Isolation and characterization of haemoporin, an abundant haemolymph protein from *Aplysia californica*

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In the present study, we show the isolation and characterization of the protein haemoporin, which constitutes the second most abundant protein fraction in the haemolymph of the marine gastropod *Aplysia californica*. Although *Aplysia* is commonly used to investigate the molecular basis of learning, not much is known about the proteins in its haemolymph, which is in contact with the neurons owing to the open circulatory system of molluscs. In the native state, haemoporin is a macromolecular complex forming a cylinder with a central solvent-filled pore. The native complex most probably is a homopentamer made up from 70 kDa subunits with a molecular mass of 360 kDa and a sedimentation coefficient of 11.7 S. Prediction of the secondary structure by CD spectroscopy revealed that haemoporin contains 36 % α -helices and 19 % β -strands. An absorption band in the 300–400 nm region indicates that haemoporin probably contains a bound substance. Haemoporin also contains a below average amount of tryptophan as evident from absorption and fluorescence spectra.

The specific absorption coefficient at 280 nm ($a_{280\text{ nm}, 1\text{ mg/ml}}$) varies between 0.42 and $0.59\text{ l}\cdot\text{g}^{-1}\cdot\text{cm}^{-1}$ depending on the method. The function of the protein is not yet known, but there are structural parallels between haemoporin and a pore protein reported previously in the haemolymph of another marine gastropod *Megathura crenulata*. The alanine-rich N-terminal sequence (AAVPEAAAEATAEAPVSEF) is unique among protein sequences and indicates an α -helical structure. Whereas one side of the helix is hydrophobic and faces the interior of the protein, the other side contains a glutamic cluster, which may form the channel of the pore in the quaternary structure. Thus both proteins might belong to a new class of haemolymph proteins present in the haemolymph of marine gastropods.

Key words: *Aplysia*, channel protein, helix, haemolymph, polyaniline, pore protein.

INTRODUCTION

Haemolymph in marine invertebrates performs many important tasks as it distributes oxygen, nutrients, hormones, components of the immune system and often also vitellogenic and storage proteins. Metabolic wastes and harmful substances are transported to the excretory organs by haemolymph. In many species, specialized transport proteins facilitate the distribution of such substances. The major protein fraction in the haemolymph of many marine gastropods is the giant oxygen carrier haemocyanin, but little is known about other haemolymph proteins [1].

The ionic composition of the haemolymph of *Aplysia* is similar to seawater [2]. Besides haemocyanin, other proteins such as acetylcholinesterase, haemagglutinin and erythrocurorin have been reported as haemolymph constituents in *Aplysia californica* [3]. Up to six prominent protein bands, with molecular masses from 13 to 300 kDa, have been described in the haemolymph depending on the age of the animal [4]. When the animals are fed on a diet of red algae, the haemolymph has a red colour due to the red algal pigment phycoerythrobilin, which is transported from the digestive gland to the ink gland via the haemolymph [5]. Several hormones and neuropeptides that alter the behaviour of *Aplysia* have also been found in the haemolymph [6,7].

Although *A. californica* is a model animal for neurophysiology, very little information is available about other physiological processes in these animals. This lack of details limits the description of how nerve function is affected by the internal physiological state of the animal. It seems especially useful to pay attention to the composition of the haemolymph, since in these

animals, with an open circulatory system, haemolymph is in contact with all neurons. A previous study already reported that haemolymph and the acetylcholinesterase contained therein is neurotrophic to *Aplysia* neurons in culture [8,9].

We describe a new protein from *A. californica* which constitutes the second most abundant protein fraction in the haemolymph besides haemocyanin. The newly described protein was purified and characterized and we have called it 'haemoporin' because it is a cylindrical protein with a central pore.

EXPERIMENTAL

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

Preparation of haemolymph

Laboratory cultured *A. californica* (Gastropoda/Opisthobranchia/Anaspidia) were obtained from the NCRR National Resource for *Aplysia* at the University of Miami (Miami, FL, U.S.A.). Animals were kept in circulating seawater at 14 °C and fed ad libitum with red seaweed (*Gracilaria* sp.). Animals having an average weight of 50 g were used for the experiments. Haemolymph was collected by puncturing the pedal blood sinus with a syringe needle. Approx. 10 ml of haemolymph was collected from each animal. Haemocytes and cellular debris were removed by centrifugation at 10 000 g for 20 min at 4 °C. The haemolymph was stored at –20 °C until further use.

Abbreviation used: GdnHCl, guanidine hydrochloride.

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Protein purification

Cell-free haemolymph was concentrated in centrifugal filters (Biomax 30K; Millipore, Eschborn, Germany) to a protein concentration of approx. 10 mg/ml. Then 2 ml of concentrated haemolymph was applied to a Sephacryl S-300 16/60 HR size-exclusion column (Bio-Rad, Munich, Germany) and eluted with stabilization buffer (100 mM Tris/HCl/100 mM NaCl/15 mM CaCl_2 /15 mM MgCl_2 , pH 7.8) at a flow rate of 0.6 ml/min at room temperature (25 °C). Elution of proteins was monitored by measuring the absorbance of the eluate at 280 nm in a flow cell. For analytical size-exclusion chromatography, a Superose 6 HR 10/30 column (Pharmacia, Freiburg, Germany) was used at a flow rate of 0.2 ml/min with stabilization buffer at room temperature.

Electrophoresis

SDS/PAGE was performed using the system of Laemmli [10] as modified by See and Jackowski [11]. Before electrophoresis, the samples were denatured at 100 °C for 5 min in sample buffer containing SDS and 2-mercaptoethanol. Wide Molecular Weight Standard (Sigma) was used as the molecular-mass standard. Gels were stained using the silver stain method of Blum et al. [12].

Protein sequencing

Protein bands from SDS/PAGE [10 % (w/v) gel] were transferred to a Problot PVDF membrane in 10 mM 3-(cyclohexylamino)-1-propanesulphonic acid buffer (pH 11.0) at 12 V for 4 h. The membrane was stained by Ponceau S (0.1 % in 1 % acetic acid) to locate the protein bands and de-stained in water. The protein band was excised from the membrane and sequenced on a Procise 491 protein/peptide sequencer (Applied Biosystems, Foster City, CA, U.S.A.) using Edman degradation chemistry.

Analytical ultracentrifugation

Sedimentation velocity and sedimentation equilibrium experiments were performed in a Beckman Optima XL-I analytical ultracentrifuge (Palo Alto, CA, U.S.A.) using an An-50Ti rotor. Velocity runs were conducted at 20 °C at 25 000 rev./min, using 1.2 mg/ml protein in stabilization buffer. Sample cells with 12 mm double-sector charcoal-filled epon centrepieces and quartz windows were used for all experiments. The absorbance of the cells was scanned at 280 nm every 5 min during the run. Velocity data were analysed using the method of van Holde and Weischet and the finite-element analysis of Demeler and Saber as implemented in the program ULTRASCAN 5.0 [13–15]. Sedimentation equilibrium runs were conducted at 20 °C at 7000, 9000 and 11 000 rev./min, using protein concentrations between 0.48 and 1.43 mg/ml in the stabilization buffer. Sample cells with six-channel charcoal-filled epon centrepieces and quartz windows were used for all experiments. Samples were run for at least 24 h at each speed. The absorbance of the cells was scanned at 280 nm and it was assumed that equilibrium was achieved when scans taken at intervals of 2 h showed no significant change. Equilibrium data were analysed with the program ULTRASCAN 5.0, employing the global-fit routine to integrate data taken at different protein concentrations and speeds [16,17]. In all calculations, a partial specific volume of 0.72 cm³/g was assumed as commonly used [18].

Electron microscopy

Protein samples were negatively stained with 2 % (w/v) uranyl-acetate on carbon support film employing the 'droplet technique' [19]. Images were taken on a Zeiss 900 transmission electron microscope at $\times 50\,000$ magnification and recorded on Kodak 4489 film (Kodak, Germany).

Absorption spectroscopy

Absorption spectra were recorded with a U-3000 UV/Vis spectrophotometer (Hitachi, Tokyo, Japan) in quartz cuvettes with an optical pathlength of 1 mm for the 190–300 nm range and 10 mm for the 250–700 nm range. Spectra were recorded at 20 °C. Molar and specific absorption coefficients at 205 and 280 nm were calculated using the empirical method of Scopes [20]. All protein concentrations given in the paper were determined using a calculated $a_{280\text{ nm}, 1\text{ mg/ml}}$ of 0.42 litre $\cdot$ g⁻¹ $\cdot$ cm⁻¹.

Protein assay

To measure the specific absorption coefficient at 280 nm, protein concentrations were determined using the method of Bradford as provided in the Bio-Rad protein assay kit (Bio-Rad) using BSA as a standard. The microassay method was used for the experiments [21].

Acetylcholinesterase assay

Acetylcholinesterase activity in the eluted fractions was measured by the method of Ellman [22]. Briefly, 100 μ l of each fraction was mixed with a reaction solution (0.24 mM Ellman's reagent, 0.47 mM acetylthiocholine, 50 mM phosphate buffer, pH 7.2) and the absorbance was measured at 405 nm at 20 °C.

Fluorescence spectroscopy

Fluorescence excitation and emission spectra were recorded with a F-4500 fluorimeter (Hitachi) in quartz cuvettes with a pathlength of 10 mm at 20 °C. To measure the spectra of denatured protein, the protein samples were incubated in 4.0 M GdnHCl (guanidine hydrochloride) in stabilization buffer for 90 min at 60 °C and the spectrum was measured afterwards at room temperature. Then the sample was left for 24 h at room temperature and heated to 100 °C for 1 h before a second measurement at room temperature.

CD spectroscopy

CD spectra were recorded with a CD6 instrument (Jobin-Yvon, Longjumeau, France) in quartz cuvettes of 1 mm optical pathlength at 20 °C. Secondary structure was predicted with the programs SELCON3, CDSSTR and CONTINLL from the CDPro program suite [23]. The appropriate reference set of proteins was determined with the program CLUSTER included in the CDPro program suite.

RESULTS

The protein composition of haemolymph from *A. californica* was analysed by preparative size-exclusion chromatography (Figure 1). The giant oxygen carrier haemocyanin eluted in the first peak and was identified by its subunits in SDS/PAGE. As

Figure 1 Purification of *Aplysia* haemoporphin

(a) Preparative size-exclusion chromatography. Haemolymph was applied to a S-300 size-exclusion column and eluted with stabilization buffer at a flow rate of 0.6 ml/min. (b) Fractions of the size-exclusion chromatography were analysed by SDS/PAGE (7.5% gel) and stained with silver. Haemocyanin eluted in the first protein peak, whereas the second peak was from *Aplysia* haemoporphin made up from 70 kDa subunits.

reported by several authors, the haemocyanin in this peak is the typical didecamer and multidecamers with molecular masses of more than 8 MDa are also observed [3,24,25]. The 450 kDa haemocyanin subunits are made up from 7 or 8 functional units with one oxygen-binding type 3 copper-active site on each functional unit [26]. Owing to their mass, the haemocyanin subunits did not migrate far into the gel. The second protein peak was composed of subunits with a molecular mass of 70 kDa. The 70 kDa protein is the second largest protein fraction in the haemolymph, as already reported by Srivatsan et al. [4], and its abundance decreases with age. The third protein peak was composed of subunits smaller than 29 kDa (results not shown) and was not analysed further. Fractions 20–24 of the protein with the 70 kDa subunit protein were concentrated and rechromatographed once under the same conditions to remove further contaminations by the adjacent protein peaks. Afterwards only one band at 70 kDa was observed in the sample used for subsequent experiments.

The 20 N-terminal amino acids of the 70 kDa protein band were determined by Edman degradation and yielded the sequence AAVPEAAAEATAEAAPVSEF. A database search found no identical or homologous sequences, which was surprising for

Figure 2 Sedimentation velocity analysis of purified haemoporphin

A preparation of purified haemoporphin was centrifuged at 25000 rev/min at 20 °C. Protein concentration in the cell was 1.2 mg/ml in stabilization buffer. (a) The absorbance at 280 nm along the length of the cell was recorded every 5 min. (b) van Holde/Weischet extrapolation plot. (c) Distribution plot. More than 80% of the protein sedimented with 11.7 S, whereas only a small fraction sedimented either faster (15–40 S) or slower (3–8 S).

such a long peptide. Interestingly, the N-terminal sequence of the 70 kDa protein contains a remarkably high percentage (almost 50%) alanine residues.

To determine the aggregation state of the native protein complex made up from 70 kDa subunits, the sample was analysed by analytical ultracentrifugation. van Holde–Weischet analysis of sedimentation velocity data revealed that >80% of the native protein sedimented with a sedimentation coefficient of 11.7 S, whereas a small fraction either sedimented faster with a sedimentation coefficient of 15–40 S or slower with 3–8 S (Figure 2). The molecular mass of the component sedimenting with 11.7 S was analysed using finite-element analysis and a model for three non-interacting components. The molecular mass for the 11.7 S protein was found to be 360 kDa. In addition, sedimentation equilibrium experiments yielded a molecular mass of 355 kDa for the native complex (Figure 3). Therefore we conclude that the native protein complex present in the haemolymph is a pentamer made up by 70 kDa subunits. The molecular mass of the small fraction of slower and faster sedimenting proteins could not be determined by this method because their quantity was not sufficient. Most probably, the smaller component is the free 70 kDa subunit, whereas the faster sedimenting components are higher aggregates of the same protein, since this rechromatographed sample contained no contamination by other proteins as judged by SDS/PAGE (see above).

In addition, molecular mass was also determined by analytical size-exclusion chromatography on a column calibrated with arthropod haemocyanins of 1800, 900 and 450 kDa as molecular-mass markers (Figure 4). Here the protein complex made up of 70 kDa subunits eluted at the same time as haemocyanin from *Pallinurus elephas*, indicating a molecular mass of 450 kDa. Consequently, the native oligomer should be a hexamer. Electron microscopic analysis of the protein sample revealed that the sample was homogeneous and made up of cylindrical particles with a side length and diameter of 13 nm each (Figure 5). There were two predominant orientations of the protein complex. Top views along

Figure 3 Sedimentation equilibrium analysis of purified haemoporin

A preparation of purified haemoporin was centrifuged at 20 °C at 7000, 9000 and 11 000 rev./min (rpm) until equilibrium was achieved. Protein concentrations between 0.48 and 1.43 mg/ml in stabilization buffer were used. For the sake of clarity, only the results for 0.48 mg/ml haemoporin at (a) 7000 rev./min, (b) 9000 rev./min (c) 11 000 rev./min are shown. The results obtained for all protein concentrations and speeds were fitted globally and a molecular mass of 355 kDa was obtained.

the rotation axis of the cylinder showed a disc-shaped particle with a central pore of 3 nm diameter that is accessible to solvent and thus filled with stain. The side view, which corresponded to a perpendicular orientation, displayed a square particle.

The fractions of the analytical size-exclusion chromatography were assayed for acetylcholinesterase activity, since it was suggested in the literature that the 70 kDa protein in the haemolymph was identical with the acetylcholinesterase in the haemolymph [4]. This activity could obviously be excluded since all the acetylcholinesterase activity eluted together with the haemocyanin fraction at higher molecular masses (Figure 4).

The secondary structure of the protein was investigated by CD spectroscopy in the far UV region (Figure 6). The spectrum indicates that it contains α -helices as well as β -strands. The following proportions of secondary structure elements were predicted with the program CDSSTR: 20% regular α -helix, 16% distorted α -helix, 12% regular β -strand, 7% distorted β -strand, 19% turns and 26% unordered. The programs SELCON3 and CONTINLL, which use different algorithms for fitting the experimental data, produced similar results.

The protein is colourless and shows no absorption bands in the visible range (Figure 7a). In the UV region, the absorption maximum of the aromatic amino acids was at 277 nm and an additional broad absorption band in the 300–450 nm range was also observed. This absorption band could easily be attributed to contaminations of the sample by oxygenated haemocyanin, which exhibits an absorption band at 340 nm [27]. However,

Figure 4 Analytical size-exclusion chromatography of *Aplysia* haemolymph

For analytical size-exclusion chromatography, *Aplysia* haemolymph was applied to a Superose 6 column and eluted with stabilization buffer at 0.2 ml/min. Absorbance at 280 nm was recorded continuously in the eluate. To monitor the elution of haemocyanin specifically, the most abundant protein in *Aplysia* haemolymph with its specific absorbance at 340 nm was measured in each fraction. The column was calibrated with arthropod haemocyanins of *Eurypelma californicum* (1.8 MDa), *Astacus leptodactylus* (900 kDa) and *P. elephas* (450 kDa) as molecular-mass markers. The *Aplysia* haemoporin (grey shading) eluted at the same time as the haemocyanin of *P. elephas*. Note that all proteins with a molecular mass > 1000 kDa elute in the single front peak in Figure 1 owing to limitations of the column. In addition, acetylcholinesterase activity was assayed in the fractions (Δ). All of the acetylcholinesterase activity eluted in the high-molecular-mass fractions and not with the haemoporin.

Figure 5 Electron microscopy of haemoporin

(a) Electron microscopic image of purified haemoporin negatively stained with uranyl acetate. The scale denotes 100 nm. (b–d) Close-up views of haemoporin particles in the top-view orientation along the cylinder axis. The outer diameter of the protein is 13 nm, whereas the pore with a diameter of 3 nm is filled with the solvent. Scale bar, 10 nm. (e) Schematic representation of haemoporin.

this possibility can be ruled out since SDS/PAGE confirmed that no contamination with haemocyanin was present. Thus the absorption has to be attributed to some unknown substance or cofactor bound to the protein. From the far-UV spectrum (Figure 7b), $a_{205 \text{ nm}, 1 \text{ mg/ml}} = 28.64 \text{ litre} \cdot \text{g}^{-1} \cdot \text{cm}^{-1}$ and $a_{280 \text{ nm}, 1 \text{ mg/ml}} = 0.42 \text{ litre} \cdot \text{g}^{-1} \cdot \text{cm}^{-1}$ were determined by the method of Scopes [20]. Assuming a molecular mass of 70 kDa, molar absorption coefficients $\epsilon_{205 \text{ nm}} = 2004 \times 10^6 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ and $\epsilon_{280 \text{ nm}} =$

Figure 6 CD spectrum of purified haemoporphin

CD spectrum of purified pore protein in the far-UV region. Protein concentration was 0.32 mg/ml in 50 mM phosphate buffer (pH 7.5) in a 1 mm cuvette. A mean residue mass of 0.11 kDa was assumed. To predict the contents of secondary-structure elements, the experimental data (●) were fitted with the program CDSSTR (○). Haemoporphin contains approx. 36 % α -helices and 19 % β -strands.

Figure 7 Absorption spectra of purified haemoporphin

Absorption spectra were taken in (a) the 250–700 nm region in stabilization buffer at a haemoporphin concentration of 1.3 mg/ml in a 1 cm cell and (b) in the 190–300 nm region in 50 mM phosphate buffer (pH 7.5) at a haemoporphin concentration of 0.32 mg/ml in a 1 mm cell. The maximum absorption in the aromatic region was at 277 nm. Also an absorption band in the 300–400 nm range was present. In (b), the spectrum was magnified $\times 10$ in the range of 250–300 nm.

29743 litre $\cdot$ mol $^{-1}$ $\cdot$ cm $^{-1}$ were calculated. To exclude the possibility that compounds responsible for the absorption band in the 300–450 nm region might also absorb in the far-UV region and thus influence the determination of the absorption coefficient by the method of Scopes, the dye-binding procedure of Bradford was used as an independent method [21]. Using this method,

Figure 8 Fluorescence spectra of purified haemoporphin

Emission spectra of native (-----) and denatured protein (—, 90 min;, 24 h) were recorded for excitation at (a) 280 and (b) 295 nm. The maximum emission of haemoporphin when excited at 280 nm was at 302 nm for native and denatured protein in both cases. When tryptophan residues were excited selectively at 295 nm, the emission maximum red-shifted from 322 nm in the native state to 347 nm in the denatured state after 90 min of incubation and later to 351 nm after 24 h of incubation. (c) Excitation spectra were recorded for emission at 302 nm (—) and 322 nm (-----). For both wavelengths, the maximum excitation was at 279 nm. Protein concentration was 0.261 mg/ml in stabilization buffer in all experiments, except for those with denatured protein where the protein concentration was 0.0871 mg/ml. For easier comparison, the emission curves at 0.0871 mg/ml were scaled up by a factor of 3 to match the higher concentration.

$a_{280\text{ nm}, 1\text{ mg/ml}}$ of $0.591\text{ l} \cdot \text{g}^{-1} \cdot \text{cm}^{-1}$ was obtained. The rather low specific absorption coefficient in the aromatic region at 280 nm obtained with the two independent methods indicates that the protein contains a lower than average amount of aromatic amino acids.

The aromatic amino acids of the protein were characterized further by fluorescence spectroscopy (Figure 8). When tyrosine, phenylalanine and tryptophan were excited at 280 nm, the emission spectrum was similar to that of tyrosine with a fluorescence maximum at 302 nm (Figure 8a). The dominance of the fluorescence spectrum by tyrosine residues implies that only very few or no tryptophan residues are present in the protein. When

tryptophan was excited selectively at 295 nm, a fluorescence maximum at 322 nm was noted, confirming that tryptophan residues were present (Figure 8b). Denaturation of the protein in 4 M GdnHCl at 60 °C for 90 min results in a red shift of the emission maximum of tryptophan residues to 347 nm. Additional denaturation for 24 h in 4 M GdnHCl and heating to 100 °C for 1 h yields a further small shift to 351 nm at 20 °C. This total exposure of buried tryptophan residues to the polar solute strongly indicates the complete unfolding of the protein, as was confirmed by CD spectroscopy (results not shown) [28]. However, tryptophan contribution to overall fluorescence is very small, since the red shift of tryptophan fluorescence in the denatured protein is barely noticeable when all aromatic amino acids are excited at 280 nm, leaving the emission maximum unchanged at 302 nm (Figure 8a). Fluorescence excitation spectra, when monitoring emission at 302 or 322 nm, exhibited a maximum excitation at 279 nm (Figure 8c).

DISCUSSION

The second most abundant protein fraction after haemocyanin in the haemolymph of the commonly used species *A. californica* was first described by Srivatsan et al. [4] in a study on the effect of aging on haemolymph protein composition. They reported it to be a 77 kDa protein, but besides its molecular mass, nothing was known about its structure in the native state. Because of the pore-like nature of the protein (Figure 5), we propose that the protein is similar to the pore protein reported for the keyhole limpet (*Megathura crenulata*) and we specifically propose the name *Aplysia* haemoporin [29].

Using SDS/PAGE, we found the subunit molecular mass to be 70 kDa. The spatial dimensions of the purified protein in electron microscopic images suggested that the protein is a multimeric macromolecular complex in the native state. We investigated the aggregation state of the protein with analytical ultracentrifugation and size-exclusion chromatography. A sedimentation coefficient of 11.7 S and a molecular mass of approx. 360 kDa were obtained from the sedimentation velocity as well as the equilibrium experiments. In contrast with these results from analytical ultracentrifugation, the size-exclusion chromatography suggested a molecular mass of 450 kDa. Thus the protein could either be a pentamer or a hexamer from the 70 kDa subunits. However, the method best suited to determine molecular mass is analytical ultracentrifugation. On the other hand, the elution behaviour in size-exclusion chromatography is only indirectly dependent on the molecular mass, but more on hydrodynamic properties of the molecule like the radius of gyration. In globular proteins, this is normally directly related to the molecular mass. The higher molecular mass determined in analytical size-exclusion chromatography is caused, most probably, by the fact that the dimensions of the pore protein, with a diameter and length of both 13 nm, are a little longer than that of the nearly globular haemocyanin from *Palinurus elephas* which has a molecular mass of 450 kDa. Consequently, the pore protein elutes at the same time or a little earlier than haemocyanin even though its molecular mass is smaller. This behaviour on the column is therefore in full agreement with the observations made in electron microscopy. The pore protein is less densely packed with molecules than the haemocyanin because of the pore. Thus we conclude that the most likely native structure of the pore protein is a homopentamer of 70 kDa subunits, since only one band was revealed by SDS/PAGE. Prediction of secondary-structure elements revealed that the protein contains approx. 36% α -helix and 19% β -strands.

For several reasons, we believe that the *Aplysia* haemoporin is related to a 350 kDa pore protein observed in the haemolymph

of the keyhole limpet *M. crenulata* and both proteins belong to the same class of haemolymph proteins [29]: (1) both are of cylindrical shape and possess a solvent-filled pore in their middle, (2) both share the same molecular mass and sedimentation coefficient, and (3) they occur as the second most abundant protein fraction in the haemolymph of marine gastropods. However, there is an important structural difference between the two proteins: although they have the same overall mass and general structure, Harris and Markl [29] found the *Megathura* protein to be a decamer of 35 kDa subunits, whereas we found a pentamer of 70 kDa subunits. Harris and Markl [29] described a quaternary structure of two cylindrical pentamers with a 5-fold symmetry axis in the pore concatenated end-to-end along the symmetry axis. We were not able to determine the spatial arrangement of subunits in the cylinder of the *Aplysia* haemoporin. To explain these differences, we propose a gene duplication in the *Aplysia* haemoporin which may have led to a concatenation of two 35 kDa subunits to form a 70 kDa subunit. Alternatively, these subunits may be covalently linked to each other post-translationally. In any case, SDS and the reducing conditions in SDS/PAGE did not succeed in breaking up the proposed dimer excluding a disulphide bridge.

The N-terminal amino acid sequence was not similar or homologous to any known protein or class of proteins. Therefore no structural or functional insight into the possible native function of haemoporin could be gained from the N-terminal sequence. The obvious lack of similarity could indicate that the pore protein is, probably, a member of a still unknown class of proteins. Alternatively, since polymorphism at the N-terminus can be observed in many known protein classes, *Aplysia* haemoporin could be a modified member of a known protein class. It should be noted that the N-terminal sequence contains a very high proportion of alanine residues and thus is quite hydrophobic. The amino acid composition and the distribution pattern of hydrophilic and hydrophobic amino acids suggest that the N-terminus of *Aplysia* haemoporin forms an α -helix. The boundaries of the α -helix are marked by two proline residues (Pro⁴ and Pro¹⁶), which are known to be favourable for an α -helix formation [30]. The N-terminal sequence contains 11 amino acids between the two proline residues of which seven are alanine residues and three are glutamic residues. Both alanine and glutamic residues have very high intrinsic helix propensities [31–34]. Furthermore, alanine is one of the two amino acids that has a helix propagation factor > 1 and thus polyalanine sequences prefer an α -helical conformation even without helix-stabilizing side-chain interactions, as reported by Chakrabarty et al. [33]. Therefore the haemoporin sequence should form an α -helix with three turns. A closer look at the spatial arrangement of the amino acids reveals that the α -helix is amphiphilic with the hydrophobic alanine residues on one side and the negatively charged glutamic residues on the other (Figure 9). The hydrophilic side is made up from the glutamic residues forming one line almost parallel to the α -helix axis. We propose that the negatively charged glutamic residues of the α -helix are forming the central pore, and subsequently a binding site for positively charged ions or substances, as discussed for porin proteins [35]. This idea would be supported by the electron microscopic images where the uranyl cations are fixated in the central pore as indicated by the darker stain (Figure 5).

Potential functions of haemoporin

Can we infer the function of *Aplysia* haemoporin from our other data? An absorption band is present in the spectrum in the range between 300 and 450 nm, which clearly cannot be attributed to a haemocyanin contamination. Thus it must originate either from a substance bound to the protein or from an active site. The nature

Figure 9 Secondary structure of the N-terminal sequence

The N-terminal sequence most probably forms an α -helix. Two typical helix-breaker amino acids, Pro⁴ and Pro¹⁶, mark the boundary of the α -helix that consists of 13 amino acids and forms three turns. The helix is amphiphilic, since charged (Glu⁵, Glu⁹ and Glu¹³) and hydrophobic amino acids are ordered in a pattern such that all glutamic residues form a negatively charged strip along the helix. (a) Helical wheel representation. Charged amino acids are boxed. (b) Side view of the helix. The glutamic residues (●) form a negatively charged strip on the helix surface (grey shading).

of the bound substance is unknown, but it is possible that the protein could act as a transport protein for a substance with poor solubility such as lipids or hormones, since lipoproteins are commonly present in the haemolymph of many vertebrates and invertebrates [36]. Haemolymph proteins often are storage proteins that store amino acids during development and release them once a certain developmental stage is reached. As a common example, arylphorins can be found in high abundance in the haemolymph of insects as storage proteins for aromatic amino acids [37]. We believe we can rule out at least that function for *Aplysia* haemoporin, since its absorption and fluorescence characteristics indicate that it contains a below average amount of aromatic amino acids. In the past, it has been suggested that the protein in the haemolymph of *Aplysia* is identical with the haemolymph acetylcholinesterase [4]. However, we were able to rule out this possible function since no acetylcholinesterase activity was found associated with this haemoporin.

A possible clue that the haemolymph protein might play a role during development was observed by Srivatsan et al. [4], who found that the concentration of haemoporin decreases from young to old animals, but at the same time the concentration of the oxygen transporter haemocyanin increases. However, a possible role during development is not yet known, but at least it seems improbable that it is a vitellogenin since the protein is most abundant in young and not sexually mature animals.

Aplysia haemoporin shows interesting structural parallels to a pore protein described in the haemolymph of the keyhole limpet *M. crenulata* and it seems probable that the proteins of both species belong to the same class of mollusc haemolymph proteins [29]. Thus their function should be similar too. A possible role of the pore protein in the immune system of the keyhole limpet was considered, but experiments showed that it at least lacked agglutinating activity against erythrocytes [29]. However, more interestingly, the pore protein of *Megathura* possesses phospho-

tidylcholine degradation activity. On the basis of these observations, it was suggested that this property could be correlated to the ability of the protein to get inserted into a lipid bilayer and form a pore [29]. Indeed, earlier studies have reported pores of a similar size which were associated with an increase in ion conductance in lipid bilayers but were thought to originate from dissociated keyhole limpet haemocyanin [29,38,39].

In conclusion, we describe the second most abundant haemolymph protein in the gastropod mollusc *A. californica* to be a pentameric protein made from a single subunit type with an obvious pore-like structure, and call it *Aplysia* haemoporin. Its function and relatedness to known families of proteins remains to be elucidated.

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