The action of glycosylases on dopachrome (2-carboxy-2,3-dihydroindole-5,6-quinone) tautomerase

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It is shown that dopachrome (2-carboxy-2,3-dihydroindole-5,6-quinone) tautomerase (DCT) is a glycoprotein containing *N*-linked oligosaccharides. The enzymic activity can be stimulated by partial deglycosylation with a number of glycosylases such as neuraminidase, β -mannosidase and β -galactosidase. However, the stability of the enzyme after the hydrolytic treatment becomes lower. Thus total deglycosylation with peptide *N*-glycosidase F directly provokes an inactivation of DCT. The native enzyme also shows a strong affinity for concanavalin A–Sephadex. This affinity decreases after treatment with neuraminidase and/or β -mannosidase. The DCT associated with coated vesicles seems to be mostly glycosylated, since the action of glycosylases on the enzyme obtained from these vesicles produced a similar stimulation to that with the melanosomal enzyme. Treatment of cultured melanocytes with tunicamycin elicited a decrease in the amount of active DCT inside the cells. All data suggest that the structure of the carbohydrate moiety of DCT should be very similar to, if not identical with, the structure proposed for tyrosinase by Ohkura, Yamashita, Mishima & Kobata (1984) Arch. Biochem. Biophys. **235**, 63–77].

INTRODUCTION

The biosynthesis of melanin, the main pigment found in mammalian skin, hair and eyes, occurs in a cytoplasmic organelle, called the melanosome, inside melanocytes. In the absence of thiol-group-containing compounds, the pathway yields eumelanins and can be divided in two different phases. The first one, which is fairly well understood (Mason, 1948; Lerner & Fitzpatrick, 1950; Cabanes *et al.*, 1987), leads from L-tyrosine to dopachrome, and requires the presence of a single enzyme, tyrosinase (EC 1.14.18.1). This enzyme catalyses both the hydroxylation of L-tyrosine to L-dopa (3,4-dihydroxyphenylalanine) and the oxidation of this *o*-diphenol to yield L-dopaquinone. This quinone is transformed into L-dopachrome (2-carboxy-2,3-dihydroindole-5,6-quinone) through spontaneous chemical reactions. The second phase leads from L-dopachrome to eumelanins, but the enzymic control is still poorly known. In 1980, three protein factors involved in the regulation of this phase were postulated (Pawelek *et al.*, 1980), but until now only one of these has been isolated and characterized as an enzyme. Its name has been changing as the knowledge about the properties of this enzyme has been improving. The protein was called L-dopachrome conversion factor (Korner & Pawelek, 1980), dopachrome oxidoreductase (Barber *et al.*, 1984) and dopachrome isomerase (Pawelek, 1990). Once it was demonstrated that the enzyme catalyses a keto–enolic tautomerization of dopachrome to yield 5,6-dihydroxyindole-2-carboxylic acid (DHICA) (Leonard *et al.*, 1988; Aroca *et al.*, 1990a,b), it was renamed dopachrome tautomerase (DCT) (Aroca *et al.*, 1990a). In addition, the specificity and catalytic properties of this enzyme have been recently published (Aroca *et al.*, 1991). Thus it is now clear that mammalian melanogenesis is regulated by at least two different enzymes: the well-known tyrosinase (EC 1.14.18.1) and DCT (EC 5.3.2.3).

Tyrosinase is a glycoprotein (Ohkura *et al.*, 1984; Imokawa &

Mishima, 1985; Imokawa, 1990). Previous studies have shown the existence of several isoenzymic forms probably resulting from post-translational modifications during the maturation of tyrosinase (Hearing *et al.*, 1978; Imokawa & Mishima, 1982; Ferrini *et al.*, 1987; Imokawa, 1990). However, the kinetic parameters of the melanosomal enzyme are only slightly affected by the action of glycosylases (Hearing *et al.*, 1978; Martinez *et al.*, 1987).

Although it has been shown that DCT is also transferred to melanosomes via coated vesicles (Chakraborty *et al.*, 1989), no data are available on the existence of carbohydrate moieties in the molecules of this enzyme and, if so, the possible effects of several glycosylases on its stability and kinetic parameters. The aim of the present study was to analyse these points and to discuss the role(s) that the carbohydrate moiety might play in the structure of the enzyme.

MATERIALS AND METHODS

Reagents

Culture media, foetal-calf serum, Hanks iso-osmotic salt solution and other reagents for cell culture were from Flow Laboratories. β -Mannosidase, β -galactosidase, L-tyrosine, L-dopa, concanavalin A–Sephadex, *N*-acetylneuraminic acid (AcNeu), β -mercaptoethanol, phenylmethanesulphonyl fluoride (PMSF), EGTA, Mes, phenylthiourea, α -methyl mannoside and hydroxyapatite type I were from Sigma. Brij 35, EDTA, Na₂HPO₄, NaH₂PO₄, NaOH, sucrose, trichloroacetic acid, (NH₄)₂SO₄, MgCl₂, NaN₃ and NaCl were from Merck. Tunicamycin, neuraminidase, *N*-acetyl- β -glucosaminidase, α -mannosidase [from jack bean (*Canavalia ensiformis*)] and *N*-glycosidase F (peptide *N*-glycosidase F from *Flavobacterium meningosepticum*) were from Boehringer-Mannheim. Sodium periodate was from Probus. Sephacryl S-300 and DEAE-Sephadex were from Pharmacia. All reagents were of the highest purity commercially

Abbreviations used: DCT, dopachrome tautomerase; dopachrome, 2-carboxy-2,3-dihydroindole-5,6-quinone; DHICA, 5,6-dihydroxyindole-2-carboxylic acid; DHI, 5,6-dihydroxyindole; AcNeu, *N*-acetylneuraminic acid; PMSF, phenylmethanesulphonyl fluoride; MEM, minimal essential medium; Con A, concanavalin A.

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available and were used without further purification. All solutions were prepared by using double-distilled water further deionized by passage through a Milli-Q Waters deionizer system (resistance greater than $10 \text{ M}\Omega \cdot \text{cm}$).

Animals and melanomas

B16-F10 mouse melanomas were obtained by serial transplantation on hybrid mice obtained from male DBA and female C57/Bl (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 enzyme preparations.

Cell culture

B16/F10 malignant melanocytes were maintained in minimum essential medium (MEM) (Eagle; modified with Earle's salts and glutamine and without bicarbonate). The medium was supplemented with 10 % (v/v) fetal-calf serum, penicillin (100 units/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$), and the cells were grown in a water-saturated 5 % CO_2 atmosphere. They underwent routine passages every 4 days. For tunicamycin studies, the media of some flasks were supplemented with tunicamycin (0.4 $\mu\text{g}/\text{ml}$), and other flasks were used as control. After 24 or 48 h of treatment, melanoma cells were harvested by incubation with trypsin (0.1 mg/ml), EDTA (0.2 mg/ml) in Hanks solution, pH 7.2, free of Ca^{2+} and Mg^{2+} for 5 min. Then flasks were shaken and the cells were pelleted by centrifugation at 214 g for 10 min. To assay enzymic activities, cells were disrupted by resuspension in 10 mM-phosphate buffer, pH 6.8 containing 1 % of Brij 35, followed by sonication in an ice-cold water bath for 5 min. After centrifugation at 13 000 g for 10 min in an Eppendorf centrifuge, the supernatant was used to measure DCT and tyrosinase activities.

Purification of DCT

The purification of the melanosomal enzyme from solid melanoma tumours was carried out as described by 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-PMSF. The homogenate was then centrifuged at 700 g for 20 min and the supernatant was further centrifuged at 11 000 g for 30 min. The resulting melanosomal pellet was washed with the same buffer and then solubilized in 10 mM-phosphate buffer, pH 6.8, containing 1 % Brij 35. The extract was fractionated with $(\text{NH}_4)_2\text{SO}_4$, the protein pellet obtained between 35–60 % saturation of the salt being retained. This 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 and gel-filtration chromatographies. By this procedure, the specific activity of the DCT preparation was increased from 16.5 munits/mg for the crude melanosomal extract to 496 munits/mg for the purified preparation. One unit of DCT activity was defined as the amount of enzyme that catalyses the tautomerization of 1 μmol of L-dopachrome/min at 30°C. The yield was 36 %, and the purification factor 30-fold, although at this stage the enzyme is not totally pure, as judged by SDS/PAGE. These values were very similar to those published elsewhere (Aroca *et al.*, 1990a, 1991).

Coated-vesicle preparation

Coated vesicles were prepared from B16/F10 melanomas by the method of Chakraborty *et al.* (1989), with minor modifications. Briefly, melanomas were homogenized in 0.1 M-Mes

Fig. 1. Electron micrograph of the coated-vesicle preparation

The material was negatively stained with 1 % phosphotungstic acid (magnification $\times 68\,000$)

buffer, pH 6.5, containing 1 mM-EGTA, 0.5 mM- MgCl_2 and 0.02 % NaN_3 . The homogenate was centrifuged at 20 000 g for 30 min, and the supernatant was centrifuged at 55 000 g for 1 h. The pellet was resuspended in a small volume of Mes buffer containing 10 % sucrose and centrifuged at 105 000 g for 1 h. The pellet was again resuspended in a small volume and submitted to 30–60 % (w/v)-sucrose-density-gradient centrifugation for 18 h at 45 000 g in a swinging-bucket SW-28 Beckman rotor. The turbid band, close to the bottom, was collected and centrifuged at 105 000 g for 1 h. The pellet was resuspended and finally centrifuged in a 5–30 %-sucrose gradient at 105 000 g for 1 h. The pellet of purified coated vesicles was resuspended in a Mg^{2+} -free Mes buffer. This preparation was melanosome-free, as judged by electron-microscopic observation of samples submitted to negative staining using a 1 % phosphotungstic acid solution (Fig. 1).

Determination of DCT and tyrosinase activities

DCT activity was always determined by using fresh L-dopachrome as substrate by two different methods: by monitoring the increase in absorbance at 308 nm (Aroca *et al.*, 1990b), monitoring the formation of DHICA, or the decrease in the absorbance at 475 nm, monitoring the disappearance of L-dopachrome (Korner & Pawelek, 1980; Barber *et al.*, 1984). L-Dopachrome was prepared immediately before use by mixing a solution of L-dopa in 10 mM-sodium phosphate, pH 6.0, containing 0.1 mM-EDTA, with the required volume of a solution of sodium periodate to achieve a 1:2 molar ratio of L-dopa to periodate (Aroca *et al.*, 1990b). The assay mixture (1 ml) contained 0.1 mM-L-dopachrome as the standard concentration, except in experiments leading to K_m and V_{max} determinations. Routinely, 50 μl of native or glycosylase-treated DCT were added to the reaction medium. Blanks were performed without enzyme to estimate the rate of absorbance decrease at the appropriate wavelength.

Tyrosinase was determined by recording the absorbance increase at 475 nm due to L-dopachrome formation from L-dopa, as described by Mason (1948). One unit of this activity was defined as the amount of enzyme that catalyses the oxidation of 1 μmol of L-dopa/min at 30°C.

Glycosylase treatments

Purified DCT was treated at 30 °C in different media according to the characteristics of the glycosylase used in every case. Control samples of DCT were treated under the same conditions, but in the absence of glycosylase. When reagents of the incubation

media recommended for glycosylase activity strongly inhibited DCT activity, such as β -mercaptoethanol for the action of *N*-glycosidase F, they were omitted from the incubation mixture.

RESULTS AND DISCUSSION

Effect of glycosylases

Table 1 shows the stimulatory effect of several glycosylases on purified melanosomal DCT activity (first column). Preincubation with β -mannosidase provoked maximal effect, although neuraminidase and β -galactosidase also stimulated more than 2-fold. The effect of α -mannosidase from jack bean was smaller, and *N*-acetyl- β -glucosaminidase did not have any effect. When β -

Table 1. Effect of some glycosylases on DCT

All incubations with glycosylases were carried out for 2 h, using the appropriate amounts of purified melanosomal enzyme (496 munits/mg), crude coated vesicles (1.1 munits/mg) or crude melanosomal (10.7 munits/mg) preparations in order to add 5 munits of DCT in 10 mM-phosphate buffer, pH 6.8, containing 0.1% Brij 35. Values in parentheses indicate the numbers of experiments.

Treatment	DCT activity (% of control)		
	Purified melanosomal	Crude	
		Melanosomal	Coated vesicles
None (control)	100 (8)	100 (3)	100 (3)
Neuraminidase (36 m units)	238 $\pm$ 66 (6)	216 $\pm$ 27 (2)	202 $\pm$ 49 (2)
β -Mannosidase (100 munits)	262 $\pm$ 94 (4)	208 $\pm$ 64 (3)	228 $\pm$ 56 (3)
Neuraminidase (36 munits) + β -mannosidase (100 munits)	358 $\pm$ 81 (2)	—	—
β -Galactosidase (25 units)	220 $\pm$ 36 (2)	—	—
α -Mannosidase (500 munits)	160 $\pm$ 42 (2)	—	—

Fig. 2. Thermal stability at 45 °C of native DCT (○) or treated with neuraminidase (●) or neuraminidase plus β -mannosidase (■)

To treat DCT with glycosylases, 25 munits of DCT were previously treated for 3 h with 10 munits of neuroaminidase or the same amount of neuraminidase plus 50 munits of β -mannosidase. Aliquots were withdrawn at different times of incubation at 45 °C to measure residual activity. Control DCT was treated under identical conditions in the absence of glycosylases. All data points represent means of two determinations.

Table 2. Kinetic parameters of native and desialylated DCT

A_{308} and A_{475} refer to the values of kinetic parameters obtained by spectrophotometric assays at 308 or 475 nm respectively. Values are the mean of two determinations. Each data point at different concentrations of substrate was also performed in duplicate. * $P < 0.01$.

Constant	Native			Neuraminidase-treated		
	A_{308}	A_{475}	Mean $\pm$ S.D.	A_{308}	A_{475}	Mean $\pm$ S.D.
K_m (mM)	0.13	0.13	0.13	0.27	0.24	0.255 $\pm$ 0.15*
V_{max} (m units)	7.14	8.33	7.73 $\pm$ 0.59	25	20	22.5 $\pm$ 2.5*

mannosidase and neuraminidase acted together, the stimulation of the tautomerase activity was higher than that obtained when any of those glycosylases acted separately, although their effects were not totally additive. All activations showed saturating kinetics, reaching a plateau of maximal DCT stimulation after 1–2 h under the conditions described in Table 1. These results indicate the existence of sialic residues in the enzyme, as well as other carbohydrate units bound by different types of glycosylic linkages. The percentage of activation elicited by glycosylases varied by up to 30% from one preparation of DCT to another. This could suggest the presence of several forms of DCT with a heterogeneous content of oligosaccharides in different proportions among different enzymic preparations. Such heterogeneity in the carbohydrate composition has been described for tyrosinase (Ohkura *et al.*, 1984).

A more detailed study of the action of neuraminidase on DCT and the properties of the treated form demonstrated that the activatory effect shown by this glycosylase was specific to its hydrolytic activity, as indicated by the following observations: (i) boiled glycosylase was unable to affect DCT activity; (ii) the time needed to get maximal activation was dependent on the amount of glycosylase; (iii) the addition of AcNeu partially prevented the activation elicited by neuraminidase; (iv) the thermal stability at 45 °C of untreated DCT or DCT treated with neuraminidase or neuraminidase plus β -mannosidase (Fig. 2); (v) finally, the kinetic parameters of the native and treated enzymes, determined by two methods, were statistically different. The native form of the enzyme displayed a higher affinity for the substrate, but a lower V_{max} than the desialylated form, as Table 2 shows.

The decreased thermal stability of the enzyme and the variation of its kinetic parameters suggest that the conformation of DCT could be dependent on the presence of AcNeu residues in its carbohydrate moiety. Thus the 'stiffness' of DCT seems to be greatly dependent on these residues, as opposed to the case of tyrosinase, which shows a remarkable rigidity. Other experimental data, such as the resistance of tyrosinase to SDS and urea, and the great sensitivity of DCT to the same agents (results not shown), support the differing characteristics of both melano-genic enzymes.

Concanavalin A (Con A)–Sephacrose affinity chromatography

Fig. 3 shows the chromatographic pattern of native DCT, DCT partially deglycosylated by treatment with neuraminidase and DCT treated with neuraminidase plus β -mannosidase. All species have a strong affinity for Con A and they can be only partially eluted by α -methyl mannose. Table 3 shows the DCT activities obtained for the three species. Although the species treated with neuraminidase plus β -mannosidase showed less

Fig. 3. Con A-Sepharose affinity chromatography of DCT

Samples of native purified enzyme (●), after complete activation by treatment with neuraminidase (○-○) or with neuraminidase plus β -mannosidase (■) were applied to a Con A-Sepharose column equilibrated with 10 mM-phosphate buffer, pH 6.8, with 0.1 % Brij 35. The column was washed with 4 vol. of equilibrating buffer at 4 °C, and then the flow was stopped. Then the temperature was raised to 25 °C and elution was with 1 M- α -methyl mannoside (α -MM) in 100 mM-phosphate buffer, pH 7.5, with 0.1 % Brij 35. Fractions (approx. 1 ml each) were collected and assayed for DCT activity.

Table 3. DCT activity after Con A-Sepharose affinity chromatography

The values in parentheses are percentages of the total activity applied to the column.

Treatment	DCT activity (munits)		
	Total*	Unbound†	Recovered‡
Control	630	6 (1 %)	141 (22 %)
Neuraminidase (40 munits)	600	50 (8 %)	380 (63 %)
Neuraminidase (40 munits) + β -mannosidase (100 munits)	704	230 (33 %)	290 (41 %)

* Activity applied to the column.

† Amount of enzyme activity not retained by the column.

‡ Amount of enzyme displaced by α -methyl mannoside.

affinity for Con A, it still was able to bind to the column to a significant extent. α -Methyl mannoside was able to elute 41 % of the most deglycosylated species, suggesting that this form would keep some carbohydrate residues in its structure.

Con A binds most of *N*-linked oligosaccharides, although it is able to separate glycoproteins according to the number and mode of attachment of side chains to mannose residues in the core structure. Two peripheral AcNeu-Gal-GlcNAc- branches linked to a mannose-rich core or required for binding to the lectin, whereas glycopeptides with tri- or tetra-antennary complex-type oligosaccharides are weakly or not bound (Krusius *et al.*, 1976; Cummings & Kornfeld, 1982). The strong affinity of the DCT for Con A-Sepharose suggests that the enzyme is a mannose-rich glycoprotein with terminal AcNeu-Gal-GlcNAc-oligosaccharide sequences.

Effect of glycosylases on DCT from coated vesicles

The effects of β -mannosidase and neuraminidase on crude DCT solubilized with 1 % Brij 35, either from coated vesicles or melanosomes, were tested. The results obtained indicated that the pattern of activation of DCT from coated vesicles was very similar to the activation of the melanosomal enzyme, since the

Table 4. Effect of tunicamycin on DCT and dopa oxidase activity of melanoma cells in culture

The reported data are the means of two determinations, and the enzymic activities are expressed as munits/mg of protein. Values in parentheses indicate the residual activity in tunicamycin-treated cells. The activation of DCT by neuraminidase was estimated by incubation *in vitro* for 2 h at 30 °C of 3 munits of DCT from each sample with 2 munits of neuraminidase (the value for the respective DCT control being 100 %).

Period of exposure	Activity (munits/mg of protein)		DCT activity after neuraminidase treatment (%)
	DCT	Dopa oxidase	
24 h			
Control	6.32	2.07	186
Tunicamycin-treated	4.76 (75 %)	2.51 (121 %)	195
48 h			
Control	4.27	1.95	190
Tunicamycin-treated	1.19 (28 %)	0.86 (44 %)	118

differences between both forms are not statistically significant (Table 1; second and third columns). This supports the existence of complete oligosaccharide units in the molecules of DCT from coated vesicles, since it is a newly synthesized and modified enzyme. The activations of these crude preparations were slightly lower than those obtained for the purified melanosomal enzyme, probably owing to the competitive effects of other glycoproteins occurring in the crude preparations.

Effect of *N*-endoglycosidase

We also tested the effect of an *N*-endoglycosidase in order to break linkages to asparagine and to achieve the total deglycosylation of DCT. *N*-Glycosidase F cleaves the *N*-glycosylic linkage between asparagine and the carbohydrate chains of glycoproteins (Tarentino *et al.*, 1985). This hydrolysis can be also carried out on native glycoproteins in order to determine possible effects on the catalytic activity of the enzyme. In the incubation buffer recommended by Tarentino *et al.* (1985) we omitted β -mercaptoethanol, since this reagent elicited a time-dependent loss of the tautomerase activity when the enzyme was incubated at 30 °C (results not shown). In contrast with exoglycosylases, the action of *N*-glycosidase F on DCT did not enhance its enzyme activity, but provoked a time-dependent inactivation of the enzyme, with only 25 % of the control activity after 24 h of treatment. The lability of the *N*-glycosidase F-treated enzyme made it impossible to study the interaction of this species with Con A-Sepharose. These results indicate that the cleavage of the *N*-glycosylic carbohydrate chains decreases the stability of the molecule.

Effect of tunicamycin on DCT from cultured malignant melanocytes

Tunicamycin is an antibiotic that inhibits the transfer of *N*-acetylglucosamine 1-phosphate to dolichol monophosphate and blocks the formation of protein-carbohydrate linkages to asparagine (Duksin & Borstein, 1977; Mahoney & Duksin, 1979). These *N*-linked oligosaccharides appear to be required for secretion of proteins or for movement of membrane glycoproteins to the target organelle. Thus we treated cultured malignant melanocytes with tunicamycin in order to study the effect on the level of DCT activity. The effects of treatment with tunicamycin 0.4 μ g/ml for 24 and 48 h are shown in Table 4 since, in our hands, treatment with doses of 1 μ g/ml or greater in the culture

Fig. 4. Possible *N*-oligosaccharide contained in DCT

This structure has been demonstrated in tyrosinase (Ohkura *et al.*, 1984). The existence of this structure in the DCT molecular would be compatible with the observed effects of glycosylases on this enzyme. The possible bonds hydrolysed by each glycosylase are shown on the right. Abbreviations: NAcGlc, *N*-acetylglucosamine; PNG-ase F, peptide *N*-glycosidase F.

media were cytotoxic for the melanoma cells (results not shown). It has been shown that this antibiotic induces a marked pigment loss and decreases cell growth rate (Imokawa & Mishima, 1982; Mishima & Imokawa, 1983). Tyrosinase activity was also measured as an index of the action of tunicamycin, since it is known that this enzyme is also *N*-glycosylated (Ohkura *et al.*, 1984; Imokawa, 1990). After 24 h, the effect of tunicamycin was small, and 75 % of the DCT activity of control cells was conserved. In addition to that, the enzymes from control and treated cells were similarly activated by neuraminidase. However, it can be seen that, after 48 h, the effect of tunicamycin was more pronounced, the DCT activity being decreased to 28 %. This residual activity was activated only 18 % by neuraminidase treatment. Moreover, the DCT obtained from 48 h-tunicamycin-treated cells was unstable, and the preparations lost its activity overnight at 4 °C. This result supports the notion (based on results obtained for purified DCT treated with glycosylases) that deglycosylated DCT loses its rigidity and is easily inactivated.

Finally, although it is necessary to wait for a complete purification of DCT and subsequent direct analysis of the carbohydrate moiety of this enzyme, it seems very plausible that this enzyme contains antennary sugar chains very similar to, if not identical with, those found in tyrosinase, as shown in Fig. 4 (Ohkura *et al.*, 1984; Imokawa, 1990). This structure is consistent

with all the data reported above on the susceptibility of the enzyme to glycosylases. The cleavage of part of this structure produces the unstabilization of the enzyme, concomitant with a transient stimulation of the catalytic activity, whereas the cleavage of the complete structure by *N*-glycosidase F directly provokes the loss of the stability of the enzyme. Thus asparagine-linked oligosaccharides might have two different functions in the DCT molecule: the signal for the specific transfer to melanosomes and the stabilization of the active structure of the enzyme.

This work was partially supported by grant no. PB87-0698 from the Direcció General de Investigació Científica y Técnica, Spain. We thank the Servicio de Microscopía Electrónica for the staining of coated-vesicle preparations and the Servicio de Cultivos Celulares for assistance in the preparation of the cell-culture media. P. A. thanks the Comunidad Autónoma de Murcia for a fellowship.

REFERENCES

- Aroca, P., García-Borrón, J. C., Solano, F. & Lozano, J. A. (1990a) *Biochim. Biophys. Acta* **1035**, 266–275
- Aroca, P., Solano, F., García-Borrón, J. C. & Lozano, J. A. (1990b) *J. Biochem. Biophys. Methods* **21**, 35–46
- Aroca, P., Solano, F., García-Borrón, J. C. & Lozano, J. A. (1991) *Biochem. J.* **277**, 393–397
- Barber, J. I., Townsend, D., Olds, D. & King, R. A. (1984) *J. Invest. Dermatol.* **83**, 145–149
- Cabanes, J., García-Cánovas, F., Lozano, J. A. & García-Carmona, F. (1987) *Biochim. Biophys. Acta* **923**, 187–195
- Chakraborty, A. K., Mishima, Y., Inazu, M., Hatta, S. & Ichihashi, M. (1989) *J. Invest. Dermatol.* **93**, 617–620
- Cummings, R. D. & Kornfeld, S. (1982) *J. Biol. Chem.* **257**, 11235–11240
- Duksin, D. & Bornstein, P. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 3433–3437
- Ferrini, U., Mileo, A. M. & Hearing, V. J. (1987) *Int. J. Biochem.* **19**, 227–234
- Hearing, V. J., Nicholson, J. M., Montague, P. M., Ekel, T. M. & Tomecki, K. J. (1978) *Biochim. Biophys. Acta* **522**, 327–339
- Imokawa, G. (1990) *J. Invest. Dermatol.* **95**, 39–49
- Imokawa, G. & Mishima, Y. (1982) *Cancer Res.* **42**, 1994–2002
- Imokawa, G. & Mishima, Y. (1985) *J. Invest. Dermatol.* **85**, 165–168
- Korner, A. & Pawelek, J. M. (1980) *J. Invest. Dermatol.* **75**, 192–195
- Krusius, T., Finne, J. & Rauvala, H. (1976) *FEBS Lett.* **71**, 117–120
- Leonard, L. J., Townsend, P. & King, R. A. (1988) *Biochemistry* **27**, 6156–6159
- Lerner, A. B. & Fitzpatrick, I. B. (1950) *Physiol. Rev.* **30**, 91–126
- Mahoney, W. C. & Duksin, D. (1979) *J. Biol. Chem.* **254**, 6572–6576
- Martinez, J. H., Solano, F., Arocas, A., García-Borrón, J. C., Iborra, J. L. & Lozano, J. A. (1987) *Biochim. Biophys. Acta* **923**, 413–420
- Mason, H. S. (1948) *J. Biol. Chem.* **172**, 83–90
- Mishima, Y. & Imokawa, G. (1983) *J. Invest. Dermatol.* **81**, 106–114
- Ohkura, T., Yamashita, K., Mishima, Y. & Kobata, A. (1984) *Arch. Biochem. Biophys.* **235**, 63–77
- Pawelek, J. M. (1990) *Biochem. Biophys. Res. Commun.* **166**, 1328–1333
- Pawelek, J., Korner, A., Bergstrom, A. & Bologna, J. (1980) *Nature (London)* **286**, 617–619
- Tarentino, A. L., Gomez, C. M. & Plummer, T. H. (1985) *Biochemistry*, **24**, 4665–4671

Received 31 July 1991/23 October 1991; accepted 4 November 1991