Specificity of dopachrome tautomerase and inhibition by carboxylated indoles

Considerations on the enzyme active site

Pilar AROCA, Francisco SOLANO,* Jose C. GARCIA-BORRÓN and Jose A. LOZANO

Departamento Bioquímica y Biología Molecular, Facultad de Medicina, Universidad de Murcia, 30071 Murcia, Spain

Dopachrome tautomerase (EC 5.3.2.3) catalyses the tautomerization of dopachrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA) within the melanin-formation pathway. We have analysed a series of substrate analogues and related compounds as possible substrates and inhibitors of tautomerization. The enzyme appears to be highly specific since D-dopachrome, α -methyl-dopachrome, dopaminochrome, adrenochrome methyl ether and deoxyadrenochrome are not substrates. Conversely, dopachrome tautomerase catalyses the tautomerization of dopachrome methyl ester, suggesting that a carboxy group, either free or as a methyl ester, is essential for enzyme recognition. No inhibition of dopachrome tautomerization was observed in the presence of either semiquinonic compounds, such as tropolone and L-mimosine, or pyrrole-2-carboxylic acid and unsubstituted indole. However, a number of indole derivatives, including DHICA, the product of dopachrome tautomerization, and the analogues 5-hydroxyindole-2-carboxylic acid and indole-2-carboxylic acid were able to inhibit the enzyme. Furthermore, indoles with a side chain at position 3 of the ring and containing a carboxylic group at the γ -position of this chain, such as L-tryptophan or indole-3-propionic acid, are stronger inhibitors of the enzyme. Indole-3-carboxylic acid, indole-3-acetic acid and indole-3-butyric acid are very weak inhibitors, showing that the carboxylic group needs to be located at an optimal distance from the indole ring to mimic the carboxylic group at position 2 on the authentic substrate.

INTRODUCTION

The melanin-biosynthetic pathway has been extensively studied, but its regulation is still poorly known. The first part of the pathway, which is fairly well understood, consists of the oxidation of L-tyrosine to dopachrome, catalysed by the enzyme tyrosinase (EC 1.14.18.1) (Mason, 1948; Lerner & Fitzpatrick, 1950; Cabanes *et al.*, 1987). In the second part, dopachrome is oxidized to yield melanin. Some regulatory factors acting on this last series of reactions have been postulated (Pawelek *et al.*, 1980; Korner & Pawelek, 1980; Barber *et al.*, 1984; Aroca *et al.*, 1990a).

It was first thought that dopachrome oxidation and polymerization occurred spontaneously through a series of highly reactive intermediates, such as 5,6-dihydroxyindole (DHI), indole-5,6-quinone (IQ) and melanochrome, yielding an irregular melanin polymer (Mason, 1948). However, further studies led to the proposal of the existence of protein factors controlling this part of the melanogenesis pathway. Among these factors, only a dopachrome-conversion factor (Pawelek *et al.*, 1980; Korner & Pawelek, 1980), also called dopachrome oxidoreductase (Barber *et al.*, 1984), has been partially characterized. It was postulated that this factor catalysed the direct decarboxylation of dopachrome to yield DHI, since this indole was proposed to be the final product of the enzymic reaction. In turn, the spontaneous chemical decarboxylation of 5,6-dihydroxyindole-2-carboxylic acid (DHICA) to DHI does not take place (Palumbo *et al.*, 1988). More recent studies have shown that the enzymic action consists of the non-decarboxylative re-arrangement of dopachrome to DHICA, as shown in Scheme 1 (Aroca *et al.*,

1990a,b; Leonard *et al.*, 1988; Pawelek, 1990). This reaction is a tautomerization, and therefore the enzyme has recently been renamed dopachrome tautomerase (EC 5.3.2.3) (Aroca *et al.*, 1990a).

Although the nature of the substrate and product of dopachrome tautomerase is now well established, no data are available as to the structural features essential for substrate recognition by the active site, and the specificity of the enzyme remains unknown. In the present paper we report the first data

Scheme 1. Dopachrome tautomerase catalyses the tautomerization of dopachrome to DHICA

The substrate is not stable, and it undergoes a slow spontaneous decarboxylative rearrangement to DHI in the absence of enzyme.

Abbreviations used: dopachrome, 2-carboxy-2,3-dihydroindole-5,6-quinone; dopaminochrome, 2,3-dihydroindole-5,6-quinone; deoxyadrenochrome, 1-methyl-2,3-dihydroindole-5,6-quinone; adrenochrome methyl ether, 1-methyl-3-methoxy-2,3-dihydroindole-5,6-quinone; α -methyl-dopachrome, 2-methyl-2-carboxy-2,3-dihydroindole-5,6-quinone; DHICA, 5,6-dihydroxyindole-2-carboxylic acid; DHI, 5,6-dihydroxyindole; IQ, indole-5,6-quinone.

* To whom correspondence should be addressed.

on alternative substrates and on the inhibition of dopachrome tautomerase from B16-F10 mouse melanoma by a number of reagents structurally related to the product of the tautomerization. From these data, some characteristics of the enzyme active site and its structural requirements are discussed.

MATERIALS AND METHODS

Reagents

L-Tyrosine, L-tryptophan, 5-hydroxy-L-tryptophan, tryptamine, L-dopa, D-dopa, L- α -methyldopa, L-dopa methyl ester, adrenaline methyl ether, deoxyadrenaline, phenylmethanesulphonyl fluoride, EDTA, phenylthiourea, hydroxyapatite type 1, Brij-35, 3,4-dihydroxybenzylamine, 3,4-dihydroxybenzoic acid, tropolone, L-mimosine, pyrrole-2-carboxylic acid, indole, indole-2-carboxylic acid, 5-hydroxyindole, 5-hydroxyindole-2-carboxylic acid, indole-3-carboxylic acid, indole-3-acetic acid, indole-3-propionic acid and indole-3-butyric acid were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Na_2HPO_4 , NaH_2PO_4 , NaOH, sucrose, trichloroacetic acid, $(\text{NH}_4)_2\text{SO}_4$ and NaCl were from Merck (Darmstadt, Germany). Sodium periodate was from Probus (Barcelona, Spain). Sephacryl S-300 and DEAE-Sephadex were from Pharmacia (Uppsala, Sweden). DHICA was kindly given by Dr. Wyler (Lausanne, Switzerland), and was also obtained in our laboratory as described elsewhere (Wakamatsu & Ito, 1988). All reagents were of the highest purity commercially available and were used without further purification. All solutions were prepared in double-distilled water, further deionized by passage through a Waters Milli-Q deionizer system (final resistance greater than $10 \text{ m}\Omega \cdot \text{cm}$).

Animals and melanomas

B16-F10 mouse melanoma melanocytes were maintained by serial transplantation on hybrid mice obtained from male DBA and female C57/B1 (Panlab, Barcelona, Spain). Only male mice at 6–8 weeks of age were used for tumour transplantation and they were injected subcutaneously with approx. 10^5 viable cells. After 3–4 weeks, visible tumours were excised, and then some were used for new implantation and the others for enzymic preparations.

Purification of dopachrome tautomerase

The purification process was carried out by the method of Aroca *et al.* (1990a). Briefly, freshly excised tumours were weighed and washed in ice-cold 10 mM-phosphate buffer, pH 6.8, containing 0.25 M-sucrose and 0.1 mM-EDTA. The washed tumours were homogenized in the same buffer supplemented with 0.1 mM-phenylmethanesulphonyl fluoride. The homogenate was then centrifuged at 700 g for 20 min and the supernatant was further centrifuged at 11000 g for 30 min. The resulting melanosomal pellet was solubilized in 10 mM-phosphate buffer, pH 6.8, containing 1% Brij 35. The extract was then adjusted to 35% saturation with $(\text{NH}_4)_2\text{SO}_4$. After centrifugation at 11000 g for 30 min, the supernatant was adjusted to 60% saturation with $(\text{NH}_4)_2\text{SO}_4$ and again centrifuged. The pellet was resuspended in a small volume of 0.1% Brij 35 in 10 mM-phosphate buffer, pH 6.8, extensively dialysed against this buffer, and further purified by hydroxyapatite chromatography and gel-filtration chromatography. The fractions with the highest specific activities were pooled, concentrated by ultrafiltration and used for our studies.

By this procedure, the specific activity of the preparation was increased from 18 munits/mg for the crude melanosomal extract to 648 munits/mg for the final preparation, obtained after pooling the three fractions with highest activities from the gel-

filtration chromatography step. The yield of the purification was 32%, and the purification factor 36-fold, considering the melanosomal extract as the original material. These values are very similar to those published before (Aroca *et al.*, 1990a). The enzyme at this purification stage was still not totally pure, and SDS/PAGE showed the presence of four bands in the preparation. Total purification of the enzyme has yet to be achieved. However, the final preparation was totally devoid of tyrosinase, the only known enzyme that could interfere with the assay of dopachrome tautomerase. Addition to the enzymic preparation of phenylthiourea, a well-known tyrosinase inhibitor, did not affect the tautomerase activity. Other proteins most probably do not interfere with the assay since they do not affect the dopachrome stability at the concentrations used in the assay media as described by Aroca *et al.* (1990b).

Preparation of dopachrome and related compounds

Fresh solutions of dopachrome were prepared by mixing a solution of L-dopa in 10 mM-sodium phosphate, pH 6.0, and the required volume of a solution of sodium periodate to achieve a 1:2 molar ratio of L-dopa/periodate. The dopachrome solutions were prepared immediately before use because of its relative instability. Other 'chromes' and quinones were prepared by periodate oxidation of their corresponding *o*-dihydroxy precursors in the same way as dopachrome is prepared from dopa, but with the required amount of sodium periodate (1:1 for *o*-quinones).

Determination of dopachrome tautomerase activity

This enzymic activity was spectrophotometrically determined by monitoring the decrease in absorbance at 475 nm, the peak of visible absorbance for most of the possible substrates used. However, the activity was monitored at 485 nm for adrenochrome methyl ether and at 390 nm for the *o*-quinones obtained by oxidation of 3,4-dihydroxybenzylamine and 3,4-dihydroxybenzoic acid. Alternatively, the enzymic activity was monitored in the near-u.v. region, 308 nm (Aroca *et al.* 1990b), but in the case of determination of the activity in the presence of inhibitors in the assay media we always monitored the decrease in absorbance at 475 nm rather than the increase in absorbance at 308 nm since the high absorbance of all indoles in this u.v. region prevented accurate determinations at this wavelength.

One unit of dopachrome tautomerase activity was defined as the amount of enzyme that catalyses the tautomerization of $1 \mu\text{mol}$ of dopachrome/min at 30°C .

RESULTS AND DISCUSSION

Putative substrates

We have tested as substrates of the enzyme some L-dopachrome analogues, including semiquinonic structures such as D-dopachrome, dopaminochrome, L- α -methyldopachrome, L-dopachrome methyl ester, adrenochrome methyl ether and deoxyadrenochrome, and *o*-quinonic structures such as 3,4-*o*-quinone benzylamine and 3,4-*o*-quinone benzoic acid (structures in Table 1).

Since all these possible substrates are relatively unstable, we determined their rates of disappearance in the absence (blanks) or in the presence of 4 munits of dopachrome tautomerase. These rates were determined at the λ_{max} in the visible region of the spectrum corresponding to each compound. Thus the difference between the rate of disappearance in the presence of the enzyme and the blank can be taken as a measure of the enzyme action on each putative substrate.

Table 1. Structure of possible substrates of dopachrome tautomerase

Substrate	R ¹	R ²	R ³	R ⁴
Dopachrome	H	CO ₂ H	H	H
Dopachrome methyl ester	H	CO ₂ CH ₃	H	H
α -Methyldopachrome	H	CO ₂ H	CH ₃	H
Dopaminochrome	H	H	H	H
Deoxyadrenochrome	CH ₃	H	H	H
Adrenochrome methyl ether	CH ₃	H	H	OCH ₃

Table 2. Action of dopachrome tautomerase on different possible substrates

All assays were repeated three times with 0.1 mM solution of the relevant substrate in 1 ml total volume of 10 mM-phosphate buffer, pH 6.0, containing 0.1 mM-EDTA, and in the presence of 4 munits of dopachrome tautomerase (+DC-ase) or in its absence (–DC-ase). Reaction rates are expressed as nmol of each putative substrate transformed/min. Note that L-dopachrome leads to DHICA in the absence of dopachrome tautomerase, whereas in the presence of the enzyme DHICA is formed. Thus the enzymic activity is the difference between both rates of substrate disappearance. For L-dopachrome methyl ester the same product is formed in the absence and in the presence of dopachrome tautomerase, since decarboxylation is prevented.

Substrate	Rate of reaction (nmol/min)	
	+DC-ase	–DC-ase
L-Dopachrome	4.35	0.36
D-Dopachrome	0.38	0.36
L-Dopachrome methyl ester	5.84	1.40
Dopaminochrome	0.57	0.48
L- α -Methyldopachrome	1.61	1.58
Deoxyadrenochrome	0.28	0.25
Adrenochrome methyl ether	0.04	0.04
3,4-Quinone of benzylamine*	0.27	0.27
3,4-Quinone of benzoic acid*	0.12	0.14

* These rates were measured 10 min after periodate oxidation of the *o*-dihydroxy precursors to allow for colour stabilization.

Table 2 shows that L-dopachrome was the best substrate for the enzyme, the reaction being highly stereospecific since the rate of D-dopachrome evolution was unaffected by the enzyme. Only L-dopachrome methyl ester was an alternative substrate that was susceptible to tautomerization. However, the affinity of the enzyme for the methyl ester derivative was lower (the K_m for this substrate was 330 μ M as opposed to approx. 100 μ M for L-dopachrome; results not shown).

The action of dopachrome tautomerase on the substrates L-dopachrome and L-dopachrome methyl ester follows normal enzyme behaviour. Fig. 1(a) shows that the rate of reaction was constant with time for the first few minutes. The absorbance decreases underwent noticeable deviations from linearity after approx. 5 min of reaction since the substrate concentrations in the assay media were not saturating. Therefore the reaction rate was calculated in the early stage of the reaction. Moreover, Fig. 1(b) shows a linear relationship between reaction rate and the

Fig. 1. (a) Absorbance decrease versus time for the L-dopachrome and L-dopachrome methyl ester in the absence [controls, L-dopachrome (A) and L-dopachrome methyl ester (C)] or the presence of 4 munits of dopachrome tautomerase [L-dopachrome (B), L-dopachrome methyl ester (D)] and (b) relationship between reaction rate (absorbance decrease at 475 nm/min) and enzyme amount (munits) for the substrates, L-dopachrome (O) and L-dopachrome methyl ester (●)

amount of enzyme added for both the true substrate L-dopachrome and the analogue L-dopachrome methyl ester.

The fact that dopaminochrome, adrenochrome methyl ether and deoxyadrenochrome were not substrates of the enzyme suggests that the carboxylic group at position 2 of the substrate is necessary, either as free acid or methylated, for enzyme recognition. As might be expected, L- α -methyldopachrome was not a substrate since tautomerization involves the formation of a double bond between carbon atoms 2 and 3 of the indole ring.

The *o*-quinones of 3,4-benzylamine and 3,4-benzoic acid were not substrates. It should also be considered that their transformation into the dihydroxy analogues implies a net reduction rather than an internal oxidoreduction.

Indoles and related compounds as inhibitors

Some analogues of dopachrome or its tautomeric product DHICA (structures in Table 3) have been tested as inhibitors of dopachrome tautomerase, and the results in percentage inhibition are shown in Table 4.

Non-indolic structures resembling parts of the dopachrome molecule. Two different semiquinones such as tropolone and L-mimosine, resembling the structure at positions 5 and 6 of dopachrome, were first tested as inhibitors, but both of them failed to affect the rate of dopachrome tautomerization. Another reagent that mimics the pyrrolic ring of dopachrome, pyrrole-2-carboxylic acid, gave the same negative result. These assays indicated that neither *o*-semiquinones nor pyrrole rings with a

Table 3. Structures of possible inhibitors of dopachrome tautomerase

Inhibitor	R ¹	R ²	R ³	R ⁴
Indole	H	H	H	H
5-Hydroxyindole	OH	H	H	H
Indole-2-carboxylic acid	H	H	CO ₂ H	H
Indole-2-carboxylic ethyl ester	H	H	CO ₂ CH ₂ CH ₃	H
5-Hydroxyindole-2-carboxylic acid	OH	H	CO ₂ H	H
5,6-Dihydroxyindole-2-carboxylic acid	OH	OH	CO ₂ H	H
L-Tryptophan	H	H	H	CH ₂ -CH(NH ₂)-CO ₂ H
Tryptamine	H	H	H	[CH ₂] ₂ -NH ₂
5-Hydroxy-L-tryptophan	OH	H	H	CH ₂ -CH(NH ₂)-CO ₂ H
Indole-3-carboxylic acid	H	H	H	CO ₂ H
Indole-3-acetic acid	H	H	H	CH ₂ -CO ₂ H
Indole-3-propionic acid	H	H	H	[CH ₂] ₂ -CO ₂ H
Indole-3-butyric acid	H	H	H	[CH ₂] ₃ -CO ₂ H

Table 4. Inhibition of dopachrome tautomerase by structural analogues of DHI or DHICA

Inhibitor	Concn. (mM)	Inhibition (%)
Tropolone	3	0
L-Mimosine	3	0
Pyrrole-2-carboxylic acid	3	0
Indole	1	0
5-Hydroxyindole	1	0
Indole-2-carboxylic acid	1	11.5
Indole-2-carboxylic ethyl ester	1	11
5-Hydroxyindole-2-carboxylic acid	1	18.5
5,6-Dihydroxyindole-2-carboxylic acid	1	24
L-Tryptophan	2	49
Tryptamine	2	0
5-Hydroxy-L-tryptophan	2	25
Indole-3-carboxylic acid	2	2
Indole-3-acetic acid	2	16
Indole-3-propionic acid	2	47
Indole-3-butyric acid	2	15

carboxy group at position 2 incorporate sufficient structural features for enzyme recognition.

Hydroxylated and/or 2-carboxylated indoles. We performed assays with different substituted indoles. Indole and 5-hydroxyindole also failed to result in any noticeable inhibition. Further experiments were carried out with carboxylated indoles such as indole-2-carboxylic acid, 5-hydroxyindole-2-carboxylic acid and 5,6-dihydroxyindole-2-carboxylic acid. All of them decreased the rate of dopachrome tautomerization, confirming the previous suggestion that the carboxy group at position 2 of the indole ring is essential for the interaction of such reagents with the enzyme active site. Indole derivatives with hydroxy groups at positions 5 and 6 were slightly more potent inhibitors than their unsubstituted counterparts, indicating that these groups increase the affinity of the carboxylated indoles for the enzyme. However, a 1 mM concentration of DHICA, the authentic product of

dopachrome tautomerization, results in a decrease of about 25 % of the reaction rate, even though the concentration of inhibitor is ten times higher than the standard dopachrome concentration.

Indoles substituted at position 3. L-Tryptophan has been described as a competitive inhibitor of dopachrome tautomerase (Aroca *et al.* 1990a). However, 2 mM-tryptamine did not inhibit the enzymic activity at all (Table 4). This supports the absolute requirement of a carboxy group for specific interaction of the inhibitor with the enzyme. Moreover, the inhibition by L-tryptophan indicates that such a carboxy group does not need to be located at position 2 of the indole ring. 5-Hydroxy-L-tryptophan was less potent than L-tryptophan. This fact is surprising bearing in mind the former results obtained for the series of hydroxylated 2-carboxylic indoles, where the presence of the hydroxy groups increased the affinity of the inhibitor for the enzyme. Taking into account that the carboxy group of L-tryptophan is located at the γ -position of the hydrocarbon chain at position 3 on the indole ring, this carboxy group could adopt a spatial disposition mimicking the 2-carboxylic group of the authentic substrate in the interaction with the enzyme. In this case, the hydroxy group on 5-hydroxy-L-tryptophan might also interact with a different group on the enzyme and thus partially impede the adaptation of the γ -carboxylic group on the enzyme active site. Bearing this in mind, we tested the series indole-3-carboxylic acid, indole-3-acetic acid, indole-3-propionic acid and indole-3-butyric acid. At a 2 mM concentration, the first was not inhibitory, and indole-3-acetic acid and indole-3-butyric acid inhibited the enzyme-catalysed reaction weakly. The strongest inhibition was achieved by indole-3-propionic acid, a compound similar to L-tryptophan in that the carboxy group is located at the γ -position on the branched hydrocarbonated chain. Fig. 2 shows the percentage of residual dopachrome tautomerase activity versus inhibitor concentration for the two strongest inhibitors found, L-tryptophan and indole-3-propionic acid. Both show a competitive pattern, as shown in Fig. 3 for indole-3-propionic acid and described by Aroca *et al.* (1990a) for L-tryptophan. However, the affinity of dopachrome tautomerase for these inhibitors (inhibition constants 1.8 mM and 1.9 mM for L-tryptophan and indole-3-propionic acid respectively) was lower than the affinity for the authentic substrate, L-dopachrome.

Therefore the above experiments indicate that the recognition of indoles by dopachrome tautomerase needs the presence of a carboxy group at position 2, as in the substrate dopachrome, or

Fig. 2. Residual activity (%) versus inhibitor concentration (mM) for dopachrome tautomerase (3 munits) in the presence of L-tryptophan (●) or indole-3-propionic acid (○)

All assays were performed with 0.1 mM-dopachrome in 50 mM-phosphate buffer, pH 6.0, containing 0.1 mM-EDTA.

at the γ -position in a hydrocarbon chain located at position 3 on the indole ring. The enzyme is able to recognize this carboxy group either free or as the methyl ester. This clearly indicates that the interaction between the enzyme and the carboxy group is not electrostatic and therefore the negative charge is not necessary for the interaction.

Stereospecificity. As described above, the enzyme could not use D-dopachrome as a substrate for the reaction. Moreover, D-dopachrome at 0.1 mM failed to result in any measurable inhibition of L-dopachrome tautomerization, consistent with a high degree of stereospecificity of the active site, which only recognizes the L stereoisomer of dopachrome.

Recently, a protein factor isolated from insects and showing strong similarities to the specificity of dopachrome tautomerase has been described. This factor has been named dopaquinone imine conversion factor, and it was reported to catalyse the conversion of dopachrome into DHI instead of DHICA (Aso *et al.*, 1989). Taking into account our results, we consider that this point should be re-investigated, since it is plausible that enzymes catalysing the tautomerization of dopachrome could occur in different organisms of the phylogenetic scale. This enzyme offers an alternative pathway leading to the indole-2-carboxylated units instead of the spontaneous re-arrangement of dopachrome at neutral pH, which is concomitant with decarboxylation. The widespread existence of the enzyme would support the view that it might play an important role in the regulation of melanization in different organisms, a point that should be further studied.

Fig. 3. Double-reciprocal plot of the dopachrome tautomerase activity in the absence (●) or in the presence of 2 mM-indole-3-propionic acid (○)

K_m for L-dopachrome was 0.13 mM and, according to the competitive pattern, K_i for the inhibitor was 1.9 mM.

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