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MAMMALIAN TYROSINASE

STRUCTURAL AND FUNCTIONAL INTERRELATIONSHIP OF ISOZYMES

VINCENT J. HEARING, JESSE M. NICHOLSON, PAUL M. MONTAGUE, THOMAS M. EKEL and KENNETH J. TOMECKI

Dermatology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Md. 20014 (U.S.A.)

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Summary

The isozymes of tyrosinase from normal and malignant melanocytes were studied; the data indicates that each consists of a basic tyrosinase polypeptide, and differs by post-translational modifications. T_3 represents the de novo form of the enzyme; it is converted to T_1 in vivo by the addition of sialic acids and neutral sugars, and in turn, to T_4 by complexing with melanosomal membrane constituents. The T_2 isomer is suggested to be an artefact of the electrophoretic procedure, and due to deamidation of T_3. It is shown that the apparent kinetics of enzyme activity are unaffected by any of these modifications.

Introduction

The presence of multiple forms of tyrosinase in actively melanogenic tissues has been enigmatic for many years to those studying pigmentation in mammals [1—6]. The problem of whether these isozymes are products of different genetic loci, or whether they represent post-translational modifications of a single basic polypeptide, has hampered the identification of the specific function of many mutant genes affecting pigmentation [7,8]. In addition, the functional significance of the various forms of tyrosinase present in normal and malignant melanocytes is poorly understood. Initial structural studies indicated that there were significant differences in the amino acid contents and molecular weights of two of the isozymes, T_1 and T_3, [9] (suggesting that different loci coded each isozyme). More recently, evidence has been presented that these two isozymes are immunologically [10,11] and kinetically [2,4] similar, and possibly interconvertible by means of enzymatic modification [12—16] (suggesting a common basic unit, coded by a single locus). This study was initiated to characterize the interrelationships of the various isozymic forms of tyrosin-

ase more fully, and to determine the functional significance of the multiple forms of the enzyme.

Experimental Procedure

Sources of materials. The following were obtained from Sigma Chemical Co., St. Louis, Mo.: phospholipase A (EC 3.1.1.4); phospholipase C (EC 3.1.4.3); phospholipase D (EC 3.1.4.4); neuraminidase (EC 3.2.1.18); phospholidylcholine; lysophosphatidylcholine; L-3,4-dihydroxyphenylalanine. Phospholipase C and neuraminidase were also purchased from Grand Island Biological Co., Grand Island, N.Y., and from Boehringer Mannheim, New York, N.Y. Trypsin (EC 3.4.21.4), and protease (EC 3.4.4.5), were purchased from ICN Pharmaceuticals, Cleveland, Ohio (see Table I for additional information on the above biochemicals). Ampholines (Bio-Lyte 3/10) were from Bio-Rad Laboratories, Richmond, Calif. L-[3,5-^{3}H]tyrosine and L-[U-^{14}C]tyrosine were purchased from Amersham/Searle Corp., Arlington Heights, Ill.

Sources and preparation of tissues. Actively growing B-16 melanoma was used as the source of malignant melanocytes; this tumor was serially implanted subcutaneously in the thigh muscle of C57B1/6N mice. Dorsal epidermis from 5-day-old C57B1/6N mice was used as the source of normal melanocytes. Melanin granules were isolated from these tissues as previously described [17,18]. Briefly, the dissected tissues were homogenized in phosphate buffer (0.1 M, pH 7.4) at 4°C with a Waring Blendor and/or a TenBroeck glass:glass tissue grinder. These homogenates were then centrifuged at 500 × *g* for 5 min, and the supernatant was recovered and centrifuged at 10 000 × *g* for 20 min. The pellet was recovered and washed twice through 30% sucrose, and finally resuspended in 0.1% Triton X-100 for 1—5 min. The insoluble material was then sedimented at 10 000 × *g* for 20 min, and the supernatant was passed through a 0.45 μm Millipore filter. Protein determinations were performed by the method of Bramhall et al. [19]. This Triton X-100-soluble extract was then subjected to preparative polyacrylamide gel electrophoresis as described below for the isolation of tyrosinase isozymes. Purified isozymes were then treated with either water or the agents as listed in the legends of tables or figures for 30 min at 37°C; the isozymes were then either assayed or subjected to polyacrylamide gel electrophoresis as detailed below. Phospholipase A, phospholipase C, phospholipase D and neuraminidase were tested at a final concentration of 1 unit/ml; trypsin, protease, phosphatidylcholine and lysophosphatidylcholine were tested at a final concentration of 1 mg/ml. The unit of activity is the International Unit (I.U.); 1 unit of enzyme catalyzes the formation of 1 μmol product per min.

Cultured melanoma cells used were B-16 F_{10} melanoma cells [20] (courtesy of Drs. I. Fiddler and C. Dermody, of the Frederick Cancer Research Center, Frederick, Md.). These cells were harvested, washed by centrifugation, solubilized with 1% Triton X-100 for 5 min, and centrifuged at 10 000 × *g* for 20 min. These soluble extracts were applied directly to analytical gels for polyacrylamide gel electrophoresis or isoelectric focusing as detailed below.

Tyrosinase assays. Assays for both functions of tyrosinases were performed. Rates of tyrosine hydroxylation to 3,4-dihydroxyphenylalanine were carried

out with the Pomerantz [21] assay for the measurement of ^3H_2O formation; the rates of 3,4-dihydroxyphenylalanine oxidation to melanin were measured with the Kim and Tchen [22] assay, which follows the incorporation of [^{14}C]-tyrosine into acid-insoluble melanin. Both of these assays were done on a microscale as described in a previous paper [23]. Kinetic analyses of the enzymes were performed in the presence of 5 μM 3,4-dihydroxyphenylalanine as detailed in ref. 23 and reported kinetic values were estimated by a computer program using least-squares weighted multiple linear regression analysis of Eadie-Hofstee plots.

Polyacrylamide gel electrophoresis. Analytical and preparative polyacrylamide gel electrophoresis were performed on the samples as described previously [24]. This method employs a Tris/glycine buffer system similar to that initially described by Davis [25]. For routine analysis, 7.5% acrylamide gels were run with approx. 8-cm separation gels, 1-cm concentration gels, at 2 mA/tube at 20°C, until the bromphenol blue-tracking dye neared the bottom of the tube. The gels were then cut at the bromphenol blue front and stained by either the Coomassie Blue G stain for proteins [26], or for the demonstration of tyrosinase activity by incubation at 37°C for 30 min in 3,4-dihydroxyphenylalanine (1 mg/ml) in phosphate buffer (0.5 M, pH 7.4) [2]. The gels were then fixed and stored in 7.5% acetic acid.

For preparative applications, the proteins were electrophoresed in a LKB 7900 Uniphor electrophoresis apparatus as described by Hearing et al. [27]. This method employs a 7.5% acrylamide separation gel (5 cm high by 2.5 cm diameter), with a 2.5% concentration gel (4 cm high by 2.5 cm diameter). The sample, containing 40—50 mg protein in a 20 ml volume, was electrophoresed at 20°C at 15 mA. Fractions were collected from the bottom of the gel at an elution rate of 12—15 ml/h. Isozymes were then identified by radioassay and/or by analytical polyacrylamide gel electrophoresis and 3,4-dihydroxyphenylalanine staining as described above.

Isoelectric focusing was performed in the following manner: 10-cm gels were made, which contained 7.5% acrylamide, 3% bisacrylamide, 5% glycerol and 2% ampholines (pH 3—10). After polymerization, the samples (with 10% sucrose) were applied, overlayered with 5% sucrose, and electrophoresis was done at 4°C overnight (18 h) at 200 V. The upper buffer was 0.04 M NaOH, the lower buffer 0.02 M H_2SO_4. After focusing was completed, the pH gradients were measured with a Bio-Rad gel pro-Philer, and the gels were stained with either Coomassie Blue G or 3,4-dihydroxyphenylalanine as described above.

Miscellaneous. Sialic acid was measured by the thiobarbituric acid assay of Warren [28]. Statistical analyses were performed using the Student's *t*-test, assuming unequal variance.

Results

Table I shows the effects of the various agents on the enzymatic functions of purified T_1 tyrosinase; the numbers used for each type of treatment listed in Table I are maintained throughout the remainder of the figures, tables and text. A discussion of the isozyme nomenclature for tyrosinase is presented in Discussion. None of the phospholipases used (A, C or D) consistently altered either

TABLE I

EFFECTS OF ENZYMATIC AGENTS ON THE RATES OF TYROSINE HYDROXYLATION AND MELANIN FORMATION BY PURIFIED T_1 TYROSINASE

Approx. 0.002 I.U. of purified T_1 tyrosinase were pretreated with the listed agents as described in Experimental Procedure, then assayed in the presence of 5 μM 3,4-dihydroxyphenylalanine. The S.D. was less than 5% in all cases, number of experiments, 4. Sources: S, Sigma Chemical Co.; B, Boehringer Mannheim Co.; G, Grand Island Biological Co.; I, ICN Pharmaceuticals. Rates of tyrosine hydroxylation and melanin formation are reported as pmol tyrosine metabolized by the enzyme in 1 h (% of control in parentheses).

No.	Treatment	Source		Tyrosine hydroxylation	Melanin formation
1	water	—	—	132.0 (100)	29.4 (100)
2	phospholipase A	*Vibrio russeli*	S	129.3 (98)	28.1 (96)
3	phospholipase A	bee venom	S	135.8 (103)	27.5 (94)
4	phospholipase C *	Clostridium welchii	S	130.7 (99)	28.9 (98)
5	phospholipase C	*Bacillus cereus*	B	137.3 (104)	24.5 (84)
6	phospholipase D	cabbage	S	126.9 (96)	26.4 (90)
7	neuraminidase	*Clostridium perfringens*	G	114.0 (86)	27.1 (92)
8	neuraminidase	*Clostridium perfingens*	B	112.1 (85)	23.3 (79)
9	trypsin	bovine pancreas	I	129.9 (98)	24.4 (83)
10	protease	bovine pancreas	I	122.5 (93)	24.4 (83)

* Contains high neuraminidase activity.

function of tyrosinase, i.e. tyrosine hydroxylase activity measured by the [^{3}H]-tyrosine assay or the 3,4-dihydroxyphenylalanine oxidase activity measured by the [^{14}C]tyrosine assay. However, both neuraminidase preparations decreased the rates of both enzymatic functions by about 10—20%; both proteases produced a similar decrease, although to a lesser extent (approx. 5—20%). When similar treated samples were separated by polyacrylamide gel electrophoresis to determine the effects of these agents on T_1's electrophoretic behavior, the 3,4-dihydroxyphenylalanine positive banding patterns shown in Fig. 1 were obtained. The R_m values (relative mobilities versus bromphenol blue) observed after treatment with all the agents were the same as untreated T_1 ($R_m = 0.555 \pm 0.006$, $n = 24$), with the exception of phospholipase C-4 * and the two samples of neuraminidase (7 and 8), which had R_m values significantly less than T_1 ($R_m = 0.380 \pm 0.008$, $n = 12$). This effect has been previously reported for neuraminidase [10,13,14], but not for phospholipase C. Since both enzymes gave similar results and were prepared from *Clostridium*, we sought to determine if either preparation was contaminated with significant amounts of the other. While the neuraminidase samples were found to be free of phospholipase C, the phospholipase C-4 was determined to have significant amounts of neuraminidase, as determined by the sialic acid assay [28]. In fact, the amount of neuraminidase present in this sample of phospholipase C-4 was ten times higher than the phospholipase C concentration, on a per unit basis. The minimal amount of neuraminidase necessary to cause the observed decrease in R_m was 1 I.U./ml; higher concentrations (up to 100 I.U./ml) had no additional effect.

The physical characteristics of the isozymic forms of tyrosinase are shown in

* Numbers following enzymes throughout the text refer to enzyme sources as detailed in Table I.

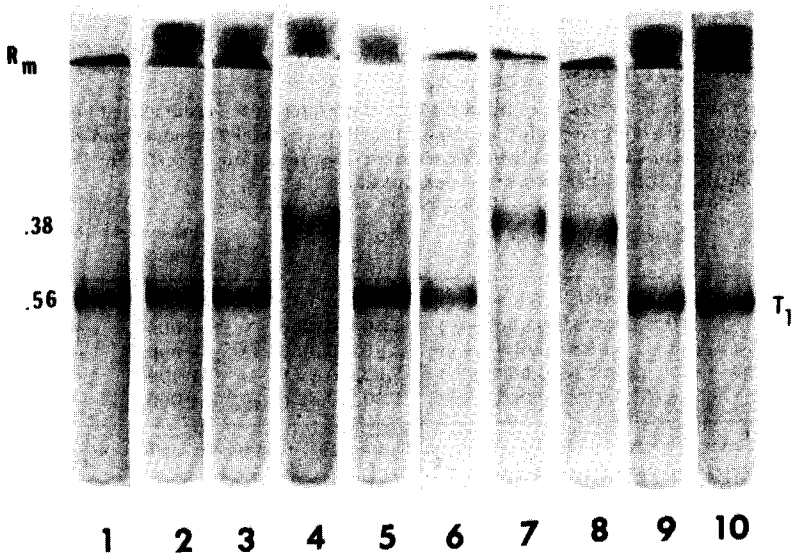

Fig. 1. Purified T_1 isozyme banding patterns after treatment with various agents. Approx. 0.010 I.U. of purified T_1 tyrosinase were treated with agents as listed in Table I: 1, water; 2, phospholipase A-2; 3, phospholipase A-3; 4, phospholipase C-4 *; 5, phospholipase C-5; 6, phospholipase D; 7, neuraminidase-7; 8, neuraminidase-8; 9, trypsin; 10, protease. The samples were then electrophoresed on 7.5% acrylamide gels and stained with 3,4-dihydroxyphenylalanine. Origin is at the top, Bromphenol Blue front is at the bottom.

Table II. The K_R (retardation coefficient, negative slope of a Ferguson plot) of the T_1 isozyme indicated a molecular weight of about 83 000. Treatment of the T_1 isozyme with phospholipase A had no significant effect on the K_R. The K_R of T_3 isozyme of tyrosinase differed significantly from the K_R of T_1; T_3 had a molecular weight of approx. 56 000. Treatment of the T_1 isozyme with phospholipase C-4 or neuraminidase yielded a K_R different from T_1 but not significantly different from T_3, although the values were consistently slightly higher than that of T_3. Similarly, the Y_o (electrophoretic free mobility, Y intercept of the Ferguson plot) was not significantly different between T_1 and phospholipase A-treated T_1, indicating a valence of about −18; the Y_o of T_3, and phospholipase C- and neuraminidase-treated T_1 were also statistically similar, indicating a valence of about −11. A determination of the isoelectric point (pI) of these same preparations gave similar results (Table III). T_1 and T_3 differed with respect to their isoelectric points, which were 3.30 and 4.24, respectively. Phospholipase A-treated T_1 was identical in pI to T_1, and neuraminidase-treated T_1 was statistically similar to T_3 (and significantly different from T_1). Fig. 2 shows isoelectric gels with banding patterns as would be expected from the data in Table III.

Determination of the kinetic constants of these various isozymes was carried out. The resultant data is presented in Table IV. The apparent K_m values of all three isozymes, T_1, T_3 and T_4, were all similar as measured by each radioassay. Treatment of T_1 isozymes with phospholipases, neuraminidase or phospholipids

* Contains high neuraminidase activity.

TABLE II

COMPARISON OF THE PHYSICAL CHARACTERISTICS OF T_1, T_3, AND ENZYMATICALLY TREATED T_1 ISOZYMES OF TYROSINASE

Approx. 0.010 I.U. of tyrosinase isozymes were treated with either water or the agents listed before electrophoresis and construction of a Ferguson plot, as detailed in Experimental Procedure and ref. 24. n, number of experiments, minimum of four points per experiment; K_R, retardation coefficient ± S.D.; Y_o, electrophoretic free mobility ± S.D.; valence in protons per molecule.

Isozyme	Treatment	n	K_R	Y_o	Molecular weight	Valence
T_1	water	18	0.086 ± 0.007 (+)*	2.48 ± 0.36 (+)	83 000	−19
T_3	water	10	0.070 ± 0.005 (+)	1.56 ± 0.13 (+)	56 000	−10
T_1	phospholipase A-3	4	0.083 ± 0.006 (— +)	2.35 ± 0.30 (— +)	78 000	−17
T_1	phospholipase C-4**	9	0.073 ± 0.006 (+ —)	1.67 ± 0.39 (+ —)	61 000	−11
T_1	neuraminidase-7	8	0.075 ± 0.005 (+ —)	1.60 ± 0.42 (+ —)	64 000	−11

* Statistical analysis in parentheses, from T_1 on left, from T_3 on right; +, indicates significant difference at $P < 0.01$, —, indicates no significant difference at $P > 0.05$.
** Contains high neuraminidase activity.

TABLE III

ISOELECTRIC FOCUSING DATA OF T_1, T_3 AND ENZYMATICALLY TREATED T_1 ISOZYMES OF TYROSINASE

Approx. 0.010 I.U. of tyrosinase isozymes were treated with either water or the agents listed before isoelectric focusing was performed as detailed in Experimental Procedure. n, number of experiments; pI, isoelectric point ± S.D.

Isozyme	Treatment	n	pI
T_1	water	11	3.30 ± 0.08 (+) *
T_3	water	9	4.24 ± 0.04 (+)
T_1	phospholipase A-3	4	3.25 ± 0.07 (—+)
T_1	phospholipase C-4 **	6	3.96 ± 0.12 (+ +)
T_1	neuraminidase-7	8	4.20 ± 0.10 (+—)

* Statistical analysis in parentheses, from T_1 on left, from T_3 on right; +, indicates significant difference at $P < 0.01$, —, indicates no significant difference at $P > 0.05$.
** Contains high neuraminidase activity.

TABLE IV

KINETIC VALUES FOR T_1, T_3, T_4 AND ENZYMATICALLY TREATED T_1 ISOZYMES OF TYROSINASE

Approx. 0.001 I.U. of tyrosinase isozymes was treated with either water, the enzymes listed or phosphatidylcholine or lysophosphatidylcholine, before kinetic analyses were performed as detailed in Experimental Procedure. K_m, Michaelis constant in molar ($\times 10^5$); V, maximal velocity in mol/h ($\times 10^{11}$).

Isozyme	Treatment	[^{3}H]Tyrosine		[^{14}C]Tyrosine	
		K_m	V	K_m	V
T_1	water	2.13	7.70	1.45	2.63
T_3	water	2.22	3.70	1.25	0.77
T_4	water	1.90	6.25	1.25	1.67
T_1	phospholipase A-3	2.24	7.72	1.22	2.21
T_1	phospholipase C-4 *	2.38	8.59	1.22	1.80
T_1	neuraminidase-7	3.25	10.13	1.10	1.63
T_1	lysophosphatidylcholine	1.94	8.09	1.12	2.75
T_1	phosphatidylcholine	2.00	7.93	1.36	2.71

* Contains high neuraminidase activity.

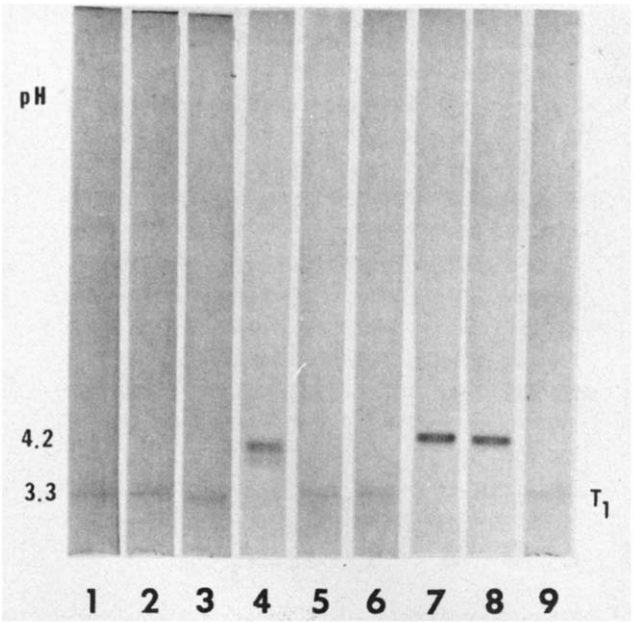

Fig. 2. Isoelectric focusing patterns of purified T_1 tyrosinase after treatment with various agents. Isoelectric focusing was carried out as described in Experimental Procedure on the following samples (approx. 0.010 I.U. each of tyrosinase), which had been pretreated with the agents as listed in Table I: 1, water; 2, phospholipase A-2; 3, phospholipase A-3; 4, phospholipase C-4 *; 5, phospholipase C-5; 6, phospholipase D; 7, neuraminidase-7; 8, neuraminidase-8; 9, trypsin. After isoelectric focusing, the gels were stained with 3,4-dihydroxyphenylalanine.

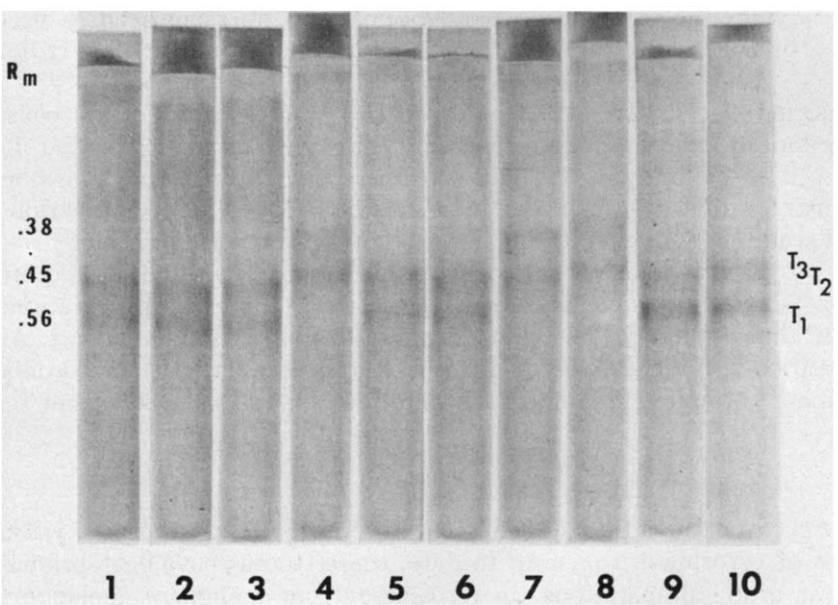

Fig. 3. Isozyme banding patterns of F_{10} melanoma cells after treatment with various agents. Approx. 0.010 I.U. of melanoma cell extract was treated with agents as listed in Table I: 1, water; 2, phospholipase A-2; 3, phospholipase A-3; 4, phospholipase C-4 *; 5, phospholipase C-5; 6, phospholipase D; 7, neuraminidase-7; 8, neuraminidase-8; 9, trypsin; 10, protease. The samples were then electrophoresed on 7.5% acrylamide gels and stained with 3,4-dihydroxyphenylalanine. Origin is at the top, Bromphenol Blue front is at the bottom.

* Contains high neuraminidase activity.

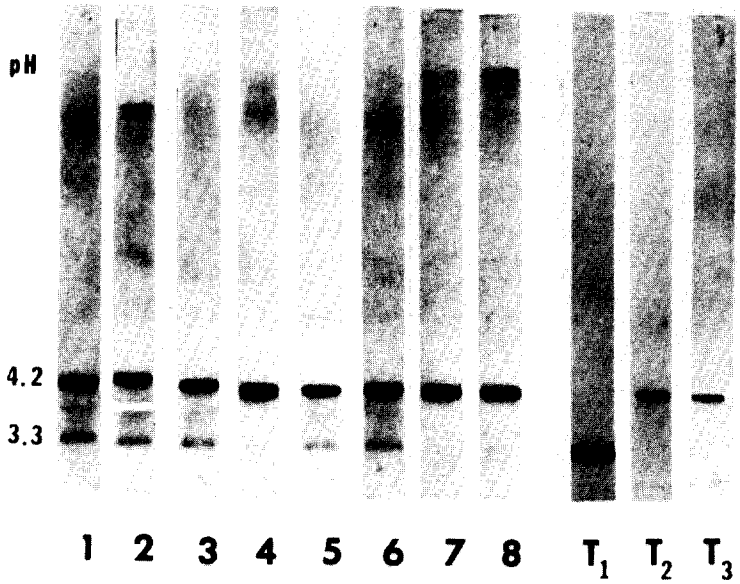

Fig. 4. Isoelectric focusing patterns of F_{10} melanoma cell extract after treatment with various agents. Isoelectric focusing was carried out as described in Experimental Procedure on the following samples (approx. 0.010 I.U. each of tyrosinase), which had been pretreated with the agents as listed in Table I: 1, water; 2, phospholipase A-2; 3, phospholipase A-3; 4, phospholipase C-4 *; 5, phospholipase C-5; 6, phospholipase D; 7, neuraminidase-7; 8, neuraminidase-8; 9, trypsin. In addition, on the right are the isoelectric focusing gels of purified T_1, T_2, and T_3; all gels were stained with 3,4-dihydroxyphenylalanine.

(lysophosphatidylcholine and phosphatidylcholine) did not significantly alter either the K_m or the apparent V of the isomers, as measured by each radioassay.

We also carried out similar experiments with F_{10} melanoma culture cells; these cellular homogenates contain relatively large quantities of T_2 and T_3 isozymes as well as the isozyme T_1. The tyrosinase banding patterns shown in Fig. 3 demonstrate that treatment of this cellular extract with the various agents left T_2 and T_3 unchanged in electrophoretic mobility, while T_1 was altered only by phospholipase C-4 and neuraminidase; the altered T_1 is quite distinct from T_2 and T_3. Isoelectric focusing of similarly treated preparations (Fig. 4), show that T_2 and T_3 are unchanged in pI by any of these agents. As shown for purified T_1 (Fig. 2), the pI of T_1 in this homogenate is changed only by phospholipase C-4 and neuraminidase, where the pI is the same as T_2 and T_3.

Discussion

A wide variety of enzymatic agents have been reported to affect the physical characteristics of tyrosinase isozymes; to date, these studies have been primarily directed at crude preparations of tyrosinase from malignant melanoma [13—16,39,41]. Most reports concentrated on the effects of only one or two of these agents and their effects on one or two physical characteristics of tyrosin-

* Contains high neuraminidase activity.

ase. We attempted to examine the effects of all the pertinent enzymatic agents on the same samples of purified tyrosinase, not only from malignant melanocytes, but also from normal melanocytes, and, hopefully, to arrive at a reasonable and plausible explanation for the varied phenomena reported.

There is some irregularity in the nomenclature of tyrosinase isozymes in the literature, which we feel arises primarily from the inconsistent observation of two closely migrating species of tyrosinase at the T_3 position. For clarity, we shall follow the established procedure of numbering isozymes with respect to their migration toward the anode. Therefore, the isozymes were numbered as follows, (with approximate R_m values in parentheses in 7.5% T, 3% C gels and 7.0% T, 2.5% C gels,(the Davis system [25]), respectively): T_1 (0.56, 0.69), T_2 (0.49, 0.57), T_3 (0.45, 0.53), and T_4 (0.10, 0.16). Evidence to be discussed later will argue that T_2 is actually an artefact of the polyacrylamide gel electrophoresis procedure.

Our first set of experiments was designed to survey the effects of phospholipases, proteases, and neuraminidase on the enzymatic functions and migration patterns of the purified T_1 isomer of tyrosinase. The results of our experiments with normal tyrosinase are presented throughout this report; essentially identical data was obtained with purified T_1 tyrosinase from B-16 melanoma melanocytes. Our data show no enhancement of enzyme activity by any of the agents tested (Table I); the neuraminidase and proteases actually decreased enzyme activity slightly. The stimulation of tyrosinase activity caused by the action of phospholipases and proteases on subcellular fractions is the result of increased enzyme solubilization and improved substrate permeability [29—31].

Treatment of the T_1 isomer with neuraminidase (also present as a contaminant of phospholipase C-4) resulted in decreased electrophoretic mobility as previously reported [10,13,14]. However, the evidence presented in this paper does not support the theory that this neuraminidase-treated T_1 is identical with native T_3. Although the p*I* of both these enzymes is indistinguishable (Table III, Figs. 2 and 4), there is a constant, significant difference in their R_m values (T_3 $R_m = 0.461 \pm 0.007$, $n = 11$ compared to neuraminidase-treated T_1 $R_m = 0.380 \pm 0.008$, $n = 12$). Since the pI values are identical and the overall charge is identical (Table II and III), the difference in R_m values must be due to a difference in molecular size. While the K_R values (and thus molecular weight) of T_3 and neuraminidase-treated T_1 are not statistically significantly different (Table II), the difference (around 8000) is real. An effective argument against identity between these two enzymes can be seen in Fig. 3, where a sample containing both T_1 and T_3 isozymes can be seen to have the T_3 unaffected by neuraminidase, while T_1 is converted to a form visibly different from the T_3.

There is evidence that the alteration in electrophoretic mobility of T_1 caused by the phospholipase C-4 is due to neuraminidase contamination. (1) The effect on the electrophoretic mobility of T_1 by both enzymes is identical, even though the enzymatic functions of neuraminidase and phospholipase C are completely different. (2) There are significant levels of neuraminidase contamination of phospholipase C-4. (3) A phospholipase C preparation which is neuraminidase negative (phospholipase C-5) shows no such effect on electrophoretic mobility of T_1.

The closely migrating isozymes T_2 and T_3 have been intriguing. The soluble isozymes T_1 and T_3 have been consistently described in the literature; T_2 has not been observed in many reports [2,8,9,11,12,18], but is a major 3,4-dihydroxyphenylalanine-positive band in other reports [1,3,7,15,29]. Karn et al. [32] have shown with other proteins that at an alkaline pH (upper buffer is pH 9.15) and elevated temperatures, significant amounts of protein deamidation may occur. Such deamidation has the effect of increasing the net negative charge while the gel run is in progress and thereby increasing the electrophoretic mobility. The reports that demonstrated T_2 in their samples have one fact in common, the gels were run at 20°C at 3—5 mA/tube [1,3,7,15,29]. On the other hand, most of the reports that did not resolve T_2 in their samples ran their gels at lower current (<2 mA/tube) and/or lower temperatures (<5°C) [8,11,12,18]. It has been reported previously that T_2 and T_3 are size isomers that differ only in charge [7]. When an F_{10} melanoma cell extract was electrophoresed at 10°C and 2 mA/tube, little T_2 was evident, but when the same sample was run at 20°C and 5 mA/tube, much more T_2 was visible (unpublished data). This evidence indicates that T_2 is an artifact of polyacrylamide gel electrophoresis, due to sample heating and high pH, and probably results from deamidation of T_3 isozyme, similar to that described for amylase [32].

The molecular weight reported here for C57B1 tyrosinase isozymes agrees closely with that in previous reports of 70 000—85 000 for T_1 and 54 000—70 000 for T_3, maintaining a difference in all cases of at least 10 000 [7,9,33].

We are aware of only one report on the pI of the various isozymes [11]; these workers found pI values for T_1 and T_3 in the acidic range, but higher than observed here. In light of the fact that they ran their isoelectric focusing only 3 h which is sufficient (in our experience) to allow pH gradient formation but not sample equilibration, we feel our data (based on more than 10 experiments electrophoresed for at least 18 h) probably reflect values closer to the true pI of these isozymes. Tyrosinase is relatively stable at the pH of 4.0, but approx. 80—90% of enzyme activity is lost at a lower pH; such inactivation accounts for the fainter bands visible at this pH in the figures.

The evidence presented in this report, when combined with data from other laboratories, is compatible with the following relationship among the tyrosinase isozymes: T_3 appears to be the de novo form of tyrosinase. In vivo labelling of tyrosinase has shown that T_3 is labelled before T_1 [6,34]. Sequential examination of melanogenic tissues has revealed that T_3 appears in the melanocyte prior to T_1, and that T_1 persists after the disappearance of T_3 [1,8].

In the next step in the processing of tyrosinase, T_3 is modified post-translationally by the addition of carbohydrate moieties. While sialic acid has been shown to be present on T_1 [10,13,14,41], there is evidence that neutral sugars are also present (mannose and galactose) [10,13,35]. Based on our molecular weight data (Table II), it would appear that 1 mol of T_1 may be associated with as much as 60 mol of sialic acid. Neutral sugars (which have no effect on the isoelectric point) probably constitute the other 8000 molecular weight difference between neuraminidase-treated T_1 and T_3, (or approx. 44 mol of neutral sugar per mol T_1). Digestion of neuraminidase-treated T_1 with β-galactosidase or α-mannosidase results in an increase in electrophoretic mobility approaching that of T_3, although exact coincidence of the bands has not yet

been achieved (unpublished data); this may be due to incomplete digestion resulting from carbohydrates that are inaccessible to these enzymes, or possibly from the presence of other types of molecules. Work is underway to resolve this question.

The function of these carbohydrate entities is at present unknown. However, it is possible that they function in the binding of the enzyme to the melanosomal membrane, and/or assist in the stabilization of the enzyme. Evidence for the former includes the fact that only the T_1 form associates with other moieties to form T_4 in vitro, while T_3 does not (unpublished data). Evidence for the latter is the fact that T_1 is a slightly more stable enzyme than T_3. It seems reasonable to assume that the sialic acid groups do not affect the enzymatic function of tyrosinase, since the removal of sialic acid does not significantly affect the apparent kinetic parameters of T_1 (Table IV); Herrmann and Uhlenbruck [13] have shown that complexing concanavalin A with the mannose groups of tyrosinase does not affect enzymatic activity, which suggests that mannose is not involved in the modification of enzyme activity as well. In fact, the data in Table IV indicate there are no significant differences kinetically between any of the isozymic forms of tyrosinase.

Lastly, T_4 consists of T_1 complexed to melanosomal membrane constituents. It has been shown that the melanin granule, when isolated and subjected to analytical polyacrylamide gel electrophoresis without prior solubilization, shows a tyrosinase band only at the top of the separation gel. T_4 falls outside the range of mobilities of proteins stacked with the Tris/glycine buffer system, and accumulates at or near the top of the separation gel [2,15,16,29]. In earlier experiments (unpublished data), we used polyacrylamide gel electrophoresis system 4059.I.VII [36], and were able to demonstrate R_m values in 7.5% acrylamide gels for the various isozymes in that buffer system as follows: T_1, 0.951, T_3, 0.937, and T_4, 0.885. Thus, T_4 does not fail to migrate in the Tris/glycine system due to its large size as previously assumed, but rather, due to its lack of intrinsic charge. However, when T_4 is treated with a variety of agents which can disrupt membrane structure, such as trypsin [29,37,41], lipase [38], urea [2], and to a lesser extent the non-ionic detergents (such as Triton X-100 [18] and isooctylphenoxypolyoxyethylene ethanol) [2], it is possible to disperse the T_4 aggregate allowing the demonstration of the exclusive presence of T_1 in this fraction. The association of T_1 and its complexed molecules to form T_4 must be very strong, although not covalent in nature.

Thus, it is possible to essentially reverse the normal process of tyrosinase "maturation", i.e. isolate a melanosomal fraction containing only T_4; disperse this with Triton X-100 (or one of the other agents), to release T_1, which can then be purified and converted to T_3 by means of removing sialic acid residues with neuraminidase, and neutral sugar residues with glycosidases.

In melanogenic tissues which have a low rate of pigment production, such as adult epidermal melanocytes, it is common to find the bulk of tyrosinase activity in the melanosomal fraction in the form of T_4. Only a small percentage of the enzyme is demonstrable as T_1 and even less as T_3. In melanocytes that undergo rapid rates of melanin formation, such as newborn mouse epidermis or regenerating hair follicles, the percent of T_1 demonstrable is significantly increased, as is that of T_3. In tissues with disrupted mechanisms of pigmenta-

tion, such as various melanomas in vivo and in vitro, the percent of T_3 demonstrable is increased even further since the intracellular rates of post-translational glycosylation are insufficient to keep up with the increased production of the tyrosinase molecule.

The reports showing the varied effects of phospholipases on demonstrable tyrosinase activity in subcellular fractions of melanocytes [30,31,39] were obviously due to secondary effects of these agents on the enzyme, such as increased substrate permeability. The lack of effect of the phospholipases on the electrophoretic or kinetic behavior of purified tyrosinase is evidence that there is no direct effect on the enzyme. The addition of a variety of synthetic phospholipids (only phosphatidylcholine and lysophosphatidylcholine are shown) similarly had no visible effect on the purified enzyme.

In conclusion, we feel we have arrived at two primary considerations of tyrosinase structure and function. (1) Each of the isomers of tyrosinase have a common subunit. The isomers vary only with regard to post-translational modifications, and such variations require only a single genetic locus to determine the enzyme's primary structure. (2) While the addition of carbohydrates and other moieties to tyrosinase undoubtedly plays a part in the interactions of the enzyme with its microenvironment, it is clear that a significant alteration in the enzymatic function of the enzyme does not depend on such an addition. Post-translational repression and activation of tyrosinase apparently require other mechanisms for the control of pigmentation, such as allosteric controls dependent on cofactor concentration [23], and/or a protein kinase system under hormonal control [40].

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