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Evidence that dopachrome tautomerase is a ferrous iron-binding glycoprotein

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Dopachrome tautomerase (DT) (EC 5.3.2.3) is a melanocyte-specific, membrane-associated, heat-labile, non-dialyzable, protease-sensitive factor which catalyzes the isomeric rearrangement of dopachrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA), apparently through a tautomerization reaction. Metal ions such as Cu, Ni, Co, Zn, Mn, Ca, Al, and Fe can also catalyze the dopachrome/DHICA isomerization. How is the reaction regulated in vivo? An attractive possibility would be that DT is a metalloenzyme. Here we present evidence that this may indeed be the case. Purified preparations of DT and tyrosinase, obtained from Cloudman S91 mouse melanoma cells, were assayed in the presence of a variety of metal chelators including EDTA (predominantly Ca and Mg), EGTA (predominantly Ca), phenylthiourea (PTU) (predominantly Cu), 2,2'-dipyridyl (predominantly Fe); 1,10-phenanthroline (predominantly Fe), and 2,3-dihydroxybenzoic acid (predominantly Fe). In addition, DT activity was assayed in the presence of two non-chelating structural analogs of 1,10-phenanthroline. Results were as follows: (i) iron chelators inhibited DT activity with no effects on tyrosinase activity; (ii) inhibition by the chelators was reversible with the addition of ferrous iron; (iii) 1,10-phenanthroline pre-complexed to ferrous iron was not inhibitory to DT; (iv) non-chelating analogs of phenanthroline were not inhibitory to DT; (v) PTU was inhibitory to tyrosinase but not DT; (vi) Ca^{2+} and Mg^{2+} chelators had little effect on either enzyme activity. Finally, studies with glycosylation inhibitors, glycosylase enzymes, and immobilized lectins, indicated that DT is a glycoprotein. The results suggest that DT is a metal-containing glycosylated enzyme, possibly with ferrous iron at its catalytic center.

Dopachrome tautomerase; Fe-Glycoprotein; Melanogenesis

1. INTRODUCTION

In 1980 it was reported for the first time that a dopachrome conversion factor (DCF) present in 2 different mouse melanomas and a hamster melanoma was capable of converting orange-red dopachrome into a colorless compound [1–2]. Dopachrome containing a ^{14}C-labelled carboxyl group was prepared, and it was noted that while DCF completely decolorized dopachrome within 10 min, during that same period 90% of the ^{14}C-labelled carboxyl groups remained intact. From this observation, it was surmised that DCF converted dopachrome to DHICA (5,6-dihydroxyindole-2-carboxylic acid). This was later verified by Korner and Gettings who identified DHICA as the product of the reaction through the use of mass spectroscopy and nuclear magnetic resonance [3]. Soon after, a number of laboratories reported that dopachrome spontaneously loses its carboxyl group to from DHI unless an isomeric rearrangement to DHICA is catalyzed, either by metal ions or by the proteinaceous, melanocyte-specific DCF of Korner and Pawelek [4–9]. There is now general agreement that the isomerization of dopachrome to DHICA occurs through a tautomerization reaction and that the most appropriate name for DCF thus appears to be 'dopachrome tautomerase' (DT) (EC 5.3.2.3), the term used throughout this report [10–12].

Since in a test tube metal ions and a melanocyte-associated enzyme can each catalyze the tautomerization of dopachrome to DHICA, how is this step regulated in vivo? From the data reviewed above there are 5 possibilities: (i) exclusively by metal ions; (ii) exclusively by DT; (iii) by DT and separately by metal ions (i.e. DT is a metalloenzyme); (iv) by DT in conjunction with metal ions; and (v) none of the above (i.e. by an as yet undiscovered mechanism).

In this report we present evidence suggesting that DT is a metalloenzyme with ferrous iron at its active site. Furthermore, we show that DT has the properties of a glycoprotein. Our results are consistent with the recent discoveries by Tsukamoto, Jackson, Hearing et al. that DT is part of the tyrosinase-related gene family and contains putative iron-binding sites and glycosylated domains [13–15]. Together, these observations could reconcile the evidence regarding 'metal vs. enzyme' in the biological conversion of dopachrome to DHICA [9].

2. MATERIALS AND METHODS

Dopachrome was synthesized by mixing ice-cold L-dopa (0.75 mg/

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Table I

Effects of chelators on tyrosinase and DT activities*

Chelators	mM	Tyrosinase activity (%)	Dopachrome tautomerase activity (%)
None		100 ± 18	100 ± 3
EDTA	10.0	76 ± 5	99 ± 2
EGTA	10.0	84 ± 21	102 ± 2
Phenylthiourea	0.5	1 ± 0.1	99 ± 2
2,3-Dihydroxy-benzoic acid	20.0	99 ± 11	77 ± 2
2,2'-Dipyridyl	20.0	106 ± 6	44 ± 5
1,10-Phenanthroline	0.1	99 ± 15	71 ± 7
	0.5	N.D.	69 ± 1
	20.0	96 ± 15	41 ± 4
Deferoxamine	0.1	83 ± 12	95 ± 1
	1.0	96 ± 1	100 ± 2

*Incubation conditions are described in Materials and Methods. Results represent average ± S.D. of triplicate samples. The experiments were repeated several times with similar results.

ml) in sodium phosphate (0.1 M, pH 6.8) with solid Ag_2O (30 mg Ag_2O : 1 mg L-dopa) for 1 min and then filtering the mixture twice through Gelman Acrodisc Disposable Filters (#4192, 0.2 μm diameter pore). The resulting orange-red solution of dopachrome contained about 33% residual L-dopa, although this had no influence on DT activity [7].

DT and tyrosinase were prepared from cultured Cloudman mouse melanoma cells through the DEAE cellulose salt gradient steps as described previously [1,7]. The steps include detergent extraction, centrifugation, differential adherence to $CaPO_4$, dialysis, and DEAE elution and result in a 600-fold reduction in starting protein content with a 200-fold increase in the specific activities of both enzymes.

DT activity was assayed spectrophotometrically as the conversion of dopachrome to DHICA at 475 nm [1].

Tyrosinase activity was measured as the formation of ^3H_2O from 3,5-[^{3}H]tyrosine by a modification of the method of Pomerantz [16].

Protein content was measured using the Pierce Protein Assay Kit (Rockford, IL), with bovine serum albumin as a standard, based on the method of Bradford [17].

Neuraminidase (from *Vibrio cholerae*) and tunicamycin were obtained from Sigma Chemicals. Ethyleneglycol-bis-(β-amino-ethyl ether)*N,N'*-tetra-acetic acid (EGTA); 2,2'-dipyridyl; 2,3-dihydroxy benzoic acid; phenanthroline-Fe(II) complex; and 1,10-phenanthroline were obtained from Sigma Chemical Co., St. Louis, MO. 1,7-phenanthroline and 4,7-phenanthroline were obtained from Aldrich Chemicals, Milwaukee, WI. Phenylthiourea (PTU) was obtained from Eastman Kodak Co., Rochester, NY. Ethylenediamine tetraacetic acid (EDTA) was obtained from J.T. Baker Chemical Co., Phillipsburg, NJ. Deferoxamine was obtained from CIBA-Geigy Corp., Summit, NJ. Stock solutions were made as 0.1 M, except for PTU which was made as 0.01 M in 50% ethanol.

Incubation with chelators was as follows: partially purified DT (10–20 μg protein) was incubated with chelators (0.1–20 mM) or appropriate solvent blanks for 30 min at 37°C in a total volume of 300 μl.

All assays were performed in triplicate and repeated several times.

3. RESULTS AND DISCUSSION

The activity of partially purified DT was decreased by the iron chelators 2,2'-dipyridyl, 1,10-phenanthroline, and 2,3-dihydroxybenzoic acid, whereas under the same incubation conditions the activity of tyrosinase was unaffected by these compounds (Fig. 1, Table I). On the other hand, tyrosinase was strongly inhibited by phenylthiourea, and to a lesser extent by EDTA, while these compounds had no affect on DT activity. Deferoxamine, which preferentially complexes ferric ion, had no effects on DT activity, however deferoxamine could not be used over a wide concentration range because its red color obscured the DT assay. The 1,7- and 4,7-structural analogs on 1,10-phenanthroline were only weakly inhibitory to DT, consistent with their reduced ability to chelate ferrous iron. In addition, the inhibitory effects of 1,10-phenanthroline on DT were largely overcome when the chelator was pre-complexed to ferrous iron (Table II). Finally, inhibition of DT by the chelators 2,2' -dipyridyl and 1,10-phenanthroline was reversible by ferrous iron after the chelators were removed by dialysis (Table III).

It is well established that tyrosinase is a glycoprotein, and evidence has also been presented that the same is

Table II

Effects of phenanthroline analogs and iron-complexed phenanthroline on DT activity*

Treatment	Dopachrome tautomerase activity (% control)
None	100 ± 1
1,10-Phenanthroline	38 ± 3
1,10-Phenanthroline-Fe^{2+} (pre-complexed)	89 ± 6
1,7-Phenanthroline	80 ± 6
4,7-Phenanthroline	89 ± 1

*The effects of 1,10-phenanthroline, iron-complexed 1,10-phenanthroline, and the non-chelating 1,7- and 4,7-isomers of 1,10-phenanthroline on DT activity. All agents were used at concentrations of 10 mM. DT was incubated with or without additions for 1 h, 37°C, dialyzed exhaustively, and assayed for activity. Results represent average ± S.D. of triplicate determinations. The experiments were repeated 3 times with similar results.

Table III

Activation of chelated DT by ferrous iron*

Chelator	Addition following dialysis	DT activity (% control)
2,2'-Dipyridyl	None	57 ± 3
	$FeCl_2$	86 ± 4
1,10-Phenanthroline	None	36 ± 5
	$FeCl_2$	89 ± 4

*DT was incubated with the designated chelator (10 mM, 1 h, 37°C), dialyzed exhaustively, incubated with $FeCl_2$ (5 mM, 1 h, 20°C), dialyzed again, and assayed for activity. Control preparations of DT were treated in the same manner without the additions of chelators of ferrous iron. Results represent average ± S.D. of triplicate determinations. The experiments were repeated 3 times with similar results.

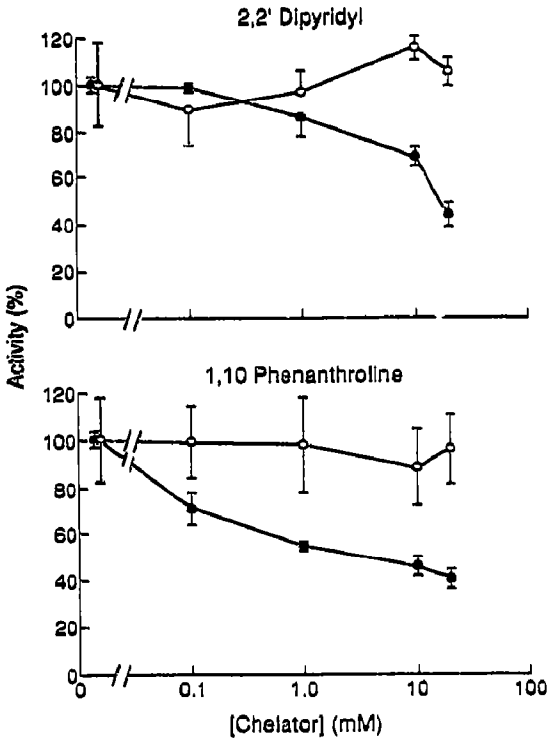

Fig. 1. Effects of 2,2'-dipyridyl and 1,10-phenanthroline on tyrosinase (○) and dopachrome tautomerase (●) activities. Incubation conditions and enzyme assays are described in Materials and Methods. Results represent average ± S.D. of triplicate determinations. The experiments were repeated several times with similar results.

true for DT [18–20]. Our evidence for the glycosylated nature of DT is as follows: (i) DT adhere to wheat germ agglutinin and could be eluted with *N*-acetylglucosamine with a recovery of approximately 30% of starting material; (ii) treatment of intact Cloudman melanoma cells with tunicamycin (0.2 mg/ml culture medium for 24 h), an inhibitor of *N*-glycosylation, inhibited both tyrosinase (44% of control) and DT (22% of control) activities in broken cell preparations; (iii) treatment of partially purified DT and tyrosinase with neuraminidase (0.05 mg/ml, 2 h, 37°C), which hydrolyzes terminal sialic acid residues from glycoconjugates (glycoproteins, glycolipids), inhibited tyrosinase activity (43% of control) and stimulated DT activity, (157% of control). Stimulation of DT activity by neuraminidase was also reported by Solano et al. [20].

The results suggest that iron, possibly in its ferrous (Fe(II)) state, is important for the function of DT, and that DT is a glycoprotein. This evidence is substantiated by the genetic analyses of Tsukamoto, Jackson, Hearing et al. which show that DT contains both potential iron binding sites and glycosylated domains [13–15]. Whether or not DT actually contains iron and sugars remains to be demonstrated. Similarly, direct proof for the involvement of iron in the catalytic tautomerization of dopachrome awaits further experimentation, since little is known regarding the reaction mechanism. Nonetheless, our results suggest that DT functions as a ferrous iron-binding glycoprotein and provide direction for future research into this import new aspect of the regulation of melanogenesis [11].

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