

Review

Opiomelanins synthesis and properties

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Summary. Opiomelanins represent a new class of synthetic pigments produced by the tyrosinase-catalyzed oxidation of opioid peptides and other tyrosine aminoterminal peptides. In contrast with dopamelanin, these polymers are fully soluble in hydrophilic media, due to the presence of the peptide moiety. Opiomelanins show paramagnetism as demonstrated by the EPR spectrum identical to that of dopamelanin. The presence of the aminoacidic chain linked to the melaninic moiety, influences the electron transfer properties of opiomelanins i.e. the ability to oxidize NADH. Like dopamelanin Tyr-Gly-melanin exhibits this behaviour whereas leuonmelanin does not show any oxidizing potential. Opiomelanins UV-Vis spectrum is characterized by an absorption band at 330 nm which disappears upon acid hydrolysis, H₂O₂ treatment and under simulated solar illumination. Opiomelanins exhibit a fluorescence emission peaked at 440 and 520 nm if excited at 330 nm. These fluorescence bands are due to the oligomeric units and high molecular weight units, respectively. When opioid peptides are allowed to react with tyrosinase in the presence of an excess of cysteine, cysteinyl dopaenkephalins are synthesized. These peptides are furtherly oxidized giving rise to pheoopiomelanins. Reactive oxygen species also are able to oxidize non enzymatically both enkephalins and cysteinyl dopaenkephalins, producing the corresponding melanin pigments.

Key words: Melanins, Opiomelanins, Enkephalins

Introduction

Melanins are amorphous pigmented polymers occurring in all living organisms (Nicolaus, 1968; Prota, 1992). The pigments are normally synthesized either by auto-oxidation of catechols or by tyrosinase-catalyzed oxidation of tyrosine or dopa. The first step of the

Mason-Raper pathway entails the formation of dopaquinone, a highly reactive compound that undergoes an internal cyclization, giving rise to dopachrome and thereafter to dihydroxyindole that is polymerized to eumelanin (Prota, 1988). In the presence of cysteine, dopaquinone produces cysteinyl dopa which yields pheomelanin, through the intermediate production of benzothiazine derivatives (Prota, 1992) (Fig. 1).

During the past years, our group was engaged in studying novel pathways for melanin production involving enzymes and substrates different from those already known. Regarding additional enzymes we showed that, in the presence of H₂O₂, lipoxygenase and xanthine oxidase as well as cytochrome c, can form eumelanins and pheomelanins in vitro (Rosei et al., 1994a, 1998a,b; Mosca et al., 1996; Foppoli et al., 1997; Blarzino et al., 1999).

As regards additional substrates, we have found that the tyrosinase-catalyzed oxidation of tetrahydroisoquinolines (TIQs) and opioid peptides produces melanin-like pigmented polymers, named TIQmelanins and opiomelanins, respectively (Rosei et al., 1992; Rosei and Mosca 1995; Mosca et al., 1998). TIQs are endogenous compounds formed by the Pictet-Spengler addition of catecholamines with an aliphatic or aromatic aldehyde and acting on dopamine receptors (Dietrich and Erwin, 1980). Opioid peptides form the well-known group of neurotransmitters and neurohormones sharing the common feature of an amino-terminal tyrosine residue essential for receptor binding (Roda et al., 1986). In the present paper the results obtained about opiomelanins synthesis and properties are reviewed.

Tyrosinase-catalyzed oxidation of opioid peptides

When leuonkephalin (Fig. 2) reacts in vitro with tyrosinase a dopachrome-like spectrum with maxima at 305 and 470 nm rapidly appears. The kinetics of this reaction has been investigated for a number of opioid peptides and tyrosine aminoterminal peptides, and enzyme affinity for the peptidic substrates, with few exceptions, has been found to be higher with respect to free tyrosine. This is due to the presence of the carboamidic linkage that can help radical formation on

the phenolic ring, thereby facilitating tyrosinase action (Rosei et al., 1989).

HPLC analysis of leu-enkephalin reaction products, coupled to electrospray ion mass spectrometry, reveals that the tyrosinase-catalyzed oxidation of the peptide generates a compound in which the tyrosine residue is converted to dopa (Rosei et al., 1989; Larsimont et al., 1994). The dopa-enkephalin peptide can bind the peptidergic receptor, exhibiting a decreased activity compared with the parent tyrosine-containing peptide (Larsimont et al., 1994). Oxidation of the peptide further proceeds giving rise to a brownish pigment that can be easily collected by mild acidification and centrifugation (Rosei et al., 1992).

Acid hydrolysis of the pigment produced by leu-enkephalin oxidation reveals the presence of glycine, phenylalanine and leucine, in the same ratio occurring in the parent enkephalin precursor (Rosei et al., 1992). Tyrosine is not recovered because of its conversion into the melaninic portion. These new synthetic pigments presenting the melaninic portion linked to the aminoacidic structure have been called enk-melanins or opiomelanins, according to their source: i.e. whether they originate from enkephalins or opioid peptides. The same results are obtained when other peptides, such as Tyr-Gly, Tyr-Ala, Tyr-Arg or Tyr-Gly-Gly undergo

tyrosinase action. From our experiments it can be argued that any peptide presenting an amino terminal tyrosine residue is able to generate a peptide-melanin upon tyrosinase action. The sequence of reactions which is postulated to explain the oxidation of enkephalins and related peptides by tyrosinase follows the Mason-Raper pathway (Fig. 1).

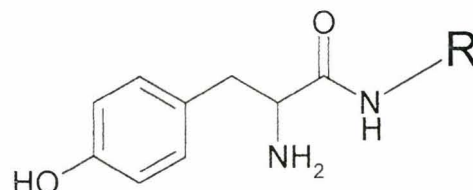
Solubility properties of opiomelanins

A relevant characteristic of opiomelanins is their solubility. These melanopeptides differ from synthetic melanins produced from tyrosine or dopa because they are thoroughly soluble in hydrophilic solvents at neutral and basic pH (Rosei et al., 1992). The hydrophilicity is shown by all the pigments tested and is retained also in the melanins synthesized from simple dipeptides such as Tyr-Gly, Tyr-Ala, Tyr-Arg, and Tyr-Glu. This behaviour suggests that an essential role in determining the solubility has to be attributed to the terminal carboxylic group. It is noteworthy that when opiomelanins are chemically hydrolyzed in 6N HCl, the resulting pigments are very similar to dopamelanin. They are converted into black, insoluble, amorphous pigments (Rosei et al., 1994b).

In the presence of tyrosinase, products coming from opioid peptides are able to cooxidize with intermediates generated by tyrosinase oxidation of dopa, thereby forming mixed type pigments that, in contrast to dopamelanin are soluble in hydrophilic media. The easy incorporation of oxidized products from opioid peptides into dopamelanin, allows also to think that any peptide with an amino terminal tyrosine, may give rise to a long list of possible soluble synthetic polymers by cooxidation with other products coming from tyrosinase action.

Redox properties of eumelanins and opiomelanins

It has been demonstrated that dopamelanin can



R = Gly	Tyr-Gly
R = Gly-Gly	Tyr-Gly-Gly
R = Gly-Gly-Phe-Leu	Leu-enkephalin
R = Gly-Gly-Phe-Met	Met-enkephalin
R = Arg	Kyotorphin

Fig. 2. Molecular structure of various opioid peptides and tyrosine NH₂-terminal peptides.

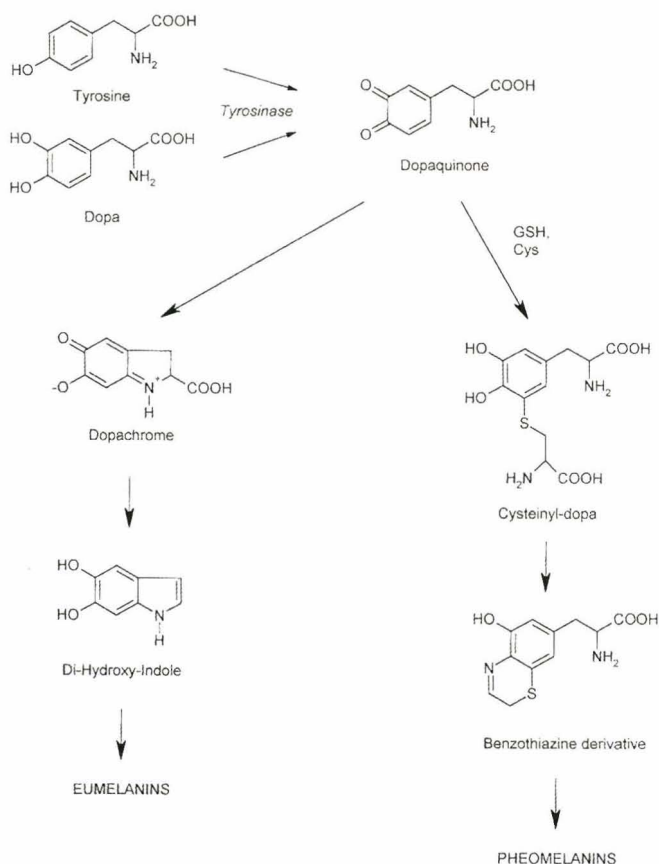


Fig. 1. Biosynthetic pathway for eu- and pheomelanins synthesis.

oxidize reduced nicotinamide adenin dinucleotide (NADH) *in vitro* (Gan et al., 1974, 1976) whereas melanoma melanin cannot (Gan et al., 1974). The oxidizing ability of dopamelanin has been ascribed to the presence of free radicals in the pigment (Sealy, 1984), responsible for the well documented Electron Paramagnetic Resonance (EPR) signal (Sarna and Sealy, 1984). Though opiomelanins show an EPR spectrum not different from that of dopamelanin, the oxidizing behaviour towards NADH differs among the various melanopeptides (Rosei et al., 1994b). Indeed, the presence of the peptide chain appears to influence heavily the NADH oxidizing behaviour. Both the electron transfer properties and the oxidizing potential seem to be inversely related to the chain length, so that, in contrast with tyrosine-melanin or dopamelanin, leuonkmelanin does not show any oxidizing activity. The above mentioned behaviour is in agreement with the loss of the transfer action demonstrated by melanoma melanin, formed by an ensemble of melanin and proteins which are covalently linked to the pigment moiety (Gan et al., 1974).

Opiomelanins stability and photooxidation

As regards opiomelanins stability it has been established that the pigments are stable in the dark, in the pH range 5.5-7.0. At higher pH values or under simulated solar illumination, in the presence or in absence of H₂O₂, pigment bleaching occurs more easily than in dopamelanin. After the irradiation, opiomelanins absorption spectrum undergoes a marked modification, being the peak at 330 nm replaced by a shoulder at 280-350 nm. The higher bleaching rate of opiomelanins compared with dopamelanin leads to hypothesize for these pigments a different polymerization degree and structure (Fig. 3) (Rosei et al., 1995a,b).

UV-Vis and fluorescence analysis of opiomelanins

The UV-Vis spectrum of the melanopeptides synthesized from leuonkephalin shows a continuous absorption pattern increasing over the range 600-230 nm (Fig. 4) (Rosei et al., 1994b). In the visible region, the leuonkmelanin spectrum is similar to that of dopamelanin, whereas in the UV domain, a peak at 330 nm can be observed. Though a clear attribution for this

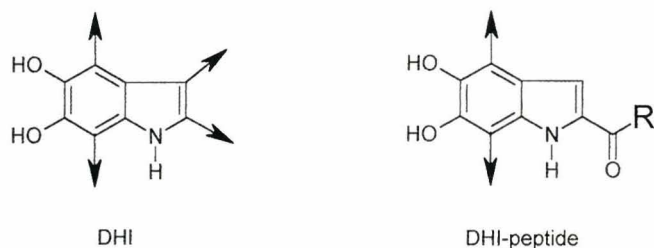


Fig. 3. Structure of dihydroxyindole (DHI) and dihydroxyindolpeptide (DHI-peptide). The arrows indicate the polymerization sites.

peak is difficult to assign, the absorption can be related to the presence of the peptide chain. In fact it has been found in all the melanopeptides, regardless of the pH medium utilized to dissolve the pigment. As previously stated, acid hydrolysis of leuonkmelanin releases aminoacids and the resulting black, insoluble precipitate analysed by UV-Vis spectroscopy exhibits a spectrum identical to that of dopamelanin i.e. a continuous, monotonic increase in absorption without any characteristic band (Rosei et al., 1995b).

However a partial attribution of this 330 absorption band has been inferred by fluorescence studies (Mosca et al., 1999).

Leuonkmelanin dissolved in aqueous medium exhibits a characteristic emission peaked at 440 and 520 nm when excited around 330 nm, where a maximum is observed in the absorption spectrum of the soluble pigment. Kinetic measurements carried out on the tyrosinase-catalyzed oxidation of leuonkephalin demonstrate that the 440-nm fluorescence band arises in the early stages of peptide oxidation, whereas the 520 nm band appears in late stages of oxidation e.g. in the course of indolequinone units polymerization. Furthermore, molecular sieve fractionation shows that in the leuonkmelanin portion with a molecular weight lower than 10 kDa, the 440 nm band is dominant in the fluorescence spectrum. The breakdown of the polymer induced by hydrogen peroxide and light (i.e. the photobleaching of melanin pigments) causes a remarkable enhancement of the 440 nm band while the 520 nm band disappears. In conclusion, our findings suggest that the observed fluorescence contains contributions from both oligomeric units (440 nm band) and high molecular weight polymers (520 nm band) (Mosca et al., 1999).

Pheomelanins from opioid peptides

When enkephalins are allowed to react with

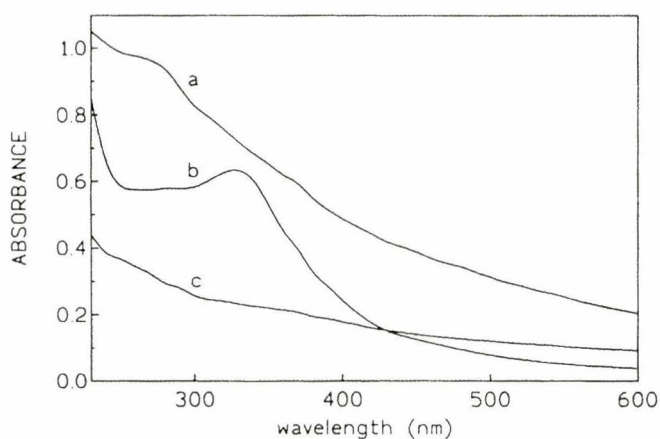


Fig. 4. Absorption spectra of different melanin peptides: a) dopamelanin; b) leuonkmelanin; and c) leuonkmelanin subjected to acid hydrolysis.

tyrosinase in the presence of an excess of cysteine, sulfhydryl adducts of enkephalins (cysteinyldopaenkephalins (CDEnks)) (Fig.5) are synthesized (Rosei et al., 2000). The major product is represented by 5-S-CDenk (80%) while the minor one is the isomer 2-S-CDenk (20%). The above mentioned peptides have been isolated by HPLC and identified by amino acid analysis and electrospray ion mass spectrometry. Then 5-S-isomer has been further characterized. This peptide is able to bind opioid peptide receptors in bovine brain synaptosomes (Rosei et al., 2000). The binding affinity is higher for δ than for μ receptors but the bond strength is 8-fold lower than that exhibited in the same conditions by leu-enkephalin.

In the presence of reactive oxygen species (ROS) both enkephalins and cysteinyldopaenkephalins yield melanin pigments i.e. opiomelanins and pheo-opiomelanins respectively (Coccia et al., 2001; Fontana et al., 2001).

Actually enkephalins and CDEnks are good scavengers of superoxide anion, hydroxyl and peroxy radicals and are able to reduce the lipid peroxidation rate induced by 2-2'-azobis-(2-amidinopropane) (ABAP). In particular the scavenger activity of the sulfhydryl derivatives of enkephalins is surprising if one considers that these peptides are more active than powerful radical scavengers such as Trolox and mannitol. Though opiomelanins and pheo-opiomelanins can be also produced enzymatically (Rosei et al., 2000), the oxidative interaction between ROS and the peptides provides evidence of a non enzymatic route of formation for both species of melanopeptides (Coccia et al., 2001; Fontana et al., 2001).

Concluding remarks

At present opiomelanins and pheo-opiomelanins can be considered good models for investigating melanin's

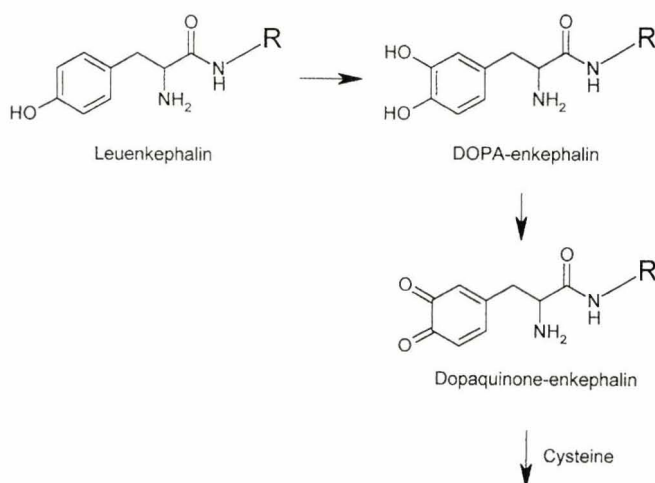


Fig. 5. Tyrosinase-catalyzed formation of CDEnks. R= $\text{CH}_2\text{CH}(\text{NH}_2)\text{CO-Gly-Gly-Phe-Leu}$.

structure and behaviour because their hydrophilicity allows spectroscopic investigations. The existence in vivo of these particular melanopeptides is at present only speculative, however their occurrence in vivo cannot be excluded; the elevated number of opioid peptide receptors in the same brain subregions in substantia nigra (Zamir et al., 1984) suggests a possible involvement of these neurotransmitters and their sulfhydryl adducts in the neuromelanization.

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