Molecular mechanism for catalysis by a new zinc-enzyme, dopachrome tautomerase

Francisco SOLANO,* Celia JIMÉNEZ-CERVANTES, José H. MARTÍNEZ-LIARTE, José C. GARCÍA-BORRÓN, José R. JARA and José A. LOZANO

Department of Biochemistry and Molecular Biology, School of Medicine, University of Murcia, 30100 Murcia, Spain

Dopachrome tautomerase (DCT; EC 5.3.3.12) catalyses the conversion of L-dopachrome into 5,6-dihydroxyindole-2-carboxylic acid in the mammalian eumelanogenic biosynthetic pathway. This enzyme, also named TRP2, belongs to a family of three metalloenzymes termed the tyrosinase-related proteins (TRPs). It is well known that tyrosinase has copper in its active site. However, the nature of the metal ion in the active site of DCT is under discussion. Whereas theoretical predictions based on similarity between the protein sequences of the TRPs suggest the presence of copper, the different inhibition pattern of DCT with some metal chelators compared with that of tyrosinase suggests that the nature of the metal ion could differ. Direct estimations of the metal content in purified DCT preparations show the presence of around 1.5 Zn atoms/molecule and the absence of copper. Apoenzyme preparation by treatment of DCT with cyanide or *o*-phenanthroline followed by reconstitution experiments of tautomerase activity in the presence of different ions confirmed that the metal cofactor for the DCT active site is zinc. Our results are consistent with Zn^{2+} chelation by the highly conserved histidine residues homologous to the histidines at the classical copper-binding sites in tyrosinase. This finding accounts for the reaction catalysed by DCT, i.e. a tautomerization, versus the copper-mediated oxidations catalysed by tyrosinase. Based on the predicted tetrahedrical coordination of the zinc ions in the enzyme active site, a molecular mechanism for the catalysis of L-dopachrome tautomerization is proposed. From the present data, the existence of additional ligands for metal ions other than zinc in the DCT molecule, such as the proposed cysteine iron-binding sites, cannot be completely ruled out. However, if such sites exist, they could be subsidiary binding sites, whose function would be likely to stabilize the protein.

INTRODUCTION

Melanin is a pigment that is widely distributed throughout all living organisms. In mammals the skin is its major tissue of synthesis and accumulation, although it is also found in hair, eyes and the inner ear. The first enzyme of the melanogenesis pathway is tyrosinase (monophenol,dihydroxyphenylalanine: oxygen oxidoreductase; EC 1.14.18.1), an enzyme catalysing the initial two steps of the oxidation of L-tyrosine to L-dopaquinone [1]. In addition to this enzyme, the regulation of the pathway involves at least two other proteins, termed tyrosinase-related proteins, TRP1 and TRP2 [2–8]. TRP1 is a second tyrosinase with a specific 5,6-dihydroxyindole-2-carboxylic acid (DHICA) oxidase function [5], and TRP2 functions as a dopachrome tautomerase (DCT; dopachrome $\Delta^2\Delta^7$-isomerase; EC 5.3.3.12), since it catalyses the conversion of L-dopachrome (DC) into DHICA [6]. The retention of the carboxyl group on the indole unit has important implications for the structure and properties of the melanin polymer formed [9].

Tyrosinase and its related proteins are glycometalloenzymes that are bound to the melanosomal membrane. They have the bulk of the polypeptide chain towards the melanosomal lumen, with a hydrophobic fragment spanning the melanosomal membrane and a relatively short C-terminal domain, around 30–40 amino acids long, facing the cytosolic side [1]. The three proteins tyrosinase, TRP1 and TRP2 have two homologous metal binding sites at the respective active sites [1,7]. Based on the corresponding amino acid sequences obtained from the cDNA, each metal ion is bound to the polypeptide chain through chelation by three conserved histidines (shown in bold in Figure 1).

1st Metal Binding site

Tyrosinase:	^{162}	**H**YYVSRDTLLGGSEI-WRDIDFA**H**EAPGFLPW**H**^{193}
TRP1:	^{168}	**H**YYSVKKTFLGTGQESFGDVDFS**H**EGPAFLTW**H**^{200}
TRP2:	^{167}	**H**YYSVKDTLLGPGRP-YKAIDFS**H**QGPAFVYW**H**^{198}

2nd Metal Binding site

Tyrosinase:	^{345}	**H**NAL**H**IFMNGTMSQVQGSANDPIFLLH**H**^{372}
TRP1:	^{353}	**H**NLA**H**LFLNGTGGQTHLSPNDPIFVLL**H**^{380}
TRP2:	^{347}	**H**NLA**H**SFLNGTNALPHSAANDPVFVVL**H**^{374}

Figure 1 Amino acid sequences of the metal-binding sites of the murine tyrosinase family

Conserved histidine residues that bind metal ions are shown in **bold** type. Data taken from [7].

Abbreviations used: DC, dopachrome (2-carboxy-2,3-dihydroindole-5,6-quinone); DCT, dopachrome tautomerase; DHI, 5,6-dihydroxyindole; DHICA, 5,6-dihydroxyindole-2-carboxylic acid; dopa, 3,4-dihydroxyphenylalanine; MBTH, 3-methyl-2-benzothiazoline hydrazone; TRP, tyrosinase-related protein.

* To whom correspondence should be addressed.

The existence of a pair of copper ions chelated by these histidines in the active site of tyrosinase is well established [10–13]. The metalloprotein nature of DCT was assumed from the first reports concerning this enzyme [4], and subsequently this was supported by the ability of several metal ions to catalyse the rearrangement of DC to DHICA with variable yields depending on the nature and concentration of the metal ion [14]. Because of its sequence identity with tyrosinase, some workers assumed that TRP2 would also contain copper [1,8,15]. However, the actual nature of the metal ion at the active site of DCT remained unclear, and the possible presence of iron-binding sites in the amino acid sequence of TRP2 and the tyrosinase family was also proposed [16]. In addition, because of the specific inhibition of DCT by metal chelators with a preferential affinity for ferrous iron, such as 1,10-phenanthroline and 2,2′-dipyridyl, the involvement of ferrous iron at the active site was proposed [17]. However, we have recently reported the reconstitution of tautomerase activity from apo-DCT with Zn^{2+} [18].

The different catalytic capabilities of tyrosinase and TRP2 indicate that their respective active sites should show specific features in spite of their identity. The differences could involve the side chains of the amino acids at the catalytic centre as well as the nature of the metal cofactor within it. To gain insight into the latter point, three approaches were used: (a) to explore the differences between tyrosinase and DCT concerning their inhibition by metal chelators; (b) measurement of the metal content in purified DCT preparations; and (c) preparation of apo-DCT and reconstitution of the enzymic activity with appropriate metal ions.

As result of these approaches we present definitive evidence demonstrating that Zn^{2+} is the metal ion cofactor in the active site of DCT. To date we know of 12 zinc-enzymes whose Zn^{2+} ligands and modes of co-ordination have been identified; these represent examples of oxidoreductases, transferases, hydrolases and lyases [19]. However, to our knowledge this is the first zinc-enzyme isomerase for which the amino acid sequence and positions of the histidines acting as chelators for the metal cofactor at the enzyme active site have been identified.

MATERIALS AND METHODS

Preparation of DCT

DCT was purified from B16 mouse melanoma cells according to the procedure described by Aroca et al. [6,20] with slight modifications. Briefly, freshly excised B16/F10 mouse melanomas were weighed and washed in ice-cold 10 mM phosphate buffer, pH 6.8, containing 0.25 M sucrose, 0.1 mM EDTA and 0.5 mM PMSF. Then, the tumours were homogenized with a Polytron homogenizer using a ratio of buffer volume to tumour mass of 2:1 (ml/g). The slurry was centrifuged at 700 ***g*** for 10 min at 4 °C, and the supernatant was centrifuged again at 11000 ***g*** for 30 min to obtain the melanosome-rich pellet. This was extracted with 1 % Nonidet P40 in 10 mM phosphate buffer, pH 6.8, using a volume of solution equal to the starting tumour mass (ml/g). The purification procedure for DCT was initiated from the solubilized melanosomal preparation, which was submitted to differential precipitation with ammonium sulphate and gel filtration chromatography on a Sephacryl S-300 column (52 cm × 2.6 cm). The two fractions with the highest DCT activity were pooled, concentrated and used as purified DCT. The final preparation was purified 55-fold and displayed a specific activity of 756 m-units/mg of protein, and the yield was 21 %. Further methods of characterization of DCT purity included SDS/PAGE and immunoreactivity on Western blots against the specific antibody αPEP8 [2,3], which was a gift from V. Hearing, NIH, Bethesda, MD, U.S.A. Details of the electrophoretic and Western blotting procedures are described elsewhere [5]. The protein preparation was homogeneous on SDS/PAGE, showing a molecular mass around 70 kDa, and this band was recognized by αPEP8 (results not shown).

Reagents

All biochemicals and chelators were of analytical grade and were used without any further purification. L-3,4-Dihydroxyphenylalanine (L-dopa), D-dopa, Nonidet P40, 3-methyl-2-benzothiazoline hydrazone (MBTH), EDTA, *o*-phenanthroline, 2,2′-dipyridyl, dithizone, PMSF and hydroxyapatite (type I suspension in 1 mM phosphate buffer, pH 6.8) were from Sigma (St. Louis, MO, U.S.A.). Brij 35, SDS, Tris and BSA were from Merck (Darmstadt, Germany). Chelex 100 and SDS/PAGE reagents were from Bio-Rad (Richmond, CA, U.S.A.). Sodium periodate, potassium cyanide and metal ions (always used as the sulphate salt) were supplied by Probus (Barcelona, Spain). Fe^{2+} was prepared as Mohr's salt (ferrous ammonium sulphate) to stabilize the ferrous ion; this solution was prepared daily. Cadmium was used as the chloride salt (Aldrich, Gesellschaft, Germany). The bicinchoninic acid kit for protein determination was purchased from Pierce. All solutions were prepared in double-distilled water passed through a Milli-Q system (Millipore) (resistance higher than 18 MΩ/cm). In addition, buffer solutions used for the preparation of apo-DCT and for reconstitution were also passed through Chelex 100 to eliminate traces of metal ions.

Determination of DCT activity

DCT activity was assayed by two different methods, as follows. (a) HPLC determination of DHICA formation from 0.5 mM L-DC after 12 min (except when stereospecificity experiments with D-DC were carried out). We used an isocratic elution buffer consisting of 50 mM potassium acetate/citric acid, adjusted to pH 4.4, containing 0.1 mM EDTA and 10 % (v/v) methanol. Typical retention times for DC, DHICA and 5,6-dihydroxyindole (DHI) are 2.05, 10.6 and 21.3 min respectively. They were detected using a Waters absorbance detector model 440 with a 313 nm filter. (b) Spectrophotometrically, by measuring the decrease in absorbance at 475 mm of a 0.1 mM L-DC solution in 10 mM phosphate buffer, pH 6.0 ($\epsilon = 3700\ M^{-1}\cdot cm^{-1}$). Both incubations were always carried out at 37 °C. The spectrophotometric method was used because it is very easy and convenient for following rapid processes, such as inactivation by chelators, and the HPLC assay was used for more accurate measurements, such as the reconstitutions. In the presence of certain inhibitors, such as *o*-phenanthroline, only the spectrophotometric assay could be performed, since this chemical produces a large peak in HPLC that masks DHICA formation. For both methods the incubation mixtures of samples and L-DC contained a large excess of EDTA (5 mM) to prevent any effect of free metal ions on L-DC. This substrate and its D-isomer were prepared by stoichiometric oxidation of the appropriate dopa isomer with sodium periodate [21]. One unit of enzyme acrivity was defined as the amount of enzyme that catalyses the tautomerization of 1 μmol of L-DC/min at 37 °C, measured by HPLC. Note that these units are not totally equivalent to those defined in former papers [20], since the concentration of substrate used in the spectrophotometric and HPLC methods are neither identical nor saturating, taking into account the K_m of the enzyme [21].

The dopa oxidase activity was determined spectrophoto-

metrically using L-dopa and MBTH at 500 nm, according to Winder and Harris [22] with minor modifications. One unit was defined as the amount of enzyme catalysing the oxidation of 1 μmol of dopa/min at 37 °C.

Protein determination

Protein was measured by the bicinchoninic acid test (Pierce) to avoid the interference of detergents. The standard curve was prepared with BSA solution (2 mg/ml).

Metal content

Zn, Fe and Cu were determined in a Varian AA-20 atomic absorption spectrophotometer. Appropriate controls of the buffer solutions used to purify these enzymes were analysed and their metal contents subtracted. This background ion concentration was always lower than 20 % of those measured in the purified enzyme samples.

Preparation of apo-DCT

DCT was incubated with 10 mM *o*-phenanthroline or KCN in 100 mM phosphate buffer, pH 9.0, for 3 h at 25 °C. The different conditions tested to remove excess chelator and to obtain the apoenzyme are described in the following section. They are based on gel filtration, desalting chromatography or extensive dialysis. All buffer solutions for these procedures were rendered metal free by passage through Chelex 100.

Reconstitution experiments

Reconstitution of the enzymic activity of apo-DCT preparations was assessed by removal of excess chelator (cyanide or *o*-phenanthroline) and incubation with metal ions according to three different methods.

(a) Method 1

A 1 ml sample of the treated DCT preparation was extensively dialysed against 1 litre of 10 mM phosphate buffer, pH 6.8, containing 0.1 % Nonidet P40 and 50 μM EDTA at 4 °C, with two changes of dialysis buffer (after 4 h and overnight). EDTA was added to minimize reconstitution by traces of metal ion during the dialysis procedure. Enzyme reconstitution was performed at 25 °C by addition to an aliquot of the apo-DCT preparation of a freshly prepared water solution containing 1 mM of the appropriate metal ion until reaching a 75 μM concentration of the corresponding cation [18].

(b) Method 2

A 1 ml sample of the treated DCT preparation was applied to a Sephadex G-25 column (PD-10 pre-packed desalting column; Pharmacia) equilibrated with 50 mM phosphate buffer, pH 6.8, containing 0.1 % Nonidet P40 and 50 μM EDTA at 4 °C. The chromatography step was around 10 min, so that the time needed for chelator-free apo-DCT preparation was greatly reduced. The apo-DCT, eluted at the void volume, was reconstituted as described for method 1.

(c) Method 3

A 1 ml sample of the treated DCT preparation was extensively dialysed overnight against 1 litre of 2 mM phosphate buffer, pH 6.8, containing 0.1 % Nonidet P40, 20 mM potassium nitrate and 100 μM $ZnSO_4$ at 4 °C. The phosphate molarity in the dialysis buffer was decreased to avoid zinc precipitation, but the ionic strength was conserved with KNO_3. By this procedure, the removal of the chelator agent was simultaneous with introduction of Zn^{2+} into the enzyme active site.

RESULTS

Inactivation by cyanide and *o*-phenanthroline

We recently reported the enzymic inactivation of DCT and the preparation of apo-DCT by incubation of the holoenzyme with cyanide at alkaline pH [18]. Inactivation can also be carried out with *o*-phenanthroline [17]. Table 1 shows the comparative efficiency of the two agents in inactivating DCT at different pH values. The enzyme was incubated with 10 mM of the appropriate chelator, and then aliquots of this mixture were taken and diluted into the assay mixture for activity determination. Although both inactivations were accelerated under moderately alkaline conditions, the pH dependence was more pronounced for inactivation by cyanide than by *o*-phenanthroline. Therefore *o*-phenanthroline is more useful to inactivate DCT at slightly acidic or neutral pHs, but cyanide is more effective in totally inactivating the enzyme.

Metal content in DCT

The metal content of purified DCT preparations (Table 2) supports the hypothesis that zinc is the metal bound to the active site of DCT. The purified preparation of DCT contains around 1.5 zinc atoms per protein molecule, and it is virtually free of copper. The existence of a certain amount of iron (less than 1 atom/molecule) makes it unlikely that this metal ion participates in the structure of the protein or is directly involved in the catalytic mechanism of tautomerization.

Table 1 Inactivation of DCT by 10 mM cyanide or *o*-phenanthroline

Incubations were carried out for 3 h at 25 °C, in triplicate; results are means ± S.E.M. The initial DCT activity in the assay medium was 20 m-units/ml.

	Residual activity (%)	
pH	Cyanide	*o*-Phenanthroline
6	100 ± 3	49 ± 8
7	95 ± 3	36 ± 5
7.5	65 ± 5	29 ± 3
8	32 ± 3	26 ± 6
9	1 ± 1	24 ± 8

Table 2 Metal content in purified DCT preparations

Copper, iron and zinc were determined, in duplicate, by atomic absorption spectrophotometry. Values are the means ± S.E.M. of two determinations using different purified DCT preparations. Ion contents in the reagent or buffer blank were subtracted from the content of the DCT sample.

Metal ion	Content (ions/protein molecule)
Cu^{2+}	0.025 ± 0.015
Fe^{2+}	0.32 ± 0.08
Zn^{2+}	1.45 ± 0.13

Table 3 Preparation of apo-DCT and reconstitution of the tautomerase activity

Values are the means ± S.E.M. of two determinations. The initial DCT activity in the purified preparation was 756 m-units/ml.

Sample	Residual or recovered DCT activity (%)
Apo-DCT preparation	
DCT + cyanide	2.5 ± 1.4
Dialysed apo-DCT	11.0 ± 2.4
Reconstitution	
+ Water	10.0 ± 1.9
+ Cu^{2+}	8.5 ± 1.2
+ Fe^{2+}	9.0 ± 1.3
+ Ni^{2+}	10.5 ± 1.3
+ Cd^{2+}	11.5 ± 2.0
+ Co^{2+}	22.0 ± 3.2
+ Zn^{2+}	33.0 ± 3.5

Table 3 shows the results obtained when DCT was treated with 5 mM KCN at pH 8.5 for 5 h, followed by extensive dialysis to remove the excess cyanide and the metal-ion–cyanide complexes formed, according to method 1 described in the Materials and methods section. The activity was almost completely suppressed by cyanide, and accordingly the metal content in apo-DCT preparations after cyanide treatment and extensive dialysis was negligible. However, around 10 % of the initial DCT activity was recovered after dialysis against sodium phosphate buffer, in spite of pretreatment of this buffer with Chelex 100 and the presence of 50 μM EDTA to prevent recovery of DCT activity due to the presence of traces of metal ions. Therefore this slight reactivation achieved after cyanide removal but in the absence of exogenous metal ions suggests that the endogenous metal ions are tightly bound to the native enzyme, and that a small proportion cannot be removed from the protein molecule even by cyanide treatment.

Table 3 also shows the recovery of enzyme activity on incubation with 75 μM of several bivalent metal ions (to yield approx. 50 μM free ion, after chelation of the other 25 μM by the EDTA present in the samples). DCT activity was determined in the presence of a large excess of EDTA (5 mM) to prevent the effect of free metal ions on L-DC. After incubation of apo-DCT with metal ions only Zn^{2+}, and to a lesser extent Co^{2+}, were able to recover tautomerase activity, but Ni^{2+}, Cd^{2+} and the physiologically occurring Cu^{2+} and Fe^{2+} were totally ineffective in recovering any more DCT activity compared with the control.

The fraction of DCT activity recovered by Zn^{2+} was significant but was only about 35 % of the total, probably due to the instability of the apo-DCT molecule and the long time required for extensive dialysis and reconstitution [18]. Taking this into account, two alternative reconstitution procedures were tested. When reconstitution was attempted by rapid removal of the chelator in a gel-filtration desalting column, very low activity could be restored (around 4 % of the initial DCT activity; Table 4). Similar data were obtained after *o*-phenanthroline treatment, but in this case no activity was recovered at all. This suggests that the formation of the DCT–inhibitor heterocomplex through chelation of the metal ion by the apoprotein and the inhibitor is a fast reaction, but the removal of the metal ion sequestered by chelator molecules is the slow and rate-limiting step of the inactivation process.

To improve the degree of reconstitution of tautomerase activity by addition of Zn^{2+} to apo-DCT, we tested a third alternative method. It consisted of dialysing directly the chelator-treated enzyme against a solution containing 100 μM Zn^{2+}, so that removal of cyanide and the hypothetical entry of new Zn^{2+} ions into the active site could occur simultaneously. Table 4 shows a comparison of enzymic activities recovered by the three methods, showing that the efficacy of the reconstitution was greatly enhanced by this last procedure. This confirms the poor stability of apo-DCT compared with the holoenzyme, so that the sooner Zn^{2+} goes into the active site to restore the architecture of the holoenzyme, the higher the percentage of activity recovered.

Table 4 DCT activity recovered from apo-DCT and Zn^{2+} by three different methods

In all cases, apo-DCT was obtained by cyanide treatment, but the excess cyanide was eliminated slowly by dialysis (methods 1 and 3) or rapidly by gel filtration desalting chromatography (method 2). Zn^{2+} was added after cyanide removal (methods 1 and 2), or simultaneously (method 3). For further details, see the Materials and methods section.

Method	Activity recovered (%)
1. Dialysis plus incubation with Zn^{2+}	33 ± 3.5
2. Gel filtration chromatography plus incubation with Zn^{2+}	4 ± 2
3. Dialysis against Zn^{2+}	67 ± 5

Table 5 Effect of the order of addition of Zn^{2+} and a second metal ion on the reconstitution of DCT activity

The first incubation of apo-DCT was carried out with 75 μM Me^{2+} for 8 h at 25 °C, and the second by further overnight incubation (15 h) supplemented with 300 μM of the second metal ion. In the first series, apo-DCT was reconstituted with Zn^{2+} and then supplemented with other ions; in the second series apo-DCT was incubated with other ions and then supplemented with Zn^{2+}. Values are the means ± S.E.M. of two determinations.

	Activity recovered (%)			
	First series		Second series	
Metal ion(Me^{2+})	+ Zn^{2+}	+ Me^{2+}	+ Me^{2+}	+ Zn^{2+}
None (water)	19.5 ± 0.9	18.8 ± 1.2	6.3 ± 0.4	10.7 ± 1.0
Fe^{2+}		19.1 ± 3.3	6.0 ± 0.7	6.5 ± 0.5
Cu^{2+}		9.6 ± 0.9	4.2 ± 0.6	4.2 ± 0.9
Co^{2+}		16.5 ± 0.6	10.0 ± 1.1	11.3 ± 1.4

The effects of a second metal ion on the activity of reconstituted DCT were also investigated. Two different series of experiments were carried out (Table 5). In the first, apo-DCT was reconstituted by incubation with Zn^{2+} and then the effect of a further incubation with Fe^{2+}, Cu^{2+} or Co^{2+} was investigated. The effect of iron displayed particularly high variability between the different experiments, suggesting some role for this ion at other possible sites on the enzyme that are not essential for enzymic activity. On the other hand, Cu^{2+} always produced a reproducibly significant inhibition. In the second series of experiments, apo-DCT was first incubated with Fe^{2+}, Cu^{2+} or Co^{2+} to reconstitute Me^{2+}-DCT species, and then incubated with Zn^{2+}. Consistent with the data described in Table 3, Co^{2+} was the only ion, other than Zn^{2+}, that partially restored enzymic activity. The increase in enzymic activity due to the subsequent addition of Zn^{2+} was negligible in all cases, suggesting that other metal ions can bind

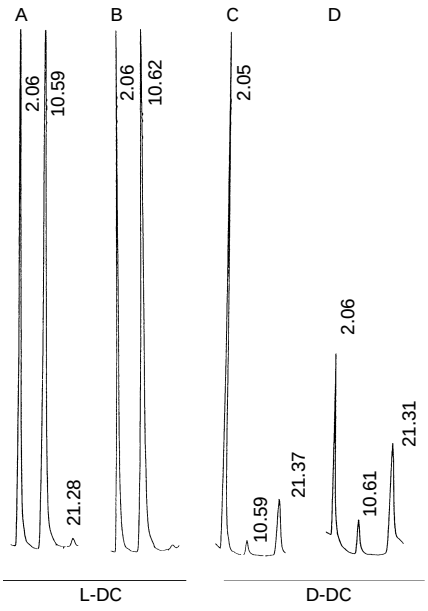


Figure 2 HPLC analysis of the stereospecificity of native DCT and reconstituted Zn^{2+}-DCT

L-DC (**A** and **B**) and D-DC (**C** and **D**) were incubated at 0.5 mM for 12 min with native DCT (**A** and **C**) and reconstituted Zn^{2+}-DCT (**B** and **D**). The peaks eluting at 2.05, 10.6 and 21.3 min are DC (irrespective of the isomer), DHICA and DHI respectively. Both enzymes are able to tautomerize L-DC to DHICA. However, the reconstituted enzyme catalysed the rapid disappearance of D-DC to yield mainly DHI. Note that the absorption coefficient at 313 nm is much higher for DHICA than for DHI.

to the active site and that their displacement from it by Zn^{2+} does not take place easily.

Both native DCT and reconstituted Zn^{2+}-DCT showed stereospecificity, being able to tautomerize only the L-DC isomer into DHICA (Figures 2A and 2B). However native DCT was virtually unable to act on D-DC (Figure 2C), while Zn^{2+}-DCT catalysed the rapid disappearance of this isomer by a decarboxylative rearrangement to yield DHI (Figure 2D). This suggests the existence of some differences in the architecture of the reconstituted active site.

DISCUSSION

The structural differences at the active sites of tyrosinase and DCT are clearly reflected by the different inhibitory effects of some metal chelators on these enzymes. On one hand, phenylthiourea and L-mimosine are powerful inhibitors of tyrosinase that have no noticeable effect on DCT [20,23]. On the other hand, *o*-phenanthroline and 2,2′-dipyridyl inhibit DCT but not tyrosinase [17]. Cyanide is a wide spectrum chelator, inhibiting both tyrosinase and DCT. It is well known that tyrosinase is a copper-containing protein [10,11], but the metal ion at the DCT active site has been proposed to be copper [1,13], iron [17] or zinc [18]. Although *o*-phenanthroline inhibits a number of iron-containing enzymes [24], it cannot be considered a specific chelator of iron since it also inhibits the copper-enzyme laccase [25] and a number of zinc-enzymes including carbonic anhydrase [26], bacterial proteases [27] and glutamyl-tRNA synthetase [28]. Dithizone, a well known chelator showing great affinity for Zn^{2+} [29], inhibits DCT to a significant extent in spite of its low solubility in aqueous media. When DCT was incubated with 50 μM chelator for 15 h at 25 °C and pH 7, cyanide was ineffective but *o*-phenanthroline and dithizone produced enzymic inactivations of 24 % and 36 % respectively.

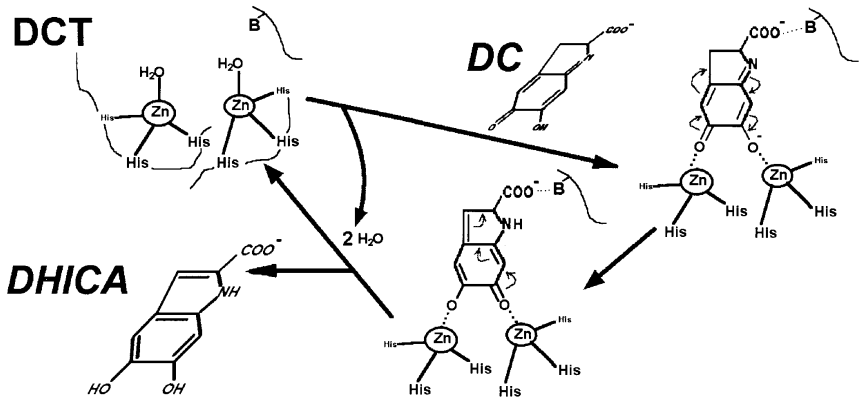
Inhibition studies with metal chelators cannot unequivocally prove the nature of the metal ion at the active site of DCT due to the absence of metal chelators showing an absolute specificity for any metal ion but, taken together, these data did not support the possibility of Cu being the cofactor for DCT, and suggested the presence of Zn rather than Fe. This suggestion was strongly supported by the content of zinc in purified DCT preparations compared with the low content of Fe and Cu found. The zinc ions were removed by cyanide treatment accompanying the loss of enzymic activity, supporting the assumption that the Zn^{2+} ions are chelated by histidines at the two metal-binding sites of DCT.

The preparation of an inactive metal-free apoenzyme that can be re-activated by the addition of an appropriate metal ion has been used as confirmatory evidence for the metal requirements of the enzyme. Our reconstitution data for apo-DCT showed that Zn^{2+} was the appropriate metal to reconstitute DCT activity. It is well known that melanin-containing tissues are especially rich in Zn^{2+} [30,31], but this accumulation is not related to the chelating capacity of melanin, since the content of Zn^{2+} is higher in poorly pigmented melanoma tissues than in heavily pigmented tissues [31,32]. In addition, Zn^{2+} is quite abundant in premelanosomes, its level being similar to that of iron and much higher than that of copper [33]. In spite of these data, the function of Zn^{2+} in melanogenesis has remained unknown. The possible involvement of this ion in accelerating the rearrangement of DC to dihydroxyindoles was initially suggested by a number of reports [34,35]. However, a role for Zn^{2+} as the metal cofactor required by DCT to yield DHICA seemed unlikely, in view of the effectiveness of free metal ions in catalysing DHICA formation from L-DC [4,14]. These reports showed that Zn^{2+} was poorly efficient in catalysing the non-decarboxylating rearrangement, with Co^{2+}, Ni^{2+} and Cu^{2+} being far more effective. Nevertheless, it was also reported that L-DC treated with 2 % zinc acetate yielded 91 % DHICA [36], as far as we know the highest percentage ever reported for any free metal, and very close to the yield obtained with DCT [15]. Although such a high concentration of zinc is not present in living cells, these chemical conditions could be mimicked at the enzyme active site using much lower concentrations of metal cofactors.

The fact that Co^{2+} could restore some tautomerase activity when added to apo-DCT also suggests that Zn^{2+} is the metal at the active site of native DCT for two major reasons. Firstly this is a isomorphic replacement, and according to the Irving–Williams series, which predicts a trend in the formation constants of metal chelators based on the ratio of charge to radius and the ligand field energy [29], Co^{2+} is the ion most similar to Zn^{2+}. Cobalt is rare in living systems, but it is a very valuable probe atom since its chemical similarity to zinc is such that it can replace it specifically [29], as in carbonic anhydrase [26]. Secondly, it has been shown that Co^{2+} can replace Cu^{2+} in *Neurospora* tyrosinase [37], indicating that the histidines at the active site of the tyrosinase family enzymes can bind Co^{2+}.

If it is assumed that DCT is a zinc-enzyme and tyrosinase is a copper-enzyme, and that the two enzymes have very similar amino acid sequences at their metal-binding sites, it is interesting to analyse the physico-chemical reasons for this difference. Two main factors can be discussed: (a) the nature of the reactions to be catalysed: tautomerization versus oxidation; and (b) the structural requirements at the respective active sites.

Concerning the first point, the existence of zinc at the active site of DCT clearly accounts for the different catalytic ability shown by DCT in contrast to the reactions catalysed by tyrosinase. The mechanism of catalysis of tyrosinase is well



Scheme 1 Proposed molecular mechanism for the DCT-catalysed tautomerization of DC

The active site of DCT, which contains two tetrahedral Zn^{2+} ions, would bind one L-DC molecule. The absence of redox properties of the metal ion induces field effects on the substrate that lead to its electronic rearrangement, and subsequent hydrogen migration consistent with tautomerization to DHICA.

established [11,12]. Copper ions at the enzyme active site may undergo a redox reaction between two states (Cu^{+}/Cu^{2+}) due to the binding of oxygen and subsequent electron transfer that results in the oxidation of *o*-diphenolic substrates to *o*-quinones. On the other hand, Zn^{2+} has some characteristics in common with Cu^{2+} and both ions are good Lewis acid catalysts, but Zn^{2+} has no redox chemistry associated with it under biological conditions. Thus Zn^{2+} is preferable to almost all other cations as an acid–base catalyst in living systems on the grounds of availability, power and absence of redox properties [19]. On comparing DCT to tyrosinase, the presence of Zn^{2+} in the first enzyme is probably due to the absence of redox reactions.

Concerning the second point, metal ions generally are ligated to proteins by a shell of hydrophilic groups that are embedded within a larger shell of hydrophobic groups [38]. This situation is common to the TRPs, since metal ions are bound to hydrophilic histidines contained in α-helix hydrophobic shells [8], but looking at the sequences of the metal-binding sites (Figure 1), there is an important difference at the first binding site. DCT contains the motif $G^{177}PGRP^{181}$, characteristic of a β-turn, but this is absent from tyrosinase and TRP1. The importance of this motif is demonstrated by the unique mutation $R \rightarrow Q$ found in mutant *slaty* mouse [7,39], which leads to a DCT showing only residual activity.

Moreover, in the co-ordination sphere of copper ions in oxytyrosinase, Cu^{2+} adopts a tetragonally distorted octahedral stereochemistry [12]. However, desoxytyrosinase (containing Cu^{+}) and the reconstituted Co^{2+}-tyrosinase have a tetrahedral structure [37]. In carbonic anhydrase, Zn^{2+} is ligated by three histidine residues in a distorted tetrahedron, with the fourth co-ordination site presumably occupied by a water molecule [19,26]. Thus Zn^{2+} and Co^{2+} are two ions that prefer tetrahedral rather than octahedral geometry at their chelates, and the co-ordination geometry of Zn^{2+} at the DCT active site should be similar to that of the desoxy form of tyrosinase (Cu^{+}). Thus we propose that each Zn^{2+} at the DCT active site is co-ordinated to three histidines and that the fourth position is occupied by a water molecule. The role of Zn^{2+} in stabilizing DCT would explain the reported data on the instability of apo-DCT [18]. Scheme 1 depicts a proposal for the catalytic mechanism of DCT. The displacement of the water molecule by the substrate, L-DC, very likely results in a rearrangement of double bonds in the indolequinone ring, so that the tautomerization of L-DC to DHICA should be the final action of the enzyme. From the present data, however, the existence of additional ligands for other metal ions in the DCT molecule, such as the proposed iron-binding sites [1,16], cannot be ruled out. These sites could be subsidiary binding sites, whose function would be to stabilize the protein. They would explain the presence of a certain amount of iron in the TRPs and the effects of this ion, reported herein and by Chakraborty et al. [17].

It is interesting to put forward some final comments about the stereospecificity of the native and reconstituted enzymes. Native DCT shows a very stringent specificity, and dopaminochrome and D-DC are not substrates for the enzyme [20]. This indicates that both the presence and orientation of the carboxyl group are essential requirements for interaction at the active site with an as yet unidentified electron acceptor group (labelled B in Scheme 1). The Zn^{2+}-reconstituted enzyme shows stereospecificity too, but it is also able to catalyse the decarboxylative rearrangement of D-DC to yield DHI. In turn, the reconstituted enzyme shows higher sensitivity to metal chelators than the native enzyme, suggesting that the removal of Zn^{2+} from this species is easier than from the native enzyme. These data, taken together, indicate that the architecture of the native enzyme active site is not totally reproduced after reconstitution. One of the distal histidines, or the proposed electronic acceptor group, might not recover the correct position. It has been reported that other DCT-related enzymes obtained from insects [40], cuttlefish [41] and rat tissues [42] are able to yield DHI rather than DHICA, with different stereospecificities. Comparison of the sequences at the active site of these 'tautomerase' enzymes with that of mammalian DCT could help to give insight on this point.

This work was supported by grant no. DGICYT 95/1158 from the Fondo de Investigaciones Sanitarias. C.J.-C. is the recipient of a fellowship from the Instituto de Fomento de la Comunidad Autónoma de Murcia. We thank V. Hearing for helpful suggestions and critical reading of the manuscript.

REFERENCES

1 Hearing, V. J. and Tsukamoto, K. (1991) FASEB. J. **5**, 2902–2909
2 Tsukamoto, K., Jackson, I. J., Urabe, K., Montague, P. M. and Hearing, V. J. (1992) EMBO J. **11**, 519–526
3 Tsukamoto, K., Jiménez, M. and Hearing, V. J. (1992) Pigment Cell Res. Suppl. **2**, 84–89
4 Leonard, L. J., Townsend, D. and King, R. A. (1988) Biochemistry **27**, 6156–6159
5 Jiménez-Cervantes, C., Solano, F., Kobayashi, T., Urabe, K., Hearing, V. J., Lozano, J. A. and García-Borrón, J. C. (1994) J. Biol. Chem. **269**, 17993–18001

6 Aroca, P., García-Borrón, J. C., Solano, F. and Lozano, J. A. (1990) Biochim. Biophys. Acta **1035**, 266–275
7 Jackson, I. J., Chambers, D. M., Tsukamoto, K., Copeland, N. G., Gilbert, D. J., Jenkins, N. A. and Hearing, V. J. (1992) EMBO J. **11**, 527–535
8 Jackson, I. J. (1994) Annu. Rev. Genet. **28**, 189–217
9 Orlow, S. J., Osber, M. P. and Pawelek, J. M. (1992) Pigment Cell Res. **5**, 113–121
10 Kubowitz, F. (1938) Biochem. Z. **299**, 32–57
11 Winkler, M., Lerch, K. and Solomon, E. I. (1981) J. Am. Chem. Soc. **103**, 7001–7003
12 Oetting, W. S. and King, R. A. (1994) Pigment Cell Res. **7**, 285–290
13 Wilcox, D. E., Porras, A. G., Hwang, Y. T., Lerch, K., Winkler, M. E. and Solomon, E. I. (1985) J. Am. Chem. Soc. **107**, 4015–4027
14 Palumbo, A., D'Ischia, M., Misuraca, G. and Prota, G. (1987) Biochim. Biophys. Acta **925**, 203–209
15 Palumbo, P., Solano, F., Misuraca, G. Aroca, P., García-Borrón, J. C., Lozano, J. A. and Prota, G. (1991) Biochim. Biophys. Acta **1115**, 1–5
16 Halaban, R. and Moellman, G. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 4809–4813
17 Chakraborty, A. K., Orlow, S. J. and Pawelek, J. M. (1992) FEBS Lett. **302**, 126–128
18 Solano, F., Martínez-Liarte, J. H., Jiménez-Cervantes, C., García-Borrón, J. C. and Lozano, J. A. (1994) Biochem. Biophys. Res. Commun. **204**, 1243–1250
19 Vallee, B. L. and Auld, D. S. (1990) Biochemistry **29**, 5647–5659
20 Aroca, P., Solano, F., García-Borrón, J. C. and Lozano, J. A. (1991) Biochem. J. **277**, 393–397
21 Aroca, P., Solano, F., García-Borrón, J. C. and Lozano, J. A. (1990) J. Biochem. Biophys. Methods **21**, 35–46
22 Winder, A. J. and Harris, H. (1991) Eur. J. Biochem. **198**, 317–326
23 Barber, J. I., Townsend, D., Olds, D. P. and King, R. A. (1984) J. Invest. Dermatol. **83**, 145–149
24 Shiman, R. and Kaufman, S. (1970) Methods Enzymol. **17A**, 609–615
25 Malmström, B. G. (1965) in Oxidases and Related Redox Systems, vol. 1 (King, T. S., Mason, H. S. and Morrison, M., eds.), pp. 207–221, John Wiley & Sons, New York
26 Lindskog, S., Henderson, L. E., Kannen, K.K, Liljas, A., Nyman, P. O. and Stranberg, B. (1971) Enzymes **5**, 587–665
27 Ayora, S. and Gotz, F. (1994) Mol. Gen. Genet. **242**, 421–430
28 Liu, J., Lin, S. X., Blochet, J. E., Pezolet, M. and Lapointe, J. (1993) Biochemistry **32**, 11390–11396
29 Williams, R. P. J. (1987) Polyhedron **6**, 61–69
30 Horcicko, J., Borovansky, J., Duchon, J. and Prochazkova, B. (1973) Hoppe-Seyler's Z. Physiol. Chem. **354**, 203–204
31 Jara, J. R., Solano, F., García-Borrón, J. C., Aroca, P. and Lozano, J. A. (1990) Biochim. Biophys. Acta **1035**, 276–285
32 Bowness, J. M. and Morton, R. A. (1953) Biochem. J. **53**, 620–626
33 Shibata, T., Prota, G. and Mishima, Y. (1993) J. Invest. Dermatol. **100**, 275S–280S
34 Harley-Mason, J. and Bu'Lock, J. D. (1950) Nature (London) **166**, 1036–1037
35 Vachtenheim, J., Duchon, J. and Matous, B. (1985) Anal. Biochem. **146**, 405–410
36 Napolitano, A., Chioracca, F. and Prota, G. (1985) Gazz. Chim. Ital. **115**, 357–359
37 Rüegg, C. and Lerch, K. (1981) Biochemistry **20**, 1256–1262
38 Yamashita, M. M., Wesson, L., Eisenman, G. and Eisenberg, D. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 5648–5652
39 Kroumpouzos, G., Urabe, K., Kobayashi, T., Sakai, C. and Hearing, V. J. (1994) Biochem. Biophys. Res. Commun. **202**, 1060–1068
40 Sugumaran, M. and Semensi, V. (1991) J. Biol. Chem. **266**, 6073–6078
41 Palumbo, A., d'Ischia, M., Misuraca, G., de Martino, L. and Prota, G. (1994) Biochem. J. **299**, 839–844
42 Odh, G., Hindemith, A., Rosengren, A. M., Rosengren, E. and Rorsman, H. (1993) Biochem. Biophys. Res. Commun. **197**, 619–624

Received 16 May 1995/8 August 1995; accepted 25 August 1995