Subcellular localization and function of melanogenic enzymes in the ink gland of *Sepia officinalis*

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The ink gland of the cuttlefish *Sepia officinalis* has traditionally been regarded as a convenient model system for investigating melanogenesis. This gland has been shown to contain a variety of melanogenic enzymes including tyrosinase, a dopachrome-rearranging enzyme and peroxidase. However, whether and to what extent these enzymes co-localize in the melanogenic compartments and interact is an open question. Using polyclonal antibodies that recognize the corresponding *Sepia* proteins, we have been able to demonstrate that peroxidase has a different subcellular localization pattern from tyrosinase and dopachrome-rearranging enzyme. Whereas peroxidase is located in the rough endoplasmic reticulum and in the matrix of premelanosomes and melanosomes, tyrosinase and dopachrome-rearranging enzyme are present in the rough endoplasmic reticulum–Golgi transport system, at the level of *trans*-Golgi cisternae, *trans*-Golgi network and coated vesicles, and in melanosomes on pigmented granules. These results fill a longstanding gap in our knowledge of the melanin-producing system in *Sepia* and provide the necessary background for dissection at the molecular level of the complex interaction between melanogenic enzymes. Moreover, the peculiar and complex organization of melanin in an invertebrate such as *Sepia officinalis* is surprising and could provide the basis for understanding the process in more evolved systems such as that of mammals.

INTRODUCTION

Over the last few years the regulation of melanogenesis in mammals has received a good deal of attention because of the involvement of melanin in human pigmentation and melanocyte dysfunction in certain pathological conditions, such as albinism, vitiligo and malignant melanoma [1,2]. Ultrastructural and biochemical studies have shown that pigment formation in mammals takes place in highly specialized cells termed melanocytes, within unique cytoplasmic organelles known as melanin granules or melanosomes [3,4]. The copper-containing enzyme tyrosinase is the primary requirement for the melanogenic pathway since it catalyses the initial and rate-determining step of melanogenesis, i.e. the conversion of tyrosine to dopaquinone (Figure 1) [5,6]. For a long time it was believed that the subsequent steps in pigment production proceeded spontaneously through a series of reactions involving formation of dopachrome followed by rearrangement to 5,6-dihydroxyindole (DHI) which is then oxidized and polymerized to melanin. However, evidence has emerged in recent years that melanogenesis *in vivo* is far more complex and that many other reactions in the biosynthetic pathway are under enzymic control [7].

With the advent of molecular biology many aspects of the regulation of melanin production in mammals have been clarified since many novel genes involved have been cloned and characterized. In addition to tyrosinase, which has been shown to map to the *albino* locus in mice [8–10], other genes that function in pigmentation have been cloned, including that for tyrosinase-related protein 2 (TRP2) [11,12], tyrosinase-related protein 1 (TRP1) [13,14], Pmel 17/*silver* protein [15] and P protein [16,17] which have been mapped in the mouse to the *slaty*, *brown*, *silver* and *pink-eyed dilution* loci respectively. Although TRP1 and TRP2 share significant sequence similarity with tyrosinase, they possess different enzyme activities. TRP2, termed dopachrome tautomerase, catalyses the rearrangement of dopachrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA) rather than to DHI [11,18,19], whereas TRP1 has been recently shown to possess DHICA oxidase activity [20]. A first series of studies carried out in 1994 revealed that Pmel 17/*silver* protein does not exhibit any known melanogenic activity [21] but probably serves as a structural matrix protein in the melanosome [22]. More recently, it has been found that this protein is able to catalyse the polymerization of DHICA to melanin [23,24]. Another enzyme that has also been suggested to play a role in melanin biosynthesis is peroxidase, based on an *in vitro* study showing that peroxidase is able to catalyse the formation of melanin polymer from DHI and DHICA [25] and the finding that the enzyme has been found in the subcellular compartments of melanocytes from hamster melanoma tissue [26]. However, the mechanism by which these melanogenic enzymes interact in the melanocyte is still not known.

The ink gland of the cuttlefish *Sepia officinalis* represents an excellent system for investigating the dynamic interactions of these enzymes. Ultrastructural observations [27] have shown that the ink-gland cells are characterized by the presence of particulate melanosomes which have also been found in primitive vertebrates, such as albino catfish [28], goldfish [29] and triton [30–32]. Since fibrillar melanosomes, typical of mammals, have been reported in lungfish [33] and coelacanths [34], it has been suggested that phylogenetically the transition from particulate melanosomes, which represent more primitive structures, to the more evolved fibrillar melanosomes occurs in teleost fish [28]. The peculiar organization of melanin in particulate *Sepia* melanosomes, which are produced without the complete filling of the matrix as occurs in mammalian melanosomes, provides an opportunity to investigate the process of melanogenesis in the

Abbreviations used: TRP, tyrosinase-related protein; αHRP, antiserum against horseradish peroxidase; αSP, affinity-purified *Sepia* peroxidase antibodies; αSTDRIgG, IgG from antiserum against tyrosinase and dopachrome-rearranging enzyme from *Sepia*; TGC, *trans*-Golgi cisternae; TGN, *trans*-Golgi network; DHI, 5,6-dihydroxyindole; DHICA, 5,6-dihydroxyindole-2-carboxylic acid; DAB, 3,3′-diaminobenzidine hydrochloride; NGS, normal goat serum; RER, rough endoplasmic reticulum.

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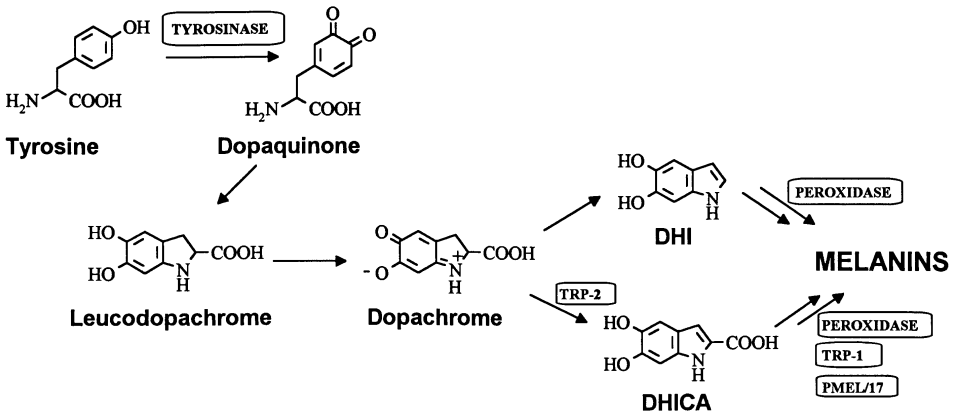

Figure 1 Schematic outline of the metabolic pathway leading to melanin formation in mammals

DHI, 5,6-dihydroxyindole; DHICA, 5,6-dihydroxyindole-2-carboxylic acid; TRP, tyrosinase-related protein.

Sepia ink gland at the subcellular level and thus gain insights into the mechanisms of interaction of the melanogenic enzymes. Parallel biochemical studies have shown that the ejected ink of *S. officinalis* contains, in addition to melanin, substantial amounts of tyrosinase [35] and a new melanogenic enzyme related to TRP2 that catalyses the rearrangement of dopachrome [36]. This latter enzyme is similar to that isolated from the haemolymph of the insect *Manduca sexta* [37] and differs from its mammalian counterpart in that it promotes the decarboxylative rearrangement of dopachrome to DHI rather than to DHICA. More recently, we reported that the ink gland of the cuttlefish exhibits intense peroxidase activity and, using PCR, we isolated and sequenced a cDNA clone encoding a protein homologous to other animal peroxidases including that of mammals [38]. The finding that the melanin-producing system of the cuttlefish contains a variety of melanogenic enzymes, including tyrosinase, dopachrome-rearranging enzyme and peroxidase, suggests that the process of melanin formation in cephalopods is much more complex than previously believed, with similarities to, and differences from, the pigmentary system of mammals.

This paper describes the characterization of polyclonal antibodies raised against *Sepia* melanogenic enzymes and their use in the immunolocalization of these proteins in the *Sepia* ink gland. Our results fill a longstanding gap in our knowledge of the melanin-producing system in *Sepia* providing the necessary background for dissection at the molecular level of the complex interaction between melanogenic enzymes. Moreover, the peculiar and complex organization of melanin in an invertebrate such as *S. officinalis* is surprising and could provide the basis for an understanding of the process in more evolved systems like mammals.

EXPERIMENTAL

Animals

Specimens of *S. officinalis* were collected in the bay of Naples, kept in a controlled environment for 2 days and subsequently killed by decapitation. The whole ink contained in the ink sac was collected and the ink glands were removed and used immediately.

Preparation of ink-gland extracts and melanin-free ink

The ink glands were homogenized in ice-cold 10 mM phosphate buffer (pH 6.8)/0.25 M sucrose/0.1 mM EDTA supplemented with 0.5 mM PMSF, using a ratio of buffer volume to tissue of 2:1 (ml/g). All subsequent manipulations were carried out at 4 °C. After centrifugation at 1000 ***g*** for 15 min, the pellet was re-extracted with the same buffer and centrifuged. The combined supernatants were treated with 1% Brij 35 at 4 °C for 1 h with occasional shaking and then ultracentrifuged at 80000 ***g*** for 1 h. The supernatant was used as ink-gland extract. The whole ink was ultracentrifuged at 80000 ***g*** for 1 h to remove melanin. The supernatant was dialysed against water and then lyophilized and used as melanin-free ink.

Antibodies

Polyclonal antibodies specific for *Sepia* peroxidase (αSP) were generated by Primm (Milan, Italy) in rabbits by injecting a mixture of two synthetic peptides which correspond to the most antigenic regions of the peroxidase sequence deduced from a *Sepia* peroxidase cDNA clone (I. Gesualdo, F. Aniello, M. Branno and A. Palumbo, unpublished work). The antiserum was affinity-purified using the peptides conjugated to Sepharose 4B–CNBr (Pharmacia LKB Biotechnology, Uppsala, Sweden) and lyophilized to give a stock solution of 1.28 mg of protein/ml. Polyclonal antibodies specific for both *Sepia* tyrosinase and dopachrome-rearranging enzyme (αSTDRIgG) were generated by Primm in rabbits by injecting a purified sample of the ejected ink which contains both enzyme activities [36]. The IgGs were purified by chromatography on a Protein A–Sepharose 6MB column (Pharmacia LKB Biotechnology) and lyophilized to give a stock solution of 1.55 mg of protein/ml. αPEP7 and αPEP8 antibodies were generated in rabbits against synthetic peptides corresponding to the unique C-terminal sequences of mouse tyrosinase and TRP2 respectively [11,39]. Rabbit anti-horseradish peroxidase serum (αHRP) and normal rabbit serum were purchased from Sigma Chimica (Milan, Italy). For pre-adsorption, αSTDRIgG and αSP were diluted in TTBS (20 mM Tris/HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20) containing the purified *Sepia* melanin-free ink (2 mg/ml) and a mixture of the peroxidase synthetic peptides (0.5 mg/ml) respectively. After incubation for 1 h at room temperature, the mixtures were used for immunodetection.

Immunoprecipitation

Samples of ink-gland extracts were adjusted to 1% Brij 35 and 0.01% SDS and preincubated for 2 h at 4 °C with a 1:1 suspension of Protein A–Sepharose 6MB in phosphate buffer at

pH 6.8 containing the same concentration of detergents. The samples were centrifuged and aliquots (40 μl each) of the supernatants were mixed with increasing amounts of antibody and brought to a final volume of 80 μl with PBS. After overnight incubation at 4 °C, 30 μl of a 1:1 suspension of Protein A–Sepharose 6MB in PBS was added and the mixture was further incubated for 1 h at room temperature with vigorous shaking. The samples were centrifuged and the supernatants examined for peroxidase, tyrosinase and dopachrome-rearranging enzyme activities. Parallel control experiments with normal rabbit serum were performed. When the immunoprecipitated extracts were examined by PAGE, the incubation with antibodies was performed for the times noted in the Figure legends. The immunocomplexes, obtained after treatment with Protein A–Sepharose, were washed twice with PBS, dissolved in electrophoresis buffer and examined as reported below.

PAGE and Western blotting

Gel electrophoresis under denaturing conditions was performed using a Pharmacia PhastSystem apparatus. Samples in 10 mM Tris/HCl, pH 8, containing 1 mM EDTA were heated at 90 °C for 3 min in the presence of 5 % 2-mercaptoethanol and 2.5 % (w/v) SDS. Electrophoresis was carried out on 7.5 % gels using 0.55 % SDS buffer strips and run at 60 V for 1 h. Molecular masses were determined by comparison with prestained molecular-mass standards from Gibco–BRL: 216 kDa, myosin; 110 kDa, phosphorylase *b*; 71 kDa, BSA; 43 kDa, ovalbumin (Life Technologies, Milan, Italy). The gels were blotted electrophoretically to Hybond–ECL nitrocellulose (Amersham Corp., Arlington Heights, IL, U.S.A.) and the blots, after saturation for 1 h with 3 % BSA in PBS buffer containing 0.04 % NaN_3, were incubated for 1 h at room temperature with primary antibodies diluted in TTBS. After three washings, bound antibodies were detected by enhanced chemiluminescence (Amersham) according to the manufacturer's instructions. Controls used included preadsorbed antibodies or substitution of the primary antibodies with normal rabbit serum or preimmune rabbit serum.

ELISA

Protein (1 μg) was bound at 4 °C overnight to Immulon II microtitre wells at pH 9.6 as previously detailed [39]. The plates were washed several times with PBS containing 0.01 % Tween 20 (PBS/Tween) to remove unbound antigen. Antisera at the dilutions noted were placed in each well and incubated for 90 min at 37 °C. The plates were then extensively washed again in PBS/Tween, then incubated for 30 min at 37 °C with peroxidase-conjugated anti-rabbit IgG (diluted at 1:1000 in PBS/Tween). The plates were extensively washed again in PBS/Tween, and specifically bound antibodies were detected using *o*-phenylenediamine substrate and quantified in a TiterTek microplate spectrophotometer at 490 mm [39].

Enzyme assays

Tyrosinase activity was determined spectrophotometrically at 27 °C by measuring the rate of formation of dopachrome (λ_{max} 475 nm) from L-tyrosine (0.4 mM) in 0.05 M phosphate buffer, pH 6.8. One unit of tyrosinase was defined as the amount of enzyme required to produce 1 μmol of dopachrome/min under the above conditions [40]. Peroxidase activity was determined spectrophotometrically at 27 °C by recording the rate of oxidation of guaiacol at 470 nm. The reaction mixture (3 ml) contained freshly prepared guaiacol (12 mM), H_2O_2 (0.33 mM) and 25 mM phosphate buffer at pH 6.

Protein determination

Protein was determined by the method of Lowry et al. [41] with BSA as standard.

Electron microscopy/enzymic cytochemistry

Fixation of ink glands was carried out in 4 % glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4 for 2 h at room temperature. Washed tissue was preincubated for 30 min in 0.05 M Tris/HCl buffer, pH 7.2, containing 0.05 % 3,3′-diaminobenzidine hydrochloride (DAB) (Sigma). Freshly prepared H_2O_2 was then added to a final concentration of 0.001 % and the material was shaken for 10 min. After incubation, the tissue was rinsed in Tris buffer and post-fixed in 1 % osmium tetroxide in sodium cacodylate buffer for 1 h and then dehydrated in a graded series of ethanol and embedded in Epon resin. Thin sections (80 nm) were picked up on 200-mesh nickel grids, and unstained sections were examined in an electron microscope. Control incubations were routinely performed in all experiments and included incubation in medium without H_2O_2 or in complete medium with added catalase (1000 units/ml).

Electron microscopy/immunocytochemistry

Fixation of ink glands was carried out in 3 % formaldehyde/0.1 % glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4 for 4 h at 4 °C. The formaldehyde solution was made fresh from paraformaldehyde immediately before use. After several washings with 0.05 M Tris/HCl buffer, pH 7.5, the tissue was dehydrated in 50 % (45 min at 4 °C), 70 %, 90 % and 100 % ethanol (1 h per wash) with progressive lowering of temperature. The dehydrated tissue was embedded in hydrophilic resin Unicryl (British Biocell International, Cardiff, U.K.) according to the manufacturer's instructions. Polymerization was performed under 360 nm long-wavelength UV light for 3 days at 4 °C. Thin sections (80 nm) were picked up on 200-mesh Formvar-carbon-coated nickel grids. The grids bearing Unicryl sections were floated face down on 10 % normal goat serum (NGS) in Tris-buffered saline (TBS; 0.05 M Tris/HCl, pH 7.5, 0.9 % NaCl) at room temperature for 1 h to block non-specific protein binding. The grids were transferred on to small drops of TBST buffer (0.01 % Triton X-100 in TBS)/NGS and incubated at room temperature for 10 min. The grids were then incubated with primary antibodies diluted in TBST/NGS at 4 °C overnight. Primary antibodies consisted of αHRP (1:1000), αSP (1:100), αSTDRIgG (1:20000), αPEP7 (1:100) and αPEP8 (1:100). Subsequently, the grids were washed with TBST and incubated with goat anti-rabbit IgG conjugated with 15 nm gold particles, diluted 1:16 in TBST/NGS buffer, for 1 h at room temperature. The grids were then washed with the same buffer for 10 min, rinsed with distilled water for 10 min and air-dried. The sections were counterstained with uranyl acetate for 15 min as described by Griffiths et al. [42], then examined ultrastructurally. Controls included sections treated with preadsorbed antibodies, omission of the primary antibodies or their substitution with normal rabbit serum or preimmune rabbit serum.

RESULTS

Characterization of antibodies

The specificities of the antibodies used in the present study were analysed by immunoprecipitation, Western-blot analysis and ELISA. Immunoprecipitation studies of peroxidase, tyrosinase and dopachrome-rearranging enzyme in the ink-gland extract of *S. officinalis* after reaction with the corresponding antibodies

Table 1 Immunoprecipitation of peroxidase and tyrosinase activities from ink-gland extract of *S. officinalis*

Samples of ink-gland extracts were incubated overnight at 4 °C with the antibodies noted at the final dilutions indicated. After treatment with Protein A-Sepharose and removal of immune complexes by centrifugation, the supernatants were examined for peroxidase and tyrosinase activities. Results are expressed as a percentage of immunoprecipitated activity calculated against the values obtained after incubation with normal rabbit serum, except for the results for αSTDRIgG, which are expressed as dilution of antiserum based on purified IgG concentration. Data are the means ± S.D. from three separate experiments.

Antiserum titre	Immunoprecipitated peroxidase activity (%)	Immunoprecipitated tyrosinase activity (%)
αHRP 1:50	72 ± 5	0
αHRP 1:100	56 ± 2	0
αHRP 1:200	38 ± 5	0
αSP 1:50	0	0
αSTDRIgG 1:80	0	72 ± 2
αSTDRIgG 1:400	0	35 ± 1
αSTDRIgG 1:800	0	20 ± 2

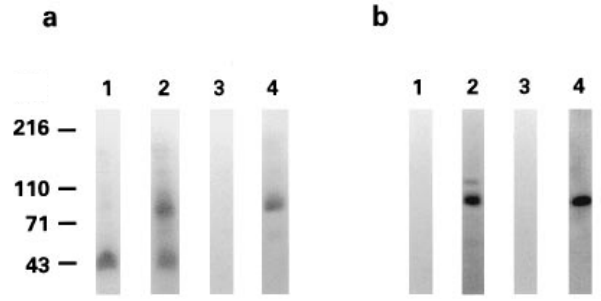

Figure 2 Immunodetection of peroxidase in the ink gland and ejected ink of *S. officinalis* by Western blotting using αHRP (a) and αSP (b) antibodies

(**a**) The ink-gland extract was immunoprecipitated for 1 h with αHRP. The immunoprecipitate was analysed by SDS/PAGE followed by transfer of the proteins to nitrocellulose, then detected with normal rabbit serum (1:1000, lane 1) or αHRP (1:1000, lane 2). The melanin-free ink was analysed by SDS/PAGE followed by Western blotting with normal rabbit serum (1:1000, lane 3) or αHRP (1:1000, lane 4). (**b**) The ink-gland extract and melanin-free ink were analysed by SDS/PAGE followed by Western blotting with preimmune rabbit serum (1:1000; gland, lane 1; ink, lane 3) or αSP (1:50; gland, lane 2; ink, lane 4). Bound antibodies were visualized by enhanced chemiluminescence. Numbers on the left indicate the positions of prestained molecular mass (kDa) standards.

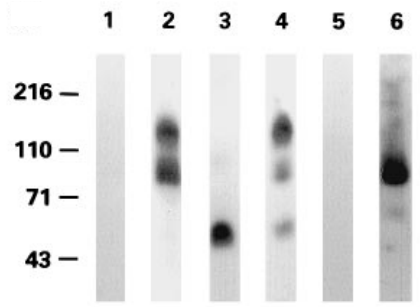

Figure 3 Immunodetection of tyrosinase and dopachrome-rearranging enzyme in the ink gland and ejected ink from *S. officinalis* by Western blotting

The ink-gland extract and melanin-free ink were analysed by Western blotting with preimmune rabbit serum (1:1000; gland, lane 1; ink, lane 5) or αSTDRIgG (1:2000; gland, lane 2; ink, lane 6). The ink-gland extract was immunoprecipitated for 30 min with αSTDRIgG (1:2000) and the immunoprecipitate was analysed by SDS PAGE followed by Western blotting with preimmune rabbit serum (lane 3) and αSTDRIgG (lane 4). Bound antibodies were visualized by enhanced chemiluminescence. Numbers on the left indicate the positions of prestained molecular mass (kDa) standards.

were carried out. Since tyrosinase cannot be readily eluted from immune complexes under conditions that preserve enzymic function, the enzyme activity remaining in supernatants was measured after immunodepletion. The same procedure was also adopted for the other enzymes. The peroxidase activity of the ink-gland extract was specifically precipitated in a concentration-dependent manner by αHRP and was unaffected by affinity-purified αSP, thus suggesting that in the latter case the epitopes were not exposed in the native protein (Table 1). Similarly, the tyrosinase activity of the ink gland was precipitated by αSTDRIgG (Table 1). As expected, peroxidase activity was not precipitated by αSTDRIgG and tyrosinase activity was not recognized by αHRP. The depletion of dopachrome-rearranging enzyme activity by these antibodies could not be observed in repeated experiments because of the low amount of the enzyme present in the gland. In spite of the failure to detect significant activity in the gland, dopachrome-rearranging activity was invariably high in the ejected ink [36]. This difference may reflect the low amount of the enzyme present in the gland compared with the ink, the limited amount of tissue available and the low sensitivity of the assay.

On PAGE analysis under denaturing conditions, αHRP detected a band of approx. 85 kDa in transblots of ink-gland extracts immunoprecipitated for 1 h with the antibody (Figure 2a, lane 2). The normal rabbit serum detected only the band at 55 kDa because of the eluted heavy IgG chains (Figure 2a, lane 1). The *Sepia* melanin-free ink exhibited the same immunoreactive band at 85 kDa with αHRP (Figure 2a, lane 4). Identical qualitative results were observed when the ink-gland extract and the melanin-free ink were incubated with αSP (Figure 2b). The molecular mass of the denatured protein recognized by anti-peroxidase antibodies agreed reasonably well with the predicted molecular mass (96.6 kDa) of the protein encoded by the complete peroxidase cDNA sequence isolated from an ink-gland cDNA library (I. Gesualdo, F. Aniello, M. Branno and A. Palumbo, unpublished work).

αSTDRIgG recognized a 85 kDa protein in the ink-gland extract and melanin-free ink with electrophoretic properties identical with that recognized by peroxidase antibodies (Figure 3, lanes 2 and 6). However, in the ink-gland extract an additional band of approx. 130 kDa was also observed. This latter band was always detected by Western immunoblotting analysis of extracts immunoprecipitated with αSTDRIgG even for times as short as 30 min (Fig. 3, lane 4). Both tyrosinase and dopachrome-rearranging enzyme migrated with an apparent molecular mass of 85 kDa. The 85 kDa protein corresponded to tyrosinase and dopachrome-rearranging enzyme, as evidenced in a previous study [36]. The 130 kDa protein present only in the extract could be an isoform or a processing form different from the final 85 kDa form found in the ejected ink. In all cases, preadsorption of the antibodies with the corresponding antigens eliminated the immunoreactivity entirely.

Since peroxidase co-migrated with tyrosinase and dopachrome-rearranging enzyme, under the experimental conditions used for SDS/PAGE, it was essential to demonstrate that the

Table 2 Reactivity of αPEP 7 and αPEP 8 for *Sepia* ink-gland extract

Sepia ink-gland extract (1 μg) was bound to ELISA plates and measured for antibody binding (A_{490}) by the peptide antibodies or by normal rabbit serum as detailed in the Experimental section. The results are presented as means ± S.E.M. for triplicate wells.

	Reactivity (A_{490})	
Antiserum titre	1:200 dilution	1:1000 dilution
Normal rabbit serum (control)	0.001 ± 0.000	0.000 ± 0.000
αPEP 7 (anti-tyrosinase)	0.869 ± 0.014	0.234 ± 0.002
αPEP 8 (anti-dopachrome tautomerase)	0.156 ± 0.010	0.008 ± 0.010

two antibodies employed recognized distinct and immunologically non-cross-reactive proteins. We therefore carried out immunodepletion experiments on the ink-gland extracts by performing three rounds of adsorption with αHRP (at a dilution of 1:10; about 92% of peroxidase activity was removed in the first adsorption, 7% in the second and 1% in the third). After this treatment, which removed peroxidase activity completely, the supernatant was treated with αSTDRIgG, and the immunoprecipitate analysed by SDS/PAGE and blotting was found to contain αSTDRIgG-reactive bands at both 85 kDa and 130 kDa (results not shown). These results demonstrate that tyrosinase, dopachrome-rearranging enzyme and peroxidase each have a molecular mass of 85 kDa.

The polyclonal antibodies αPEP7 and αPEP8, which recognize murine tyrosinase and TRP2 respectively, were examined by ELISA for their ability to recognize the equivalent *Sepia* ink-gland proteins. Both antibodies reacted specifically with the extract, αPEP7 exhibiting a stronger reaction than αPEP8 (Table 2). However, they produced no specific signal on Western blotting.

Immunogold localization of peroxidase, tyrosinase and dopachrome-rearranging enzyme in the ink gland

Both αHRP (not shown) and the affinity-purified αSP antibodies immunolabelled melanosomes with deposition of gold particles exclusively in the unmelanized matrix with no label visible on pigmented granules (Figure 4a). Gold particles also clearly decorated intraluminal regions and membranes of the rough endoplasmic reticulum (RER) (Figure 4b). Interestingly, the vesicular transport between the RER and tubular–vesicular complexes in the *cis*-Golgi did not show any immunopositivity (results not shown). Moreover, a uniform distribution of the gold particles was seen in the ejected ink at the level of the cellular components derived from the epithelial cells shed into the lumen (Figure 4c). In contrast, αSTDRIgG, which recognizes both tyrosinase and dopachrome-rearranging enzyme from *Sepia*, immunolabelled melanosomes and the ejected ink only in the pigmented granules (Figure 4d). Immunoreactivity with αSTDRIgG was also detected in the RER–Golgi transport system with a predominant localization of gold particles at the level of *trans*-Golgi cisternae (TGC), *trans*-Golgi network (TGN) and derived coated vesicles both in the intraluminal portion and on the cytoplasmic side (Figure 4e and f). Parallel experiments were performed with the antibodies αPEP7 and αPEP8, which showed a similar distribution of immunoreactivity, with gold particles localized in the RER Golgi transport system and in the melanin granules (results not shown). No gold particles were observed in any of the control sections examined.

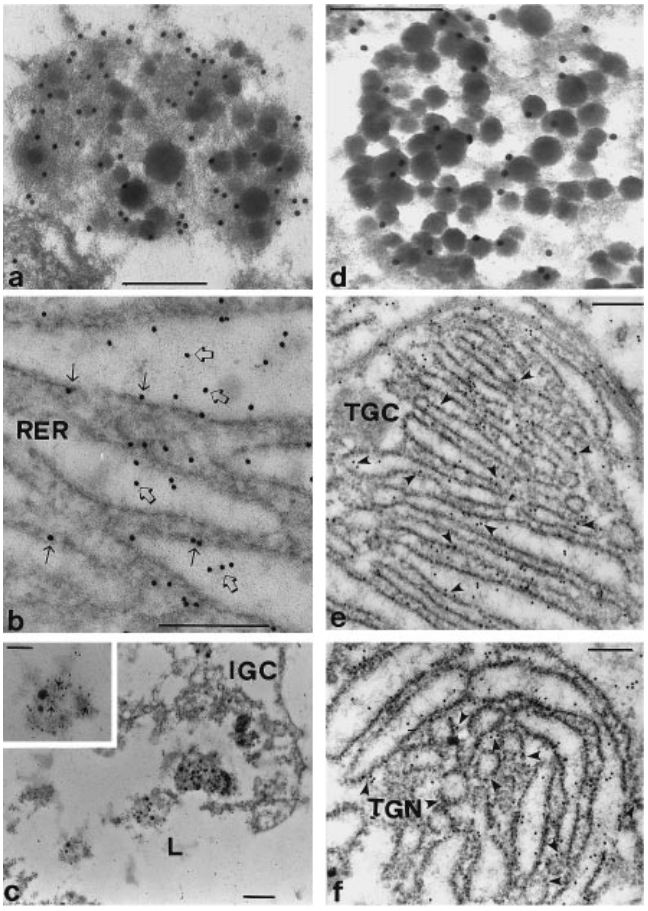

Figure 4 Immunolocalization of melanogenic enzymes in *Sepia* ink-gland cells

Tissue sections were immunogold labelled with αSP (**a**, **b** and **c**) or αSTDRIgG (**d**, **e** and **f**). (**a**) A fully melanized melanosome showing the presence of gold particles exclusively at the unmelanized matrix. (**b**) Dense gold particles can be seen in rough endoplasmic reticulum (RER) cisternae, both at the level of intralumen (open arrows) and cytoplasmic side (arrows) of the membranes. (**c**) Survey micrograph of a large area showing the shedding of the ink-gland cells into the lumen. Gold particles can be seen in the matrix of degenerated melanosomes. The inset shows a selected region at higher magnification demonstrating the specific localization of the gold particles (small arrows). (**d**) A fully melanized melanosome showing extensive labelling only at pigmented granules. (**e**) Representative immunoelectron micrograph showing gold localization in *trans*-Golgi cisternae (TGC) (arrowheads). (**f**) A selected region of *trans*-Golgi network (TGN) demonstrating coated vesicle labelling (arrowheads). IGC, ink-gland cell; L, lumen. Bars, 20 nm in **a**, **d** and in the inset, and 25 nm in **b**, **c**, **e** and **f**.

Subcellular localization of endogenous peroxidase activity

The ultrastructural localization of peroxidase activity in *Sepia* ink-gland cells was studied by enzymic cytochemistry after incubation of fixed tissue with DAB and H_2O_2. There was an intense positive reaction in the RER cisternae and in developing buds from the RER (Figure 5a). Moreover, numerous melanosomes at different degrees of melanization were observed throughout the cytoplasm characterized by DAB-positive reaction products at the level of the matrix (Figure 5b). No reaction product was detected within vesicular intermediates between the RER and Golgi or between the cisternae across the Golgi stack (results not shown). The ejected ink did not show any reaction product either (Figure 5c). No positive reaction was observed in RER cisternae or melanosomes in any of the controls (results not shown).

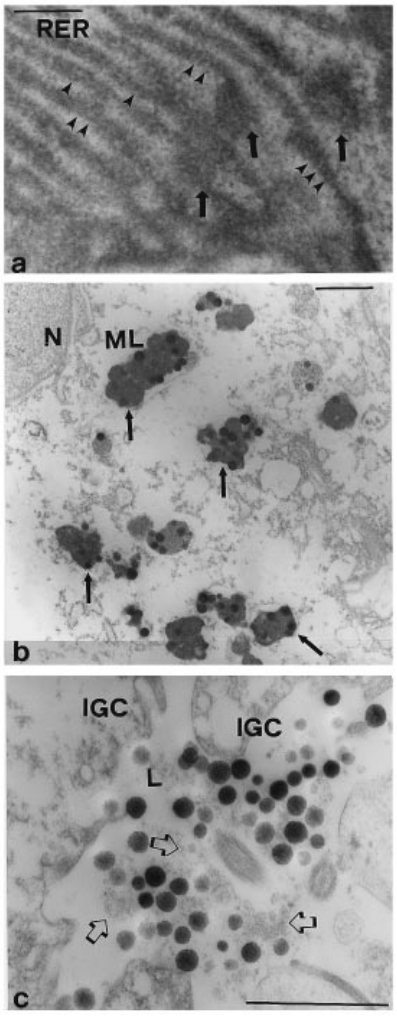

Figure 5 Intracellular localization of peroxidase activity in the *Sepia* ink-gland cells

(**a**) DAB-positive reaction product within the RER cisternae shows a granular appearance (arrowheads) which becomes compact in the developing buds (arrows). (**b**) Melanosomes at different degrees of melanization with DAB-positive matrix (arrows). (**c**) Electron micrograph showing two ink-gland cells. Note that no reaction product is detectable at the level of the cellular components (open arrows) arising from epithelial cells shedding into the lumen. IGC, ink-gland cell; N, nucleus; L, lumen; ML, melanosome. Bars, 25 nm in **a**, and 100 nm in **b** and **c**.

DISCUSSION

Little is known about the mechanisms by which the key enzymes of the metabolic pathway leading to melanin formation interact. To address this point, we raised polyclonal antibodies that recognized the melanogenic enzymes peroxidase, tyrosinase and dopachrome-rearranging enzyme, which occur in the ink-producing system of *S. officinalis*. Each of these melanogenic enzymes detected with the specific antibodies on PAGE analysis under denaturing conditions was found to migrate as a band of 85 kDa, suggesting the presence of a melanogenic complex of proteins. This finding is extremely interesting in the light of recent studies showing the existence of a multimeric complex of tyrosinase and TRP in Cloudman S 91 melanoma cells [43]. Using anti-peroxidase antibodies (αSP, αHRP) we have found that peroxidase is localized in the RER, in the large vesicles derived from it and in the matrix of the melanosomes. In contrast, it is absent from the transport vesicles responsible for the trafficking of proteins from the RER to the Golgi stack and from the TGN via coated vesicles en route to melanosomes. This observation suggests that in *S. officinalis* peroxidase is not subjected to late Golgi processing of Asn-linked carbohydrates, although it has some potential sites for N-glycosylation (I. Gesualdo, F. Aniello, M. Branno and A. Palumbo, unpublished work). The positive cytochemical reaction for endogenous peroxidase found in the RER and melanosomal matrix indicates that the enzyme is catalytically active in these locations. This finding is in line with recent histochemical and biochemical studies showing that, in melanocytes from hamster melanoma tissues, the highest levels of peroxidase activity are associated with stage-II and -III melanosomes [26].

In contrast, the use of antibodies reactive against tyrosinase and dopachrome-rearranging enzyme (αSTDRIgG) has revealed that these proteins exhibit a subcellular localization different from peroxidase. Gold particles have been found at the level of TGC, TGN, coated vesicles and pigmented granules of the melanosomes. These results are in agreement with previous studies on the localization of tyrosinase activity in the ink gland of cephalopods [27]. However, by using specific antibodies, we were able to demonstrate that tyrosinase, as well as dopachrome-rearranging enzyme, is undoubtedly localized in *Sepia* melanosomes exclusively at the level of melanin granules. This was also confirmed by immunolocalization experiments carried out with the antibodies αPEP7 and αPEP8 (data not shown).

Taken together, our results allow a more detailed characterization of the subcellular events of melanogenesis in the *Sepia* ink gland. The biogenesis of *Sepia* melanosomes involves two separate subcellular compartments (Figure 6). The process begins as vesicles bleb from the RER with concomitant removal of the ribosomes ($\xrightarrow{1}$). These vesicles, which contain peroxidase, represent premelanosomes and the matrix of the mature melanosomes. Along with the formation of RER-derived vesicles, coated vesicles containing tyrosinase and dopachrome-rearranging enzyme are released from the TGN into the cytoplasm ($\xrightarrow{2}$). At the subcellular level, we have obtained evidence that the coated vesicles then fuse with the membrane of premelanosomes and are subsequently incorporated into it ($\xrightarrow{3}$) (results not shown). The mechanism by which this incorporation occurs has been described for the formation of particulate melanosomes in goldfish [29]. It involves inversion and re-formation of the coated vesicles inside the premelanosomes with the consequence that the cellular components originally located on the internal side of the coated vesicle membrane are subsequently exposed to the melanosomal matrix. Assuming that in *Sepia* tyrosinase and dopachrome-rearranging enzyme are oriented in the membrane as in mammals [1], i.e. with the C-terminus directed towards the cytoplasm and the N-terminus and catalytic centre into the lumen of the melanosome, we can hypothesize that, after incorporation and inversion of the coated vesicles, the catalytic sites of the enzymes are exposed to the melanosomal matrix. On this basis, one can speculate that tyrosine, present within the melanosomal matrix, is converted by tyrosinase to dopaquinone. This newly formed dopaquinone undergoes intramolecular cyclization to give dopachrome which is transformed by *Sepia* dopachrome-rearranging enzyme to DHI [36]. Whether DHI is the only indole formed or whether other mechanisms responsible for DHICA production [44] are operative in *Sepia* ink gland is difficult to assess. However, a recent *in vitro* study showing that peroxidase is able to promote the formation of melanin polymers from DHI and DHICA [25] suggests that the indoles arising from dopachrome rearrangement can then be oxidized and polymerized by peroxidase which is already within the melanosomal matrix. The localization of

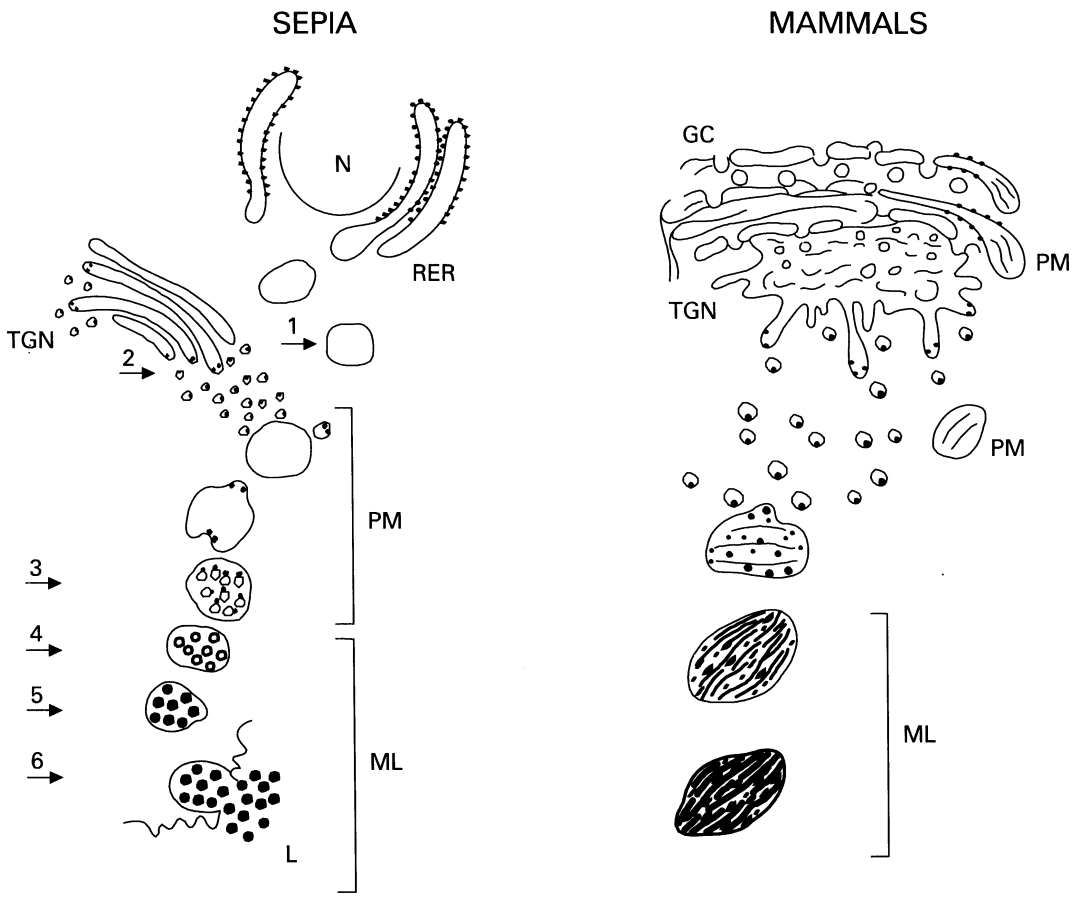

Figure 6 A comparison between melanin formation in the ink-gland of *S. officinalis* and mammals

N, nucleus; PM, premelanosome; ML, melanosome; L, lumen; GC, Golgi complex. ●, Active sites of melanogenic enzymes. The scheme of melanin formation in mammals has been adapted from Mishima [44].

peroxidase in the matrix of melanosomes, together with the polymerization properties of the enzyme, strongly suggests that peroxidase may play an important catalytic role in *Sepia* melanogenesis. Supporting evidence for this view also comes from recent histochemical studies showing that amelanotic melanoma cells do not possess any peroxidase activity [26]. After the action of peroxidase, melanin synthesis begins around the periphery of the incorporated coated vesicles ($\xrightarrow{4}$) and subsequently proceeds to completely cover their surfaces ($\xrightarrow{5}$). That melanization starts on the outer surface of the internal vesicles has been demonstrated by experiments with L-dopa carried out on goldfish [29]. On this basis, the incorporated coated vesicles not only provide tyrosinase for premelanosomes, but also function as a structural foundation for melanin synthesis. The size and number of these vesicles remain the same, even during melanin synthesis, which results in particulate melanosomes. When epithelial-cell maturation is complete, the membranes of the melanosomes fuse with those of the apical pole of the cell, and all the cellular constituents, including the melanin granules, are excreted into the lumen of the ink gland ($\xrightarrow{6}$). From the suggested scheme of melanin formation in *Sepia*, it appears that *Sepia* melanosomes possess a surprisingly complex organization for an invertebrate, which could represent an intermediate step along the phylogenetic scale in the evolution of the melanin-producing system. Although it is difficult to speculate to what extent the information obtained for *Sepia* can be extended to the mammalian system, it is evident that the major difference between the two melanin-producing systems is the mechanism of fusion of coated vesicles with premelanosomes. Whereas in *Sepia* the enzyme-filled coated vesicles are taken into the melanosomes with reversion of membrane orientation, in mammals most of the coated vesicles fuse with the limiting melanosomal membrane without any change in orientation and are subsequently incorporated into the matrix [45–47] (Figure 6). Moreover, the particulate structure of *Sepia* melanosomes offers the advantage of dissecting at the molecular level the complex interaction between melanogenic enzymes, which is not possible to investigate in mammalian melanosomes as they possess a lamellar matrix completely filled by melanin. In spite of the transition from particulate to fibrillar melanosomes. it is reasonable to hypothesize that the molecular mechanisms leading to melanin formation have remained almost unchanged in the course of evolution.

The immunolocalization of tyrosinase and dopachrome-rearranging enzyme is consistent with our previous reports on the occurrence of substantial amounts of these enzymes in the ejected ink [35,36]. The finding that the strong peroxidase immunoreactivity in the ink is not coupled with the presence of detectable peroxidase activity suggests that this enzyme is secreted in a catalytically inactive form. This enzyme-inactivation process, concomitant with the degree of melanization, is not surprising and has already been described in mammalian melanoma cells [26].

Of particular interest is the striking similarity of *Sepia* peroxidase to Pmel 17/*silver* locus protein and the *pink-eyed dilution* locus protein. Like *Sepia* peroxidase, these proteins are components of the melanosomal matrix and are absent from coated vesicles that deliver TRPs to melanosomes [22,48,49].

In conclusion, the *Sepia* ink gland provides an excellent model of a melanin-producing system in which it is possible to investigate at a subcellular level the interaction of the melanogenic enzymes localized in different compartments. Future studies will be directed at more closely characterizing this melanogenic system as a model for the regulation of pigmentation.

We thank Luigi De Martino, Gennaro Iamunno and Vincenzo Monfrecola for excellent technical support.

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Received 13 August 1996/4 December 1996; accepted 3 January 1997