyrosine (results not shown) when

Table 2. Effect of citrate on cresolase activity of tyrosinase from melanosomal fraction at various pH values at 0.0016 M-tyrosine

The assay conditions were the same as described in Fig. 3, except that in the absence of citrate, the activity was measured at 0.04 M-sodium phosphate solution, pH 5.2, and 0.04 M-sodium phosphate buffers, pH 6.2 and 6.8. The initial linear rates were taken for the calculations of specific activities. Values for '+Citrate' are taken from the data presented in Fig. 3. The same enzyme preparation was used for this study. In the absence of citrate also, no lag in cresolase activity and no inhibition by excess tyrosine of cresolase activity were observed.

pH of assay medium	Specific activity (units/mg of protein)	
	No citrate	+Citrate
5.2	1158	4718
5.7	–	4167
6.2	4355	2971
6.8	5461	2196

assayed at pH 6.2 or 6.8. Thus the conversion of a form of enzyme without lag and inhibition by excess tyrosine into the form with these properties is accomplished only by gradual increase in pH over a period of hours. Similarly, the conversion of the form of enzyme with lag and inhibition by excess tyrosine into the form without them is accomplished by gradual decrease in pH to 5.2 (Figs. 1 and 2). Thus one form of enzyme can be converted into the other form reversibly by appropriate gradual change in pH. However, rapid decrease in pH of the enzyme solution from 6.8 to 5.2 by direct addition of acid brings about an irreversible modification of the enzyme which does not exhibit either lag or inhibition by excess tyrosine either at pH 5.2 (Figs. 1 and 2) or at pH 6.8 (results not shown).

pH optimum of the form of enzyme without lag and without inhibition by excess tyrosine is 6.8

From the data presented in Fig. 3, it appears that the pH optimum of the form of enzyme which does not exhibit lag and inhibition by excess tyrosine is 5.2, whereas the tyrosine-inhibitable form of enzyme with lag, so far known in the literature [1–11] and also observed by us independently, had an optimum pH 6.8 in phosphate buffer. The results presented in Fig. 3 were obtained in citrate/phosphate buffers. Therefore, whether the shift in pH optimum was real was tested by using sodium phosphate solution, pH 5.2, and sodium phosphate buffers, pH 5.7, 6.2, 6.8 and above. These results were presented in Table 2. In the absence of citrate the pH optimum is 6.8 for the tyrosine-uninhibitable form of enzyme without lag also, and in the presence of citrate the apparent shift in pH optimum to 5.2 was due to the stimulatory effect of citrate at pH 5.2 or 5.7 and inhibition by it, at 0.022 M, at pH 6.2 or 6.8, as shown in Table 2. In sodium phosphate solution at pH 5.2, the lower activity in the absence of citrate was not due to lack of buffering, since a similar value was obtained in the presence of acetate buffer at pH 5.2.

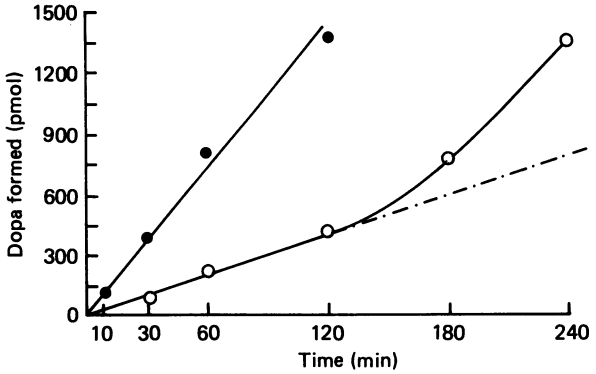

Fig. 5. Time course of cresolase activity of tyrosinase solubilized by water/detergent

The enzyme was solubilized by water/detergent and was used as such without any dialysis. The conditions of assay were the same as described in the legend to Fig. 1. The enzyme activity was measured at pH 5.2 (●) and at pH 6.8 (○). Protein concentration was 156.7 μg/ml of reaction mixture at both pH values.

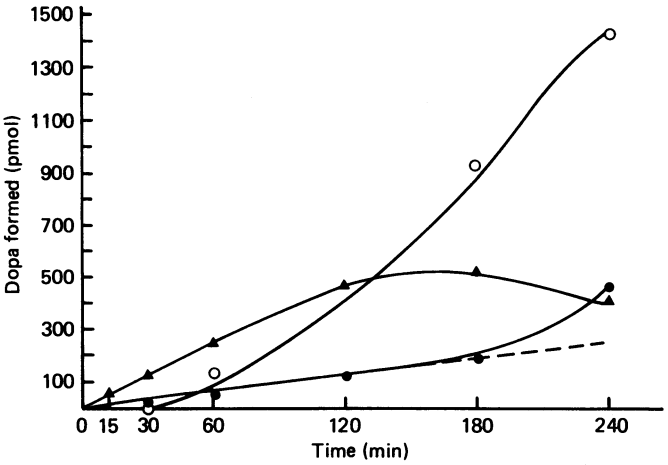

Fig. 6. Time course of cresolase activity of tyrosinase solubilized by water/detergent and dialysed against double-glass-distilled water or 0.02 M-sodium phosphate buffer, pH 6.8

The conditions of assay were the same as described in the legend to Fig. 1, except that the enzyme activity was assayed at pH 6.8 only. Protein concentration was 101.8 μg/ml (●), 92.7 μg/ml (▲) and 69.1 μg/ml (○) of reaction mixture respectively. ●, Activity measured at pH 6.8. ▲, Enzyme extracted in water/detergent, then dialysed against double-distilled water and its activity measured at pH 6.8. ○, Enzyme extracted in water/detergent, then dialysed gainst 20 mM-sodium phosphate buffer, pH 6.8, and activity measured at pH 6.8.

Time course of cresolase activity of tyrosinase solubilized in water/detergent

When the enzyme was solubilized either at pH 6.8 or at pH 5.2, only the form of enzyme with characteristic lag or the form of enzyme without lag, respectively, were observed. However, both forms may be present in the intact melanosomes, but during the process of dialysis over a period of many hours one of the forms might have been converted into the other, depending on the pH of

the buffer used for dialysis. It was thought possible that, if the enzyme was solubilized in water/detergent and its activity was measured without dialysis at pH 6.8, both the forms of enzyme could be seen, since the conversion of the form of enzyme without lag and inhibition by excess tyrosine is obtained by gradual change in pH (Figs. 3 and 4). Therefore, the enzyme was solubilized in water/detergent and its activity was measured at pH 6.8 as well as at pH 5.2. The results are presented in Fig. 5; at pH 6.8 the rate is linear up to 2 h, followed by acceleration, whereas at pH 5.2 the rate is higher than the initial rate at pH 6.8, but is linear without any acceleration up to 2 h and declines after 2 h (results on decline in rate at pH 5.2 not shown in Fig. 5). The undialysed enzyme preparation did not contain any detectable dopa, and therefore the linear activity seen in the first 2 h at pH 6.8 (Fig. 5) may actually be due to a form of enzyme seen in Fig. 3, which does not exhibit lag and is not inhibited by excess tyrosine. That this is perhaps true is indicated by the fact that, when the enzyme was dialysed against pH 6.8 buffer and then its activity was measured, the activity of the enzyme in the first $\frac{1}{2}$ h was actually zero (Fig. 6). This was not due to loss of enzyme activity owing to dialysis, since the activity at later time intervals is higher than the activity of the enzyme before dialysis. Similarly, dialysis of the enzyme against double-glass-distilled water results in more activity, and yet the activity was linear for the first 2 h and then declined, indicating that the form of enzyme without lag and inhibition by excess tyrosine still exists under these conditions.

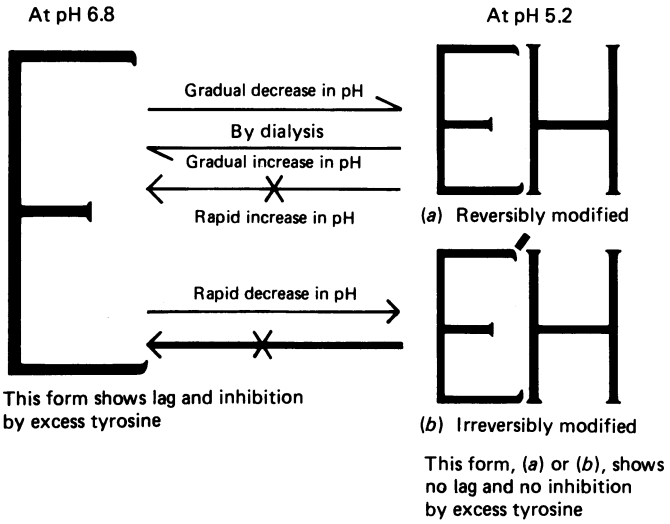

Fig. 7. Schematic representation of change in properties of tyrosinase (E) related to effects of change in pH

DISCUSSION

The lag in cresolase activity of tyrosinase was shown to be a characteristic property of this enzyme from many sources [1–11]. This lag was interpreted in the literature as being due to competition between tyrosine and dopa to bind at the dopa-activator site, based on the following evidence:

(1) if the concentration ratio of tyrosine to dopa is 10 or less, lag was abolished irrespective of the actual concentrations of tyrosine, and thus is indicative of competitive inhibition [1,6,7].

(2) The lag decreases with increasing concentration of dopa at any fixed concentration of tyrosine, whereas at any fixed concentration of dopa lag increases with increasing concentration of tyrosine [10,13,14,37].

Our studies in the present paper at pH 5.2 show linear activity with time, and the activity was not inhibited by excess tyrosine (Figs. 1 and 2), suggesting that ascorbic acid acts as reductant at this pH and dopa does not increase activity, and therefore there was no dopa-activator site at pH 5.2. Therefore at pH 6.8 also, ascorbic acid may act as reductant and activation of enzyme by dopa at pH 6.8 may be interpreted as due to association of enzyme at pH 6.8 by dopa that accumulates during the reaction, associated enzyme being more active. However, the fact that enzyme solubilized at pH 5.2 and assayed at pH 6.8 does not show lag and inhibition by excess tyrosine (Fig. 3) suggests that perhaps association of enzyme is not involved. At pH 5.2, the enzyme activity was linear with time and proportional to enzyme concentration over a wide range of the latter (42–479 μg of protein/ml of reaction mixture), and therefore the possibility of enzyme associating at this pH and resulting in properties different from that assayed at pH 6.8 is ruled out. Therefore activation of enzyme by dopa at pH 6.8, inhibition of enzyme activity by excess tyrosine (Figs. 1 and 2) and the previously published data on tyrosinase may be better interpreted as follows.

At pH 6.8, there is an allosteric site for tyrosine on the enzyme. Binding of tyrosine at this site produces inhibition, and dopa can compete with tyrosine for this site and thus relieve inhibition. The acceleration of rate with time at pH 6.8 is therefore due to the product dopa that accumulates during the reaction gradually reversing the inhibition caused by binding of tyrosine at the allosteric site, as depicted in the model (Fig. 7).

At pH 5.2, tyrosine has little or no affinity for its allosteric site and has decreased affinity at its catalytic site (Fig. 2), and therefore there is neither inhibition by excess tyrosine nor lag, since under these conditions dopa has no effect on enzyme activity. Excess ascorbic acid present in the assay mixture acts as reductant required for the tyrosine hydroxylation. A similar phenomenon was observed for tyrosinase from B-16 murine melanoma [13,14] and mushroom (C. Chaya Devi, R. K. Tripathi & A. Ramaiah, unpublished work). In the case of mushroom tyrosinase, the enzyme is interconverted by rapid change in pH. However, in the case of tyrosinase from human skin, the enzyme at pH 5.2 should be gradually adjusted to pH 6.8 by dialysis to regain the inhibitory allosteric site on tyrosinase (Fig. 4), and not by rapid increase in pH. Rapid decrease in pH from 6.8 to 5.2 results in irreversible modification of enzyme to the form without lag and inhibition by excess tyrosine. The enzyme at these two different pH values may differ in conformation, resulting in altered affinity for tyrosine at its allosteric and catalytic sites. This change in conformation may also be responsible for the higher stability of enzyme at pH 6.8 for a period of hours, as compared with the enzyme at pH 5.2–5.7, where it was not stable at 37 °C beyond 2 h (Figs. 3, 5 and 6). That these two forms of enzyme may actually exist under conditions *in vivo* is indicated by the results presented in Fig. 6.

Physiological role of these two pH-dependent interconvertible forms of tyrosinase

The initial higher activity of enzyme at low pH values and in the physiological range of tyrosine concentration, which was found to be in the millimolar range (17–19; R. K. Tripathi, C. Chaya Devi & A. Ramaiah, unpublished work), than at higher pH values may have physiological importance (Fig. 1). The intact melanosomes may have a proton gradient similar to that of lysosomes [20–28], and various agents which increase or decrease melanin biosynthesis may do so as a result of decrease or increase in pH, in addition to other mechanisms so far known [29–31]. Increased tyrosinase activity was demonstrable after a single dose of u.v. irradiation [32], and it was suggested that tyrosinase exists in an inactive or partially inhibited state and that after irradiation it was activated [33]. This increase in tyrosinase activity may be explained in terms of a decrease in pH of the intact melanosomes as a result of u.v. irradiation, and the elevated activity of tyrosinase which persists for some time after u.v. irradiation could be related to the process of conversion of the form of enzyme without lag and inhibition by excess tyrosine at low pH into the form with lag and inhibition by excess tyrosine, by increase in pH.

In addition, recently it was shown that addition of melanocyte-stimulating hormone to Cloudman S-91 mouse melanoma cells increases tyrosinase activity in intact cells by 90-fold, but the actual increase in the enzyme content was shown to be only 3-fold [34,35], suggesting the presence of inactive or less active enzyme in the cells. This confirms the previous finding by Wong & Pawelek [36], who demonstrated that this hormone promotes activation of pre-existing tyrosinase molecules in Cloudman S-91 mouse melanoma cells.

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