Tyrosinases from crustaceans form hexamers

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Tyrosinases, which are widely distributed among animals, plants and fungi, are involved in many biologically essential functions, including pigmentation, sclerotization, primary immune response and host defence. In the present study, we present a structural and physicochemical characterization of two new tyrosinases from the crustaceans *Palinurus elephas* (European spiny lobster) and *Astacus leptodactylus* (freshwater crayfish). *In vivo*, the purified crustacean tyrosinases occur as hexamers composed of one subunit type with a molecular mass of approx. 71 kDa. The tyrosinase hexamers appear to be similar to the haemocyanins, based on electron microscopy. Thus a careful purification pro-

ocol was developed to discriminate clearly between tyrosinases and the closely related haemocyanins. The physicochemical properties of haemocyanins and tyrosinases are different with respect to electronegativity and hydrophobicity. The hexameric nature of arthropod tyrosinases suggests that these proteins were the ideal predecessors from which to develop the oxygen-carrier protein haemocyanin, with its allosteric and co-operative properties, later on.

Key words: catechol oxidase, haemocyanin, oligomer, phenol oxidase, structure.

INTRODUCTION

Tyrosinases (EC 1.14.18.1) are present in almost all organisms and are involved in several biological functions [1,2]. They initiate the synthesis of melanin and, thus, are responsible for the pigmentation of skin, hair and fruit. Owing to the fungistatic, bacteriostatic and antiviral properties of melanin and its intermediates, they are also key components of the primary immune response of certain invertebrate phyla, especially arthropods [3–5]. Their central role in a common defence cascade for deuterostomata and protostomata has been reviewed [6]. Melanin also serves as a structural component in the healing of wounds in plants and arthropods [7]. Furthermore, the highly reactive quinones produced by tyrosinase serve to sclerotize the protein matrix of the arthropod cuticle after moulting [8].

Tyrosinases catalyse the *o*-hydroxylation of monophenols (cresolase or monophenolase activity) and the oxidation of *o*-diphenols to *o*-quinones (catechol oxidase or diphenolase activity). In contrast, catechol oxidases (EC 1.10.3.1) as polyphenol oxidases can only catalyse the oxidation of diphenols, although they are indistinguishable from the different kinds of tyrosinases by sequence and properties other than enzymic activity [2,9, 10]. This may be the reason why the term phenol oxidase is often used for tyrosinases and catechol oxidases of arthropods in the literature without distinguishing between them. Only three of the more than 20 reported arthropod phenol oxidases, for which the sequence is known, were proven to be tyrosinases exhibiting monophenolase activity [11–13].

Tyrosinases and haemocyanins belong to the same protein superfamily, the type 3 copper proteins, with respect to their sequences and their common features in UV–Raman resonance, X-ray absorbance, electron paramagnetic resonance and UV-visible spectroscopic properties, indicating a very similar active site [1,9,10,14,15]. Structural information about the active site is provided by a synthetic compound and the oxy structure of a

haemocyanin [16,17]. In the present study, two copper ions are co-ordinated by six histidines and reversibly bind a dioxygen molecule in a side-on co-ordination [15–18]. A crystal structure of the met form of a catechol oxidase is available from sweet potato (*Ipomoea batatas*). However, this monomeric catechol oxidase is only capable of catalysing the oxidation of diphenols [19]. Thus the structure of the exact arrangement of the atoms at the active site necessary to catalyse *o*-hydroxylation of monophenols in tyrosinase is still unknown.

Although tyrosinases and their inactive forms have been well characterized with respect to their sequences and enzymic activity, only a little is known about the native structure of the whole tyrosinase molecule. Although human and mouse tyrosinases are membrane-bound and thought to occur as dimers, tyrosinases from arthropods are reported to associate to form structures anywhere between monomers and pentamers [12,13,20,21].

Probably, there is no common tyrosinase: the enzymes found in animals, plants and fungi are different with respect to their sequences, size, glycosylation and activation. Two different types of tyrosinases can be distinguished based on sequence comparison [1,9,22]: one is more related to molluscan haemocyanin concerning the active site, and the other, which is very similar to arthropod haemocyanins, is also found in arthropods together with haemocyanins. Since much structural information is available on arthropod haemocyanins, we concentrated our study on tyrosinases from arthropods to learn more about the structure and function in comparison with the closely related haemocyanins.

In the present study, we describe the purification of tyrosinases from two crustacean species, *Palinurus elephas* (European spiny lobster) and *Astacus leptodactylus* (freshwater crayfish), characterize them physicochemically and structurally and compare them with arthropod haemocyanins. An important point in our study is the very careful differentiation between tyrosinase and the closely related haemocyanin during the purification pro-

Abbreviations used: CPC, cetylpyridinium chloride; HIC, hydrophobic-interaction chromatography; IEX, ion-exchange chromatography; MBTH, 3-methyl-2-benzothiazolinone hydrazone hydrochloride; SEC, size-exclusion chromatography.

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cedure. The results shed new light on the structural evolution of the protein superfamily that includes the arthropod tyrosinases, haemocyanins and the insect storage protein hexamerins, which have lost the ability to bind the two copper atoms at the active site [9].

EXPERIMENTAL

All chemicals were purchased from Sigma unless otherwise indicated and were of analytical grade.

Animals and preparation of haemolymph stock solution

Two crustacean species, two female *P. elephas* and approx. 150 *A. leptodactylus* of undefined sex, were used in the present study. The animals were purchased from local fish stores. They were punctured at the pericard to obtain haemolymph. The haemolymph of each species was clotted at 20 °C for a few minutes. Then larger material was pelleted at 178 000 g for 20 min at 4 °C. The supernatant was pelleted at 190 000 g for 6 h at 4 °C. The pellet containing haemocyanin and tyrosinase was resuspended in a stabilization buffer (100 mM Tris/HCl, 20 mM CaCl₂, 20 mM MgCl₂, 100 mM NaCl, pH 7.5) for several days at 4 °C. In this way, haemolymph stock solutions were obtained for the two species with a protein concentration of approx. 100 mg/ml, which could be stored for months at 4 °C without any degradation or damage to their functional properties.

Protein concentrations were estimated spectroscopically at 278 nm using $\epsilon_{278} = 1.0 \text{ ml} \cdot \text{mg}^{-1} \cdot \text{cm}^{-1}$, which is commonly used for proteins.

Haemolymph proteins were dissociated by dialysis against dissociation buffer (50 mM glycine/OH, 5 mM EDTA, pH 9.6) for 4 days at 4 °C. During dissociation, the protein solution was kept below 1 mg/ml [23].

Protein purification and analysis

Chromatography

For analytical size-exclusion chromatography (SEC), a Superose 6 HR 10/30 column (Pharmacia, Freiburg, Germany) was used. Elution occurred in stabilization buffer at 0.2 ml/min. Anion ion-exchange chromatography (IEX) was performed on a Fractogel EMD DEAE 650(S) matrix (Merck, Darmstadt, Germany) in a Superformance Universal System column (Merck) with a diameter of 10 mm and a length of 13.5 cm. Proteins were applied on to the column in IEX buffer (50 mM Tris/HCl, pH 8.5) and eluted with a 0–1 M NaCl gradient. For hydrophobic-interaction chromatography (HIC), a Phenyl-Superose HR 5/5 column (Pharmacia) was used. Proteins were bound to the column using HIC buffer (50 mM sodium phosphate buffer, 1 M (NH₄)₂SO₄, pH 7.0). For elution, a linear gradient from 1 to 0 M (NH₄)₂SO₄ was applied.

Electrophoresis

SDS/PAGE was performed on a 7.5 % (w/v) acrylamide native gel [24]. Native PAGE was performed on 7.5 % gels at 4 °C by using the same buffer system but omitting SDS from all solutions. Importantly, for native PAGE, the sample was not denatured before electrophoresis.

Electron microscopic analysis

Protein samples were stained with 2 % (w/v) uranyl acetate, and electron microscopic pictures were taken on a Zeiss EM 900

microscope. Image processing of electron microscopic images was performed using the EMAN software [25].

Measurement of enzyme activity

Dot-blot

Tyrosinase activity was semiquantitatively determined on dot-blots. Briefly, 2 µl of tyrosinase-containing solution was mixed with 2 µl of a reaction mixture [5 mM tyramine, 0.45 mM 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH), 10 mM SDS and 3 % (v/v) ethanol in 100 mM sodium phosphate buffer, pH 7.0] on a PVDF membrane (Bio-Rad Laboratories). After drying, positive samples were coloured dark red to black.

Activity staining of native PAGE

For staining of tyrosinase activity after native PAGE, gels were incubated in the reaction mixture mentioned above directly after electrophoresis. To activate the tyrosinase, either 10 mM SDS or 1 mg/ml bovine trypsin was added to the reaction mixture. Tyramine and dopamine were used to assay monophenolase and diphenolase activity respectively.

Kinetic measurements

Kinetic measurements of enzymic activity were measured on a Hitachi U-3000 spectrometer in quartz cuvettes at 20 °C. All assays were performed in 100 mM sodium phosphate buffer (pH 7.0) containing 0.5 mM MBTH as dye reagent. The time course of the reaction was measured by monitoring the absorbance at 505 nm [26]. The substrates tyramine and dopamine and the detergents SDS and cetylpyridinium chloride (CPC) used for activation were added as indicated. The reaction was always initiated by injection of the enzyme solution and mixing briefly. The dead time was approx. 10 s. Substrates and reaction mixtures were prepared fresh daily and maintained in the dark at 4 °C due to their sensitivity.

RESULTS

Tyrosinase activity in the haemolymph

The haemolymph of *P. elephas* and *A. leptodactylus* was investigated for tyrosinase activity. The haemolymph was incubated for 4 days in dissociation buffer to dissociate oligomeric protein complexes. The predominant protein in the haemolymph, haemocyanin, is effectively dissociated by this treatment and no degradation or changes to its functional properties have been observed [23,27]. The haemolymph was then subjected to native PAGE. The gel was stained for monophenolase and diphenolase activity using tyramine and dopamine as substrates respectively. To mimic the action of *in vivo* activators like lipids or protease, enzymic activity was induced by the detergent SDS and the protease trypsin. Both are well-known potent activators of tyrosinases [1,28–30]. The haemolymph of both crustacean species contained protein bands with monophenolase and/or diphenolase activity (Figure 1). For both species, there seemed to be two groups of proteins with enzymic activity: one group exhibited monophenolase as well as diphenolase activity, but obviously the gel did not contain enough of this protein to be stained by Coomassie Blue. This enzymically active protein migrates in the gel like a protein with a high molecular mass, most probably an oligomeric protein. The second group of protein bands with very weak activity exhibited only diphenolase activity and was strongly stained by Coomassie Blue. These

Figure 1 Detection of tyrosinase activity in the haemolymph of *P. elephas* (A) and *A. leptodactylus* (B)

Dissociated haemolymph samples were run on a 7.5% (w/v) acrylamide native gel at pH 8.8. The gel was stained for protein with Coomassie Blue (lane 1) and for tyrosinase activity (lanes 2–5). Dopamine and tyramine were used as substrates to stain for diphenolase or monophenolase respectively. SDS (10 mM) or trypsin (1 mg/ml) was added to activate the tyrosinase. Lanes 2–5 were stained with the following combinations of substrate and activator: lane 2, dopamine/SDS; lane 3, tyramine/SDS; lane 4, dopamine/trypsin; lane 5, tyramine/trypsin. Haemocyanin only exhibits weak diphenolase activity when activated with SDS, whereas oligomeric tyrosinase possesses monophenolase activity as well as diphenolase activity when activated with SDS or trypsin. Ty, tyrosinase; Hc, haemocyanin.

protein bands were identified as haemocyanin. Haemocyanin is the major protein component in crustacean haemolymph, constituting more than 90% of haemolymph proteins [31]. Some subunit types of haemocyanin are known to exhibit weak diphenolase activity on activation with SDS [32]. In the case of *P. elephas* only, the smaller one of the two haemocyanin subunit types exhibits a very weak diphenolase activity. On the other hand, the band with a very high molecular mass shows diphenolase as well as monophenolase activity and therefore was clearly identified as a tyrosinase. In the case of *A. leptodactylus*, six bands in the low-molecular-mass range were stained by Coomassie Blue, which are identified as haemocyanin subunits [33]. A prominent high-molecular-mass band is identified as a haemocyanin heptamer (a hexamer with an additional subunit connected via a disulphide bond). This oligomer was always observed when the 2×6 -meric *Astacus* haemocyanin was dissociated at high pH [33]. When activated with SDS, this heptamer and at least two subunit types showed diphenolase activity, as did another oligomer, which obviously occurs only in minute amounts so that it is not detected by Coomassie Blue staining. The latter band also showed monophenolase activity and was therefore identified as a tyrosinase.

Some haemocyanins have been reported to develop phenol oxidase activity upon activation with a protease such as trypsin [34]. To test the possibility that the tyrosinase is a proteolytic degradation product of haemocyanin, we used trypsin as an activator for the activity staining. For both animals, no enzymic activity of haemocyanin and its subunits was observed after treatment with trypsin (Figure 1). Therefore the possibility that the high-molecular-mass tyrosinase is a degradation product of haemocyanin was excluded.

Purification of tyrosinase

The tyrosinases in the haemolymph of *P. elephas* and *A. leptodactylus* were then purified using anion-exchange chromatography and HIC.

P. elephas

Purification was achieved in a five-step procedure (Figure 2a). Briefly, the haemolymph was applied on to an anion-exchange column and eluted with a linear gradient of NaCl to a final NaCl

concentration of 1 M. The tyrosinase-containing fractions were then rechromatographed under the same conditions to remove haemocyanin further. Then the tyrosinase-containing fractions were bound to an HIC column in the presence of 1 M $(\text{NH}_4)_2\text{SO}_4$. Elution occurred by decreasing the $(\text{NH}_4)_2\text{SO}_4$ concentration to 0 M in a linear gradient. The tyrosinase-containing fractions were then rechromatographed twice under the same conditions. The elution diagram of the last rechromatography still exhibits two distinct peaks. However, SDS/PAGE confirmed that purity had been achieved despite these two peaks, since only one protein band could be detected in all fractions containing tyrosinase activity (Figure 2c). Electron microscopy results (not shown) suggest that these two peaks occur owing to different chromatographic properties of the monomeric and multimeric forms of tyrosinase (results not shown).

A. leptodactylus

For the purification of *A. leptodactylus* tyrosinase, basically the same protocol was employed (Figure 2b). The haemolymph was applied on to an anion-exchange column. In contrast with almost all other proteins present in the haemolymph, the tyrosinase did not bind to the matrix. The bound proteins were eluted with 1 M NaCl and the tyrosinase-containing fractions were applied on to a hydrophobic interaction column in the presence of 1 M $(\text{NH}_4)_2\text{SO}_4$. Elution was achieved by lowering $(\text{NH}_4)_2\text{SO}_4$ in a linear gradient. The tyrosinase-containing fractions were then rechromatographed once more under the same conditions. The presence of only one protein band in SDS/PAGE confirmed that purity was achieved (Figure 2c).

The purity and the molecular mass of both tyrosinases from *P. elephas* and *A. leptodactylus* are shown in Figure 2(c) and the latter is determined to be approx. 71 kDa for both proteins. In both cases, only one band was detected on an SDS gel stained with silver, indicating that only one type of subunit is present.

Electron microscopy of the tyrosinases

The purified tyrosinases were negatively stained and investigated by electron microscopy. The tyrosinase molecules of both species were very similar to hexameric haemocyanin molecules (Figures 3A and 3E). The basic structural unit of arthropod haemocyanins is a hexamer made up of two stacked subunit trimers rotated by

(a)**A) Anion Exchange I** **B) Anion Exchange II** **C) Hydrophobic Interaction I** **D) Hydrophobic Interaction II** **(b)****A) Anion Exchange** **B) Hydrophobic Interaction** **Figure 2** For legend, see facing page

Figure 2 Purification of *P. elephas* (a) and *A. leptodactylus* (b) tyrosinase from haemolymph, and (c) SDS/PAGE of purified tyrosinases from *P. elephas* (A) and *A. leptodactylus* (B)

(a) Tyrosinase was purified in a five-step procedure employing IEX and HIC. Purification of the tyrosinase was monitored using a dot-blot with tyramine as substrate and activated by SDS. Panel (A): first the haemolymph was applied on to an anion-IEX column in IEX buffer. Tyrosinase was bound to the column and eluted with a linear gradient of 1 M NaCl predominantly in fraction 15. Panel (B): the tyrosinase-containing fractions 14 and 15 were rechromatographed under the same conditions, since they were still strongly contaminated by haemocyanin, which eluted later in the gradient. Panel (C): fractions 14 and 15 from panel (B) were applied on to the HIC column in HIC buffer containing 1 M $(\text{NH}_4)_2\text{SO}_4$. The bound proteins were eluted in a linear gradient with decreasing $(\text{NH}_4)_2\text{SO}_4$ concentration. Tyrosinase eluted in fractions 7 and 8, which were rechromatographed twice by HIC under the same conditions. Panel (D) shows the second rechromatography. Although two peaks elute from the HIC column in the last step, both peaks are made up of the same polypeptide, and no contamination is present that could account for the second peak (c). Electron-microscopic images suggest that the second peak is made up of tyrosinase monomers, whereas the first peak is the hexameric form of the tyrosinase (results not shown). For comparison, the elution profile of haemocyanin (— · — · —, panel C) from the HIC column is also given. The haemocyanin elutes much earlier in the gradient than tyrosinase. —, Absorbance at 280 nm; — · — · —, conductivity; · · · · ·, gradient. (b) Tyrosinase was purified in a three-step procedure by IEX and HIC. Purification of the tyrosinase was monitored using a dot-blot with tyramine as substrate and activated by SDS. Panel (A): first the haemolymph was applied on to an anion-IEX column in IEX buffer. Tyrosinase was not bound to the column and eluted predominantly in fraction 4. Most of haemolymph proteins were bound by the column and then eluted with 1 M NaCl. Panel (B): then fractions 3 and 4 of panel (A) containing the tyrosinase were applied on to the HIC column using HIC buffer containing 1 M $(\text{NH}_4)_2\text{SO}_4$. The bound proteins were eluted in a linear gradient with decreasing $(\text{NH}_4)_2\text{SO}_4$ concentration. Tyrosinase eluted in fractions 7 and 8, which were rechromatographed by HIC under the same conditions. Panel (B) shows rechromatography. For comparison, the elution profile of haemocyanin (— · — · —) from the HIC column is also given. The haemocyanin elutes much earlier in the gradient than the tyrosinase. —, Absorbance at 280 nm; — · — · —, conductivity; · · · · ·, gradient. (c) Fractions from the HIC (a, panel D; b, panel B) were run on a 7.5% gel and silver-stained. The molecular mass of the markers are given in kDa. The purified tyrosinase from both species has a molecular mass of approx. 71 kDa. OD (280 nm) = A_{280} .

45° with respect to one another around their common C3 symmetry axis [31,35]. We also observed an obvious C3 symmetry for tyrosinases (Figure 3). Only two orientations of the tyrosinase particles occurred in negative staining. Almost all tyrosinase molecules seem to be absorbed to the surface in the same orientation, with the C3 symmetry axis perpendicular to the surface. Only a very small fraction of approx. 5% was observed with the C3 symmetry axis orientated parallel to the surface. A three-dimensional reconstruction is not possible under these circumstances. To remove the noise from the electron microscopic images, we computed average images of the tyrosinase molecules from 800 and 200 single particles for *Palinurus* and *Astacus* respectively (Figures 3B and 3F). To remove irregularities due to noise that amplified during the alignment of the single molecule images, we set the C3 symmetry as a restraint during the alignment process. For comparison, a low-resolution image of a hexameric haemocyanin was used [36]. Especially when the contrast of the averaged tyrosinase images was enhanced, the images were almost indistinguishable from haemocyanin. Based on this similarity, all three domains of each of the three kidney-shaped subunits could easily be detected and identified in the tyrosinase images (Figures 3C, 3D, 3G and 3H).

Size of native tyrosinase

In addition, SEC was employed to estimate the molecular mass of the tyrosinases from both species. Owing to the minute amounts of purified tyrosinase available, only haemolymph could

be used for the experiment (Figure 4). The absorbance trace at 280 nm predominantly follows the elution of the haemocyanin, which accounts for more than 90% of haemolymph protein. In the case of *A. leptodactylus*, the haemolymph elutes as a prominent peak with a shoulder, which is identified as the covalently linked 2 × 6-mer and a hexamer [37]. The haemolymph of *P. elephas* elutes as a single peak at the same volume as the hexameric haemocyanin of *A. leptodactylus*. The elution of the tyrosinase was monitored by assaying the monophenolase activity in all fractions. Since only the tyrosinase activity of fractions was assayed, the exact elution volume of the tyrosinases within these fractions could not be determined. However, most of the activity was eluted in fraction 15, with a small portion in fraction 14. Fraction 15 corresponds to a molecular mass of 300–450 kDa. These results are in excellent agreement with the molecular mass of a hexamer made up of six subunits of 71 kDa each, resulting in a molecular mass of 426 kDa. We point out that the hexameric haemocyanin from *Palinurus*, which has essentially the same molecular mass, elutes significantly earlier from the column than the tyrosinase. This behaviour is probably due to different interactions of the haemocyanin with the gel matrix when compared with the tyrosinase.

Enzymic properties of crustacean tyrosinases

The enzymic activities of the purified tyrosinases from both crustacean species were investigated with respect to activation and time course of the activity. In arthropods, tyrosinases occur

Figure 3 Comparison of electron microscopic images of tyrosinase and haemocyanin

Electron microscopic images of negatively stained purified tyrosinase from *P. elephas* and *A. leptodactylus* are shown (**A** and **E**). Noise was reduced by averaging 800 single images for *Palinurus* tyrosinase and 200 single images for *Astacus* tyrosinase (**B** and **F**) respectively. The contrast of the reconstructed tyrosinase images was increased (**C** and **G**) to facilitate comparison with the simulated electron micrograph of *Panulirus interruptus* haemocyanin (**D**) at 2.5 nm resolution computed from crystallographic data. (**H**) The positions of the subunits and the domains within the hexamer, where ● denotes the C3-symmetry axis. (**D**) and (**H**) are reprinted from [36] © 1991 with the permission of Elsevier Science. Scale bar in (**A**) and (**E**) = 25 nm.

Figure 4 Determination of the molecular mass of the tyrosinases from *P. elephas* and *A. leptodactylus* by analytical SEC

Haemolymph was applied on to a Superose 6 HR 10/30 column and eluted with the stabilization buffer (pH 7.5) at 0.2 ml/min. The elution was monitored at 280 nm (—, *Astacus*; ---, *Palinurus*). The fractions were assayed for monophenolase activity with a dot-blot using tyramine as substrate. SDS (10 mM) was added to activate the tyrosinases for the activity staining in the dot-blot. Thyroglobulin (669 kDa), ferritin (440 kDa), alcohol dehydrogenase (150 kDa) and albumin (67 kDa) were used as marker proteins to estimate the molecular mass. OD (280 nm) = A_{280} .

as inactive proenzymes. *In vivo* activation is commonly achieved by limited proteolysis by specific serine proteases that are activated by pathogens [21]. In addition, *in vitro* experiments

showed that activation could also be achieved by fatty acids, detergents and phospholipids such as lysolecithin [29]. We investigated the activation of the purified tyrosinases by the anionic detergent SDS and the cationic detergent CPC (Figure 5). Both detergents are commonly used to activate tyrosinase *in vitro*, mimicking the activation by lipids [1,28–30]. SDS and CPC were able to activate the monophenolase as well as the diphenolase activity of our two tyrosinases and no difference in activation efficiency between the two detergents was observed. However, the kinetics were different for the two activities. Independent of the detergent used, the diphenolase activity commenced almost instantaneously (Figures 5A and 5C) after addition of the detergent, whereas the monophenolase activity showed a lag period of approx. 10 min. This lag period is typical for tyrosinases and results from the slow conversion of the met-state active sites, which are predominant in the inactive tyrosinase, into the oxy state that exhibits monophenolase activity. This conversion is not necessary for the diphenolase activity, since both the met and the oxy forms of the active site possess this specific catalytic activity [2]. To confirm that the conversion of met into oxy active sites is the reason for the lag phase in the purified crustacean tyrosinases, we tested the monophenolase activity in the presence of catalytic concentrations of diphenols (Figure 6). As expected, the lag period of monophenolase activity was totally abolished in the presence of diphenols. The turnover of catalytic amounts of diphenols quickly converts active sites in the met state into the oxy state. These results confirmed that the crustacean tyrosinases conform with the catalytic mechanism for tyrosinase activity as proposed in the literature [2,10,14]. Thus the two isolated crustacean tyrosinases behave like typical tyrosinases.

DISCUSSION

The phenol oxidases of crustaceans and insects have been the subject of detailed functional and structural studies for many years. However, most of the structural characterization has been deduced from sequence data or SDS- and native-gel electro-

Figure 5 Activation of the tyrosinase with the detergents SDS and CPC

Purified tyrosinases of *P. elephas* and *A. leptodactylus* were activated with SDS (—) or CPC (---) at a concentration of 5 mM and assayed for monophenolase (B and D) and diphenolase activity (A and C). Substrates tyramine and dopamine (1 mM) were used to assay monophenolase and diphenolase activity respectively. The assay was performed in phosphate buffer (pH 7.0) with a protein concentration of 0.92 $\mu\text{g}/\text{ml}$ for *Palinurus* and 1.22 $\mu\text{g}/\text{ml}$ for *Astacus*. Note the typical lag phase for monophenolase activity. OD (505 nm) = A_{505} .

Figure 6 Influence of catalytic amounts of diphenol on the monophenolase activity of *Palinurus* and *Astacus* tyrosinase

Tyrosinases of *Palinurus* (A) and *Astacus* (B) were activated with 5 mM SDS and incubated under the following conditions in phosphate buffer (pH 7.0): curve a, 1 mM tyramine; curve b, 2.5 μM dopamine; curve c, 1 mM tyramine and 2.5 μM dopamine. The addition of catalytic amounts of the diphenol dopamine together with the monophenol tyramine (curve c) totally abolishes the typical lag phase for the monophenolase activity that is observed when only monophenol is added (curve a). Owing to the turnover of the diphenol, met active sites are converted to the oxy state, which is capable of monophenolase activity. As a control, only catalytic amounts of the diphenol dopamine were added in (curve b), which cannot account for the increase in absorbance observed when the monophenol tyramine was also added (curve c). The assay was performed in phosphate buffer (pH 7.0) with a protein concentration of 0.46 $\mu\text{g}/\text{ml}$ for *Palinurus* and 1.26 $\mu\text{g}/\text{ml}$ for *Astacus*. OD (505 nm) = A_{505} .

phoresis. Thus the available structural information is mostly limited to the monomer. Little is known about the oligomerization state and structure of the phenol oxidase *in vivo*. The

phenol oxidase of the signal crayfish *Pacifastacus leniusculus* has been reported to exist as a tetramer based on SEC [21]. Other studies suggest that various insect phenol oxidases occur as monomers, dimers, tetramers or pentamers [12,13,20,38–43]. In addition, there is confusion concerning enzymic activity in use of the term phenol oxidase, which is used for both tyrosinases and catechol oxidases of arthropods without discriminating between them on the basis of their enzymic properties. In fact, it has only been demonstrated for three of the reported 20 phenol oxidases with known sequence that they show tyrosinase-specific activity, the monophenolase activity [11–13].

We purified tyrosinases from the haemolymph of the two decapod crustacean species *A. leptodactylus* and *P. elephas* and demonstrated that their functional properties comply fully with the enzymic mechanism proposed in the literature for tyrosinases from non-arthropods [2]. Structural characterization showed that both tyrosinases are hexamers, as are the closely related arthropod haemocyanins, but with physicochemical properties markedly different from those of haemocyanins.

Structure and physicochemical properties of arthropod tyrosinase

Electron microscopic investigations of the structures of the two purified crustacean tyrosinases revealed that native tyrosinases occur as hexamers made up of one type of subunit with a molecular mass of 71 kDa. Six subunits associate to form a hexamer closely resembling the arthropod haemocyanin hexamer.

Despite the structural similarities of tyrosinases and haemocyanins on the level of tertiary and quaternary structure and the fact that both proteins belong to the same protein superfamily, obvious differences in their physicochemical properties were revealed. These different chromatographic properties of haemocyanin and tyrosinase allowed us to rule out a possible contamination of the purified tyrosinase by haemocyanin. The surface of tyrosinases seems to be less negatively charged than that of haemocyanins, since tyrosinases exhibit only a very weak interaction with the anion-exchange matrix. In addition, the surface of tyrosinases seems to be more hydrophobic than that of haemocyanins, as indicated by its strong interaction in HIC.

Can the difference in physicochemical properties be assigned to functional properties? Haemocyanins as respiratory proteins are freely dissolved in the haemolymph at an average concentration of between 10 and 100 mg/ml. The polar and strongly negatively charged surface of the haemocyanin serves to improve the solubility and hydration of haemocyanin in the haemolymph and also to minimize interaction among haemocyanin molecules themselves and with other proteins. In addition, the negatively charged surface also helps to minimize interactions with negatively charged surfaces in the body such as the glycocalyx of cells in contact with the haemolymph. Tyrosinases are stored in haemocytes and released during the primary immune response [44]. In our experiments, the tyrosinases were probably released from the haemocytes due to the introduction of pathogens and pyrogens during the withdrawal of the haemolymph. Once released from the haemocytes and activated next to the pathogen, tyrosinases should remain in close contact with the surface of the pathogen, where the activation takes place. Thus the more hydrophobic surface combined with only low surface charge is obviously useful to improve intimate interaction with target surfaces. In this way, the different physicochemical properties of the two closely related proteins, the haemocyanins and tyrosinases, can be explained on the basis of their function in the organism.

Tyrosinase hexamers in the light of haemocyanin evolution

The present study also helps us to understand the evolutionary relationship between phenol oxidases and haemocyanins from arthropods. Phenol oxidases, as the older proteins, evolved when oxygen as a toxin had to be taken care of [9,22,45]. With time, increasing amounts of oxygen became available in the atmosphere and organisms learned to use oxygen to produce large amounts of ATP. Today most larger and complex organisms depend on a precise oxygen supply, which is guaranteed by oxygen carriers such as haemoglobin and haemocyanin. For haemocyanin, it was only a small step to evolve this function from phenol oxidases. First, phenol oxidases could already bind oxygen reversibly. Then, only a small modification in the first domain was necessary to block access to the active site for large phenolic substrates and to allow only small ligands, like oxygen, to diffuse to the active site. Secondly, the functional properties of an oxygen carrier, the co-operativity and allostery, could easily be developed from the phenol oxidase by only small structural modifications: the phenol oxidase multimer already had an intrinsic flexibility that was the prerequisite to developing different conformations. This flexibility was due to the fact that tyrosinase exists as an inactive proenzyme that is activated by proteolysis or compounds like lipids. Proteolytic cleavage of the first domain was observed in the case of the phenol oxidase from the crayfish *P. leniusculus* [11]. Structural comparison of phenol oxidases with haemocyanin revealed that cleavage of the first domain removes a phenylalanine residue that is conserved among phenol oxidases and haemocyanins from arthropods [34]. This phenylalanine residue normally blocks access to the active site and, after its withdrawal, guarantees the correct orientation of substrate at the active site [46]. An equivalent movement of the first domain relative to the second and third domains has been observed for the oxy and deoxy conformational states of haemocyanin by crystallographic analysis [17,47]. However, in the case of haemocyanin, the small movement of the first domain between oxy and deoxy states is usually not sufficient to open up access for larger substrates to the active site. This supports the hypothesis that haemocyanins are essentially phenol oxidases carrying the same active site, but inaccessible to substrates. Interestingly, in some ancient chelicerates, haemocyanin can still be activated to act as a phenol oxidase, showing that the inherent property to act as a phenol oxidase is still present even when haemocyanins function as oxygen carriers most of the time [30,32,48]. Thus arthropod phenol oxidase and haemocyanin prove to be good examples of a protein family, that has developed new functions during evolution.

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