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Function of Dopachrome Oxidoreductase and Metal Ions in Dopachrome Conversion in the Eumelanin Pathway†

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ABSTRACT: The conversion of dopachrome (DC) in the eumelanin pathway has been analyzed to determine the specific product and the role of enzyme control. 5,6-Dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA) were quantitated by HPLC with fluorescent detection, after DC incubation with heated and unheated preparations of B-16 melanoma derived dopachrome oxidoreductase (DCOR). The enzyme-catalyzed reaction produced DHICA as the major product, while DHI formed with the spontaneous reaction. It had originally been suggested that the major product of DC conversion was DHI, with DHICA being formed as a minor product of this conversion [Raper, H. S. (1927) *Biochem. J. 21*, 89-96]. Copper, nickel, and cobalt ions promoted conversion of DC, with nickel simulating DCOR activity. Removal of free ions from unheated DCOR did not alter DC conversion. We conclude that the major product of DC conversion is DHICA and that DCOR is responsible for this conversion.

Eumelanin production is known to proceed through a reaction pathway, the initial steps of which are catalyzed by the enzyme tyrosinase (Raper, 1928). Although the steps beyond the action of tyrosinase were originally believed to occur spontaneously, recent studies have shown that the conversion of DC[1] is under enzymatic control (Korner & Pawelek, 1980; Barber et al., 1984). The enzyme responsible for this conversion, DCOR, was extracted from purified melanosomes and shown to be separable from tyrosinase by enzyme activity and gel filtration (Barber et al., 1984). Initial characterization of the enzyme showed it to be protease-sensitive and heat-labile, with an optimum pH range of pH 6-8. The molecular mass of DCOR was estimated by AcA22 gel filtration to be 34 000 daltons. Further characterization awaits purification of the enzyme. The original assay for DCOR activity used light spectroscopy to measure the decrease in absorbance as DC (red color) was converted to a colorless compound, but this method was not capable of detecting the specific reaction products. According to the classical Raper–Mason pathway, the major product of DC conversion is DHI (Mason, 1955), with DHICA being formed as a minor product of this conversion (Raper, 1927); however, several recent studies have indicated that this concept of the eumelanin pathway is not correct and that DHICA may be the major product of DC conversion.

When [^{14}C]carboxy-labeled dopachrome was used as substate for DCOR, Korner and Pawelek observed that the loss of DC color did not directly correlate with $^{14}CO_2$ liberation, suggesting that DC was converted to DHICA rather than DHI (Korner & Pawelek, 1980). Korner and Gettins (1985) then analyzed the stable colorless product of DC conversion using nuclear magnetic reasonance and mass spectroscopy and identified the compound as DHICA. Finally, Ito has recently reexamined the structure of eumelanin polymer and has found that it contains a high quantity of carboxy-containing indoles. To further investigate the products of the enzymatic conversion of DC, we have developed an assay using HPLC with fluorescent detection. In this paper, the results of using the assay to quantitate the enzymatic and nonenzymatic formation of DHICA and DHI, as well as the effect of metal ions on DC conversion, are described.

Materials and Methods

Preparation of DCOR from Melanosomes. (A) Maintenance of B-16 Melanoma. Melanotic B-16 melanoma lines were maintained by serial transplantation in C57BL/6J mice. A suspension of approximately 10^5 tumor cells in minimal essential media was injected in subcutaneous tissue of the lower

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[1] Abbreviations: DC, dopachrome; DCOR, dopachrome oxidoreductase; DHI, 5,6-dihydroxyindole; DHICA, 5,6-dihydroxyindole-2-carboxylic acid; HPLC, high-performance liquid chromatography.

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extremity of tumor-free mice. Three week old tumor was harvested following cervical dislocation. The harvested tumor was washed with 0.85% saline and stored at −70 °C.

(B) Purification of Melanosomes from B-16 Melanoma. Crude B-16 tumor was homogenized in 0.25 M sucrose and centrifuged 2 × 600*g* × 10 min. The supernatant was centrifuged at 11000*g* × 10 min (JA-20 Beckman), after which the resulting pellet was resuspended in 0.25 M sucrose and spun at 11000*g* × 10 min. The pellet was again homogenized in 0.25 M sucrose and then spun at 20000*g* × 60 min through 1.7 M sucrose. Pellets (melanosomes) were washed with saline and stored at −70 °C. All centrifugation was performed at 5 °C.

(C) Extraction of DCOR from Purified Melanosomes. Melanosome pellets of approximately 0.5 mL were sonicated in 500 μL of 0.05 M phosphate buffer and 1% Chaps, pH 6.8, incubated on ice 15 mins, and centrifuged 2 min at 2000 rpm and 5 °C. All buffer solutions were passed over a column of Chelex 100 chelating ion-exchange resin (Bio-Rad Laboratories) to remove metal ions.

Preparation of Dopachrome. Dopachrome (2-carboxy-2,3-dihydroindole-5,6-quinone) was prepared by a modification of the procedure of Barber et al. (1984). A solution of 2.5 μmol of L-β-3,4-dihydroxyphenylalanine (L-dopa) per milliliter of 0.05 M Chelex-treated phosphate buffer, pH 6.8, was prepared and sonicated in ice water for 25–30 min. The solution was filtered through an 0.45-μm Millipore filter to remove undissolved dopa and then stored on ice. When needed to prepare DCOR assay reaction mixtures, the cold dopa solution was added to preweighed samples of silver oxide (6 mg of Ag_2O/mg of dopa) in plastic-capped 12 × 75 mm tubes. The mixture was vortexed for 3 min and immediately filtered through an 0.22-μm Millipore filter. The deep red DC solution was stable for approximately 30 min on ice. Barber et al. (1984) determined that this procedure results in a 77% conversion of dopa to DC. The specific modification of the procedure of Barber et al. included the preweighing of the Ag_2O and the filtering of the dopa solution.

DCOR HPLC Assay. DCOR activity was determined by using high-performance liquid chromatography (HPLC) with fluorescent detection. Reaction mixtures, prepared by using a modification of the procedure of Barber et al. (1984), contained 125 μL of melanosomal extract or buffer blank, 250 μL of dopachrome, and 625 μL of 0.05 M phosphate buffer, pH 6.8. Reactions at room temperature were started and analyzed 8 min apart. Aliquots of the reaction mixtures were diluted 1:10 with mobile phase (30 mM HPO_4, 64 mM MSA, 0.1 mM EDTA, 20% MeOH, pH 2.79), and 20 μL of the dilute samples was injected in the HPLC. The HPLC had a Beckman Model 114 M solvent-delivery module (1.5 mL/min), prefilter, guard column, and a 25 cm × 4.6 mm reverse-phase column (ODS2). The McPherson spectrofluorometer (FL-750B) was set at 308/395 nm excitation/emission, 0.1 sensitivity, slit width 16/16, and time constant 5. The HPLC was regulated with an Apple IIe computer using Chromatochart version 2.0 (Interactive Microware Inc., 1986).

For metal ion studies, the reaction mixtures contained 125 μL of a preparation mixture in place of the melanosomal extract sample. The preparation mixtures containing ions consisted of 350 μL of 0.05 M phosphate buffer, pH 6.8, 100 μL of sample (buffer, unheated melanosomal extract, or heated melanosomal extract), and 150 μL of 5 mM ion solution (copper, nickel, or cobalt) for a final ion concentration of 1.25 mM. Those without ions consisted of 500 μL of 0.05 M phosphate buffer, pH 6.8, and 100 μL of sample. Duplicate

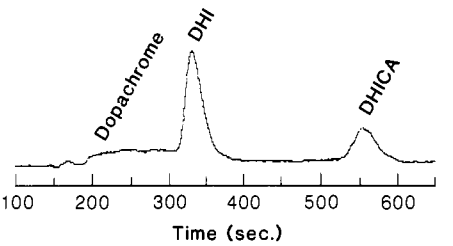

FIGURE 1: Actual chromatogram of DC and its metabolites. DCOR reaction blank was assayed at 30 min according to standard protocol. DHI and DHICA peaks were identified with standards.

mixtures were prepared, and half were mixed on a rotator for 6 min with 25 mg of Chelex, followed by 10-min centrifugation at 15000 rpm and 5 °C.

The 1.25 mM concentration of ions is an approximate 100-fold increase over the adult human serum copper level (Tietz, 1982) and likewise is expected to far exceed the level of free metal ions in mouse melanosomes. This concentration was chosen in order to clearly demonstrate the effect of each metal ion on dopachrome conversion and show that this amount of ions can be removed with 25 mg of Chelex. Adding this amount of Chelex to melanosomal extracts thereby ensured that all free metal ions would be removed, enabling the investigation of the role of free metal ions in natural eumelanin synthesis. Copper, nickel, cobalt, manganese, and zinc have all been shown to influence DC conversion (Palumbo et al., 1987). Manganese and zinc had minimal influence on the reaction in initial HPLC investigation, however, so were not selected for this study.

Other Methods. (A) Protein Determination. The protein concentration of melanosomal extracts was determined by the Pierce BCA protein assay (Pierce Chemical Co., 1986).

(B) Tyrosinase Assay. The tyrosinase hydroxylase activity of tyrosinase was determined by using a tritiated tyrosine microassay (Townsend et al., 1984).

Results and Discussion

The original light spectrophotometric assay measured the decrease in substrate absorption but had no capabilities for product identification. Analysis of DCOR reaction mixtures used HPLC-detected product peaks eluting at 320 and 540 s, which were identified with standards as DHI and DHICA, respectively. Dopachrome appeared as a broad, flat peak, spanning the interval of 250–320 s. An actual chromatogram of DC and its metabolites is shown in Figure 1. By following the reaction over time, we have found that the major product of DCOR-catalyzed DC conversion is DHICA, and only a small amount of DHI is formed, as shown in Figure 2 (representative chromatogram) and Table I (results of five experiments). In contrast, the formation of DHICA was minor in reactions containing heat-inactivated DCOR, and DHI was the major product in this conversion. We conclude from these results that the major product of enzyme-catalyzed DC conversion is DHICA, while the spontaneous, nonenzymatic reaction proceeds mainly to DHI. In contrast to what might be expected according to the Raper–Mason pathway, we were unable to detect a conversion of DHICA to DHI in unheated reaction mixtures, even with reaction times of up to 6 h, suggesting that DHICA is converted to the quinone and melanin. It remains unclear at this point whether DHICA and DHI interconvert in the eumelanin pathway, or whether they proceed directly to form melanin.

The role of metal ions in the conversion of DC was then investigated, because of the known role of copper in the function of tyrosinase and the suggestion that metal ions may

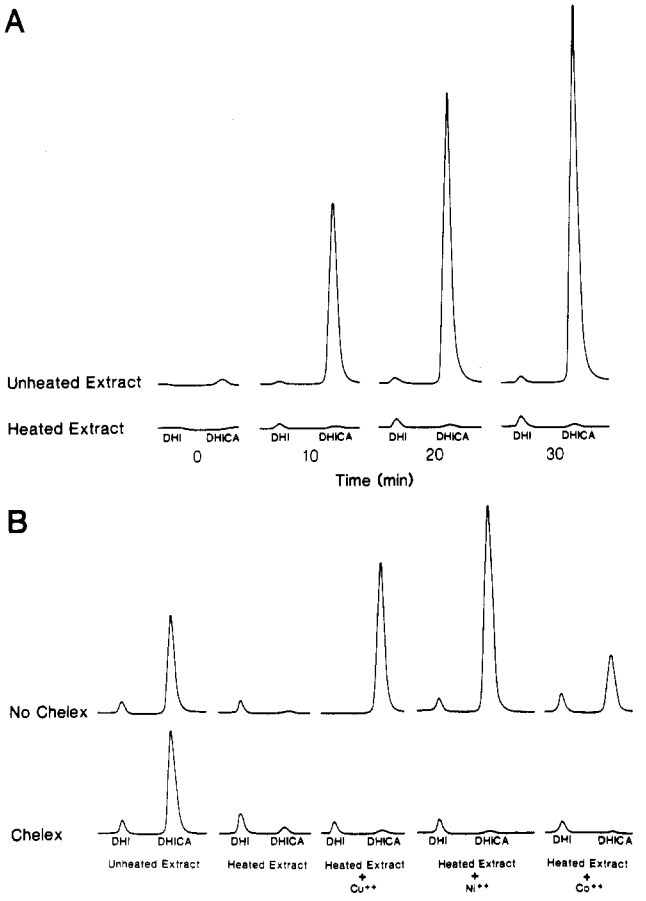

FIGURE 2: DCOR HPLC assay. (A) Purified mouse B-16 melanosomes were extracted with 1% Chaps buffer. DCOR activity was heat-inactivated in half of the extract. Aliquots of DCOR reaction mixtures containing either heated or unheated melanosomal extract were analyzed with HPLC fluorescent detection at 0, 10, 20, and 30 min. (B) Effects of metal ions on dopachrome conversion. Chromatograms show DHI and DHICA at 30 min formed for unheated melanosomal extract, heated melanosomal extract, and heated melanosomal extract plus Cu^{2+}, Ni^{2+}, or Co^{2+} with and without Chelex treatment.

Table I: Dopachrome Conversion with Heated and Unheated Enzyme[a]

sample	time (min)	DHI (area/mg of protein ± SD)	DHICA (area/mg of protein ± SD)
DCOR-H[b]	0	2.6 ± 3.2	2.8 ± 2.5
DCOR-U	0	4.1 ± 2.9	36.9 ± 3.8
DCOR-H	10	15.7 ± 1.8	7.9 ± 1.7
DCOR-U	10	18.9 ± 1.5	1100.0 ± 160.0
DCOR-H	20	34.9 ± 1.2	13.0 ± 1.8
DCOR-U	20	27.9 ± 2.3	1800.0 ± 260.0
DCOR-H	30	50.8 ± 3.3	18.3 ± 1.9
DCOR-U	30	32.9 ± 2.4	2200.0 ± 360.0

[a] Results of five experiments. [b] DCOR prepared from purified melanosomes: H, heated; U, unheated extract. Heated extract was used for calculations in order to express the results in terms of protein content.

be important in DC conversion (Napolitano et al., 1985; Palumbo et al. 1987). We found that copper, cobalt, and nickel ions caused rapid DHICA formation in reaction mixtures containing heated extract, and this effect could be removed with Chelex treatment as shown in Figure 2 (representative chromatogram) and Table II (results of three experiments). Copper was the only metal ion that virtually eliminated the DHI peak and thus could be considered the most efficient in DC conversion because of completeness. The ions also augmented the enzyme-catalyzed DHICA formation, but Chelex treatment returned the level of formation to that originally found with enzyme alone, suggesting that ions do not account for the formation of DHICA with melanosomal extracts.

Table II: Metal Ion Effects on Dopachrome Conversion, with Heated and Unheated Enzyme[a]

sample	DHI (area/mg of protein ± SD)	DHICA (area/mg of protein ± SD)
DCOR-H[b]	350 ± 62	89 ± 17
DCOR-H, Chelex	390 ± 5	190 ± 5
DCOR-U	350 ± 74	2700 ± 500
DCOR-U, Chelex	350 ± 80	2600 ± 600
DCOR-H, Cu^{2+}	35 ± 49	6100 ± 950
DCOR-H, Ni^{2+}	340 ± 68	7600 ± 630
DCOR-H, Co^{2+}	460 ± 43	3100 ± 990
DCOR-H, Cu^{2+}, Chelex	280 ± 67	110 ± 21
DCOR-H, Ni^{2+}, Chelex	320 ± 57	92 ± 14
DCOR-H, Co^{2+}, Chelex	370 ± 110	80 ± 24
DCOR-U, Cu^{2+}	0 ± 0	7700 ± 2200
DCOR-U, Ni^{2+}	290 ± 59	9600 ± 1400
DCOR-U, Co^{2+}	410 ± 55	5600 ± 1300
DCOR-U, Cu^{2+}, Chelex	310 ± 58	3000 ± 620
DCOR-U, Ni^{2+}, Chelex	300 ± 56	3000 ± 620
DCOR-U, Co^{2+}, Chelex	370 ± 31	2900 ± 720

[a] Reaction time 30 min; results of three experiments. [b] DCOR prepared from purified melanosomes: H, heated; U, unheated extract.

Table III: Comparison of Quantitated HPLC Results Shown in Table I (125-μL Extract) and Table II (21-μL Extract)

sample[a]	extract in reaction mixture (μL)	DHI area	DHI area/mg of protein[b]	DHICA area	DHICA area/mg of protein
DCOR-H[c]	125	12000	50	4500	18
	21	16000	350	4100	89
DCOR-U	125	8000	32	540000	2200
	21	16000	350	130000	2700

[a] Reaction time 30 min. [b] All values have SD no greater than 15%. [c] DCOR prepared from purified melanosomes: H, heated; U, unheated extract.

Additional proof that DCOR controls the conversion of DC to DHICA rather than DHI is shown in Table III. The amount of product formed per microgram of protein correlates only with DHICA and not DHI. The reaction containing heat-inactivated enzyme (DCOR-H) forms similar amounts of product regardless of protein concentration, whereas unheated enzyme (DCOR-U) produces an amount of DHICA directly proportional to the amount of protein in the reaction mixture. This supports the proposed model of dopachrome conversion, in which DCOR enzymatically converts DC to DHICA, while the remaining DC proceeds spontaneously to DHI.

An interesting observation made during the metal ion studies was that copper ions imitate the action of tyrosinase, while nickel ions imitate the action of DCOR. More specifically, Cu^{2+} caused a rapid conversion of the reaction mixture from red (DC) to black (melanin), while Ni^{2+} caused a rapid conversion from red (DC) to colorless (DHICA). The Ni^{2+} reaction remained colorless for at least 60 min. It has been recently demonstrated that Cu^{2+} and Ni^{2+} form stable complexes with L-dopa and related compounds (Kiss & Gergely, 1985). This is not surprising for copper, as it is a known component for tyrosinase (Solomon, 1981). It is possible that nickel may likewise be important in DCOR activity. Further investigation awaits purification of DCOR.

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Registry No. DC, 3571-34-4; DCOR, 91449-01-3; DHI, 3131-52-0; DHICA, 4790-08-3; Cu, 7440-50-8; Ni, 7440-02-0; Co, 7440-48-4.

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Primary Structure of Non-Histone Protein HMG1 Revealed by the Nucleotide Sequence†

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ABSTRACT: The isolation and sequencing of a cDNA clone coding for the entire sequence of pig thymus non-histone protein HMG1 are described. The sequence analysis reveals a complete 2192-nucleotide sequence with a 5′-terminal untranslated region of 11 nucleotides, 642 nucleotides of an open reading frame that encoded 214 amino acids, and a 3′-terminal untranslated region of 1539 nucleotides. The HMG1 protein, deduced from the nucleotide sequence, has a molecular weight of 24 785 and a C-terminal of a continuous run of 30 acidic amino acids, encoded by a simple repeating sequence of $(GAN)_{30}$. The predicted amino acid sequence is homologous to HMG1, HMG2, and HMG-T sequences from several sources, suggesting that the protein conformation is under evolutionary constraints. Northern blot analysis reveals that another hybridizable RNA species of smaller size is present. Southern blot analyses suggest that pig genome contains several HMG1 gene equivalents.

High mobility group (HMG)[1] proteins are a family of non-histone components in chromatin of relatively low molecular weights. Proteins complying with some or all of the criteria used for identification of HMGs are ubiquitously distributed in relative abundance among various organisms of eukaryotic kingdoms. HMG1 and HMG2 are a pair of proteins with a remarkable structural similarity and presumably share a common ancestral gene (Johns et al., 1975). In vitro, HMG1 and HMG2 proteins show a preferential binding to single-stranded DNA (Bidney & Reeck, 1978; Isackson et al., 1979; Yoshida & Shimura, 1984; Hamada & Bustin, 1985) and unwind double-stranded DNA structure (Yoshida & Shimura, 1984; Makiguchi et al., 1984; Javaherian et al., 1978, 1979). The high glutamic and aspartic acid regions in the C-terminal of the proteins are the active site in the DNA-unwinding reaction (Yoshida, 1987). The functions of HMG1 and HMG2 in vivo, however, have not yet been clarified, but there are some indications of their involvement in transcription or replication of chromatin (Einck & Bustin, 1985). It is very important to know the primary sequence and the structural characteristics of these proteins to understand their actual cellular roles. Walker et al. reported the primary amino acid sequences of HMG1 and HMG2 from calf thymus analyzed by the procedure of Edman degradation (Walker et al., 1980; Walker, 1982). The sequences, however, were ambiguous in having the undetermined sequences presumably because of the microheterogeneity of the proteins, in addition to the difficulty in determining the runs of similar amino acid by the chemical procedure. Recently, Pentecost and Dixon (1984) have reported the partial amino acid sequence of the C-terminal half of bovine testis HMG1 deduced from the nucleotide sequence of the cDNA missing the 5′-half of coding region. The trout testis HMG proteins, HMG-T, a second member of the protein, have homologous sequences to HMG1 and HMG2 (Dixon, 1982; Pentecost et al., 1985).

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[1] Abbreviations: HMG, high mobility group; bp, base pair(s); cDNA, complementary DNA; SDS, sodium dodecyl sulfate.

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