Effect of an abundant human skin melanosomal 66 kDa protein (MP 66) on murine tyrosinase: Its physiological implications on melanogenesis

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A single polypeptide protein (MP 66) of molecular weight 66 kDa purified to homogeneity from melanosomes of normal human skin epidermal melanocytes, was partially characterized. The isoelectric point of MP 66 is in the range of 7·3 to 7·6. This protein, which was shown to inhibit partially purified human skin tyrosinase activity at pH 6·8, also inhibits murine tyrosinase at pH 6·8. However, at pH 5·0, it stimulates murine tyrosinase activity. The physiological implications of these results are discussed.

1. Introduction

Mammalian melanin biosynthesis is regulated by multiple biochemical reactions that are under the control of a series of melanogenic proteins such as tyrosinase, tyrosinase related proteins-1 (TRP-1), DOPAchrome tautomerase (TRP-2), p-locus product, s-locus product, MSH receptor etc. (for review, see Hearing and Tsukamoto 1991; Pawelek 1991). It was suggested (Kameyama *et al* 1993) that TRP-1 and TRP-2 play important roles in the regulation of melanin production. It is still an open question, however, as to how tyrosinase is regulated or interacts with the other melanocyte-specific proteins/enzymes that participate in melanogenesis. From our laboratory, we have reported a protein (MP 66) with molecular weight 66 kDa from melanosomes of normal human cadaver skin epidermal melanocytes (Vijayan *et al* 1982), and it constituted a significant fraction of melanosomal proteins. It was reported to be a competitive inhibitor of partially purified human skin tyrosinase at pH 6·8 (Vijayan *et al* 1982). Bhatnagar *et al* (1993) established that melanosomes, where tyrosinase is mostly located and melanin is biosynthesized, are acidic. This idea is further strengthened in the last few years based on the evidence that melanosomes are related to lysosomes (Orlow 1995). In view of this, the physiological relevance of the inhibition of tyrosinase by MP 66 at pH 6·8 can be understood after studying its effects on tyrosinase at acidic pH. Therefore, in the present communication, the effects of MP 66 on murine tyrosinase were examined and the physiological importance of MP 66 on melanogenesis are discussed.

2. Materials and methods

2. *Materials*

L-Tyrosine (disodium salt), L-dopa, L-ascorbic acid, p-amino benzoic acid (sodium salt) grade I, 1-ethyl-3-(3-dimethyl) amino propyl carbodimide HCl, and 6-amino hexanoic acid sepharose 4B were purchased from Sigma Chemical Company, St. Louis, MO, USA. Pharmalytes (pH 5–8) and broad pI calibration kit (pH 3–10) were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. B-16 murine melanoma cells and electroeluted tyrosinase from murine melanoma were kind gifts from Dr Hearing (Laboratory of Cell Biology, National Cancer Institute of Health, Bethesda, MD 20892, USA).

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Human cadaver skin was taken from accident cases, which were kept in the cold, and the material was removed within 48 h after the accident.

2.2 *Preparation of human epidermal homogenate*

This was done according to the procedure described by Vijayan *et al* (1982). It was used for purifying human tyrosinase and MP 66.

2.3 *Preparation of murine melanoma 1,00,000 g homogenate*

B-16 murine melanoma cells (obtained from Dr Hearing) were propagated in our laboratory using C57 BL/6 mice. The melanoma homogenate was prepared according to the method of Yurkow and Laskin (1989). This was used for the purification of murine tyrosinase.

2.4 *Preparation of affinity column*

CH-Sepharose 4B, coupled with p-amino benzoic acid (PABA), was used for the purification of mammalian tyrosinase (Vijayan *et al* 1982; Devi *et al* 1987). The same single step affinity column was employed to purify human skin tyrosinase, MP 66 and murine melanoma tyrosinase.

2.5 *Protein estimation*

Protein was estimated by the microassay method of Peterson (1977) by using BSA as the standard reference.

2.6 *Estimation of monophenolase activity of tyrosinase*

The monophenolase activity of tyrosinase was assayed fluorometrically at 37°C by the estimation of dopa formed from tyrosine as described by Husain *et al* (1982) and modified by Devi *et al* (1987). Freshly made dopa was used as standard. One unit of enzyme is defined as one pmol of dopa formed/min.

2.7 *Electrophoresis*

SDS-PAGE (10%) was done as described by Laemmli (1970) except that β-mercaptoethanol was not added to the sample buffer. The gels were developed for protein with Coomassie blue.

2.8 *Isoelectric focusing of MP 66*

Isoelectric focusing in cylindrical gels was carried out according to the method of Awdeh *et al* (1968). The 5% gels were cast in 12·5 cm length and 0·4 cm internal diameter glass tubes containing the following chemicals at final concentrations as mentioned—urea (9 M), Triton X-100 (1·25%), Pharmalytes, pH 5·0 to 8·0 (1·66%, v/v), ammonium persulphate (0·043%, w/v). Phosphoric acid at a concentration of 0·01 M was used as anolyte and 0·02 M sodium hydroxide solution was used as catholyte. Initially 1500 volt-hours pre-run was applied to form a gradient pH. Then the protein sample and the pI marker proteins were loaded on to the individual gel tubes. The voltage was increased gradually from 500 to 1000 volts with a total of 10,000 volt-hours.

After the termination of the run, the gels were transferred to fixing solution (13% Trichloro acetic acid, 3·5% 5-sulphosalicyclic acid, and 35% methanol) over night, with a change of solution twice. Then the gels were stained in Coomassie blue R-250. Destaining was done with 10% acetic acid and 40% methanol.

3. Results

3. *Purification of human skin tyrosinase and MP 66*

The human skin tyrosinase and abundant human skin 66 kDa protein (MP 66) were purified from the human epidermal homogenate by the method established by Vijayan *et al* (1982) with the following modification. The pH of the human skin epidermal homogenate, pH 6·8, was brought to 4·7 by dialysis against 1 L of 25 mM citrate and 50 mM phosphate buffer, pH 4·7 for 6 h at 0–4°C, instead of direct addition of 0·1 M citric acid to the homogenate to bring it to pH 4·7 (Vijayan *et al* 1982). The precipitated proteins were removed by centrifugation at 27,000 *g* for 30 min at 0–4°C. The resulting supernatant, pH 4·7, was loaded on the pre-equilibrated PABA-CH-Sepharose 4B column. The bound human skin tyrosinase and MP 66 were eluted according to the procedure of Vijayan *et al* (1982).

3.2 *Purification of murine tyrosinase*

Murine tyrosinase was partially purified by the method established by Vijayan *et al* (1982).

3.3 *Effect of MP 66 on murine tyrosinase at pH 6·8*

Human skin tyrosinase and murine tyrosinase have 80% homology (Yamamoto *et al* 1987). It is, therefore, of interest to check whether this protein which was shown to inhibit tyrosinase of human skin at pH 6·8 (Vijayan *et al* 1982), is also inhibitory to tyrosinase of murine melanoma. The results are presented in table 1.

3.4 *Effect of MP 66 on murine tyrosinase at pH 5·0*

In view of the fact that melanosomal pH is acidic (Bhatnagar *et al* 1993), the effect of different concentrations of MP 66 on murine tyrosinase was studied at

pH 5·0. For this purpose, MP 66 and electroeluted murine tyrosinase were dialyzed separately against 5 mM sodium phosphate solution, pH 5·0 for 5 h at 4°C. The precipitated proteins were removed by centrifugation at 27,000 *g* for 30 min at 4°C, and the assay was done at pH 5·0. The results are presented in figure 1. From the figure, it is clear that MP 66 is stimulating the enzyme activity at pH 5·0 instead of inhibition observed at pH 6·8 (table 1).

3.5 *Isoelectric point of MP 66*

A standard graph was plotted between distance moved and pI values of known standard proteins. The isoelectric point of MP 66 was found to be in the range 7·3 to 7·6 (figure 2).

4. Discussion

In the present studies, a single step affinity chromatography, using 4-amino benzoic acid coupled to 6-aminohexanoic acid Sepharose-4B, was employed to purify human skin tyrosinase and MP 66 from epidermal homogenate, and murine tyrosinase from melanoma tissue, as described by Vijayan *et al* (1982) and Devi *et al* (1987). In View of limitations in procuring more human cadaver skin samples, we could not prepare more human skin tyrosinase enzyme for our studies at pH 6·8 and 5·0. Therefore, the effect of MP 66 at these pH were done on murine tyrosinase.

It was an unexpected observation in our laboratory to find that MP 66 also binds to the same column material (Vijayan *et al* 1982). The stoichiometric relationship between tyrosinase and MP 66 was not undertaken in the present investigation. All melanosomal proteins in the melanocytes share about 45% amino acid sequence homology. Since tyrosinase and MP 66 (Vijayan *et al* 1982) are localized in the melanosomes, it is speculated that they may have some structural homology. MP 66 at 2·6 μM concentration (170 μg/ml of reaction mixture) inhibited the urine tyrosinase also to about the same extent (table 1), as it did with partially purified human skin tyrosinase at pH 6·8 (Vijayan *et al* 1982). It is not surprising in view of the fact that there is 80% amino acid sequence homology between human and murine tyrosinases (Yamamoto *et al* 1987). MP 66 was shown to be a competitive inhibitor of partially purified human skin tyrosinase at pH 6·8 with 0·75 mM L-tyrosine. However, L-tyrosine at 2 mM concentration relieved the inhibition of tyrosinase by MP 66 (Vijayan *et al* 1982). MP 66 does not have protease-like activity. The intracellular concentration of MP 66 is sufficient to inhibit most of the human skin tyrosinase activity at the intracellular concentration of tyrosine, which is in the range of 0·4 to 2 mM (Husain 1981), if the pH of the melanosomes were to be 6·8. This is contrary to the real physiological situation where melanin cannot be made without tyrosinase being active. However, the *in vivo* melanosomal pH is acidic (Bhatnagar *et al* 1993). As both tyrosinase and MP 66 are localized in melanosomes, the effect of MP 66 on tyrosinase at acidic pH would be relevant for it to have any physiological role on melanogenesis. MP 66 was shown to actually stimulate tyrosinase when enzyme was assayed at pH 5·0 (figure 1).

The pI of MP 66 is in the range of 7·3 to 7·6 (figure 2). The broad pI range of MP 66 may have arisen due to charge microheterogeneity on the carbohydrate moiety. This was also reported to be the case with another melanosomal protein, namely gp 75 (Vijayasaradhi and Houghton 1991) whose pI ranges between 5·5 to 5·9. Based on pI values of mammalian tyrosinase (Hearing

Table 1. Effect of MP 66 on murine tyrosinase at pH 6·8.

S. No.	Tyrosinase activity (pmol dopa/min)		Inhibition (%)
	− MP 66	+ MP 66	
1. *,†	22·5		71·1
2. **,‡	23·1		98·0

The reaction mixture contained the following compounds at 37°C at a final concentration in a total volume of 55 μl: 4 mM ascorbic acid, 40 mM sodium phosphate buffer, pH 6·8, 0·75 mM L-tyrosine, and no dopa was added. Numbers in parenthesis indicate the amount of MP 66 in μg/ml reaction mixture used in the assay.

*,**Incubation period was 2 and 3 h respectively.

†,‡Electroeluted and partially purified murine tyrosinases at 145 μg/ml reaction mixture were used as enzyme sources respectively.

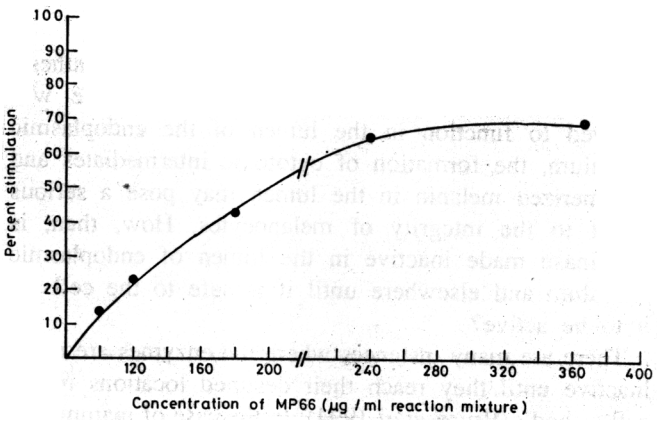

Figure 1. Percentage stimulation of murine tyrosinase by varying concentrations of MP 66 at pH 5·0.

The reaction mixture contained the following compounds at 37°C at a final concentration in a total volume of 55 μl: 4 mM ascorbic acid, 40 mM sodium phosphate solution, pH 5·0, 0·75 mM L-tyrosine, and no dopa was added. 5·3 units of electroeluted murine tyrosinase was used. The time of incubation was 2 h. Original enzyme activity without MP 66 was 3·1 enzyme units.

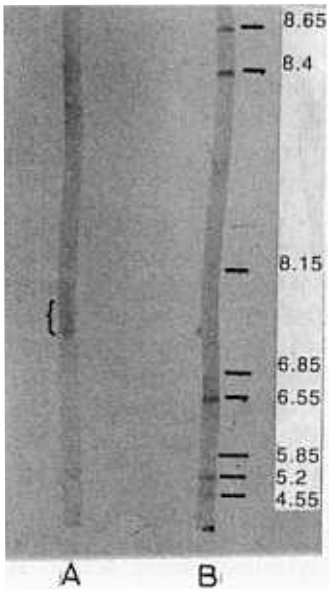

Figure 2. Isoelectric focusing of MP 66.
The purified MP 66 was focused on 5% polyacrylamide gels. (**A**) Bracket symbol shows the position of MP 66 band in the gel. (**B**) pI calibration.

et al 1981; Hearing and Jimenez 1987) and that of MP 66, it can be speculated that they can form a complex by ionic interactions at acidic pH, which is prevalent in the melanosomes. The extent of this interaction may result in altered tyrosinase activity. The stimulation of tyrosinase at pH 5·0 (figure 1), and its inhibition at pH 6·8 (table 1) by MP 66 may be related to the extent of ionic interactions between them.

4.1 *Physiological relevance of MP 66*

It is well known that intermediates of melanin biosynthesis are cytotoxic (Urabe *et al* 1992). If tyrosinase was allowed to function in the lumen of the endoplasmic reticulum, the formation of cytotoxic intermediates and polymerized melanin in the lumen may pose a serious threat to the integrity of melanocytes. How, then, is tyrosinase made inactive in the lumen of endoplasmic reticulum and elsewhere until it is safe to the cell for it to be active?

There are many instances where the enzymes are made inactive until they reach their destined locations in the cell or body (Bruce *et al* 1994). In the case of mammalian tyrosinase, it was proposed that the lag kinetics of tyrosinase at neutral pH (6·8 to 7·2) ensures that the enzyme does not function in the cytosol (Ramaiah 1996). In addition, if both tyrosinase and MP 66 are transported together from the lumen of the endoplasmic reticulum to melanosomes, MP 66 may inhibit any residual tyrosinase activity in the lumen of the endoplasmic reticulum and in the cytosol, where the pH is neutral (Seiji *et al* 1963; Burnett 1971). However, the enzyme will function when it is enclosed in an organelle like the melanosome, in which the internal environment is acidic (Bhatnagar *et al* 1993) and where the lag kinetics are abolished (Devi *et al* 1987) and where MP 66, under these conditions, would stimulate the enzyme activity (figure 1).

Winder *et al* (1995) reported that TRP-1, which is also available in abundant quantity in melanosomes, stimulates tyrosinase activity under acidic conditions. This resembles the similar stimulatory effect of MP 66 on tyrosinase at acidic pH (figure 1). Since both MP 66 and TRP-1 are available in high quantities in human skin melanosomes, and stimulate tyrosinase at acidic conditions, comparison of these proteins will reveal if they are the same similar or different proteins. Studies are under way to compare them.

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