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Astaxanthin binding and structural stability of the apple snail carotenoprotein ovorubin

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Abstract

Ovorubin (OR) is the major perivitellin of the eggs of *Pomacea canaliculata*. The astaxanthin (ASX) binding and structural stability of OR were investigated by fluorescence spectroscopy and circular dichroism (CD). The apo-OR (without astaxanthin) shows a single, high affinity binding site for ASX ($K_D = 0.5$ μM). The quenching of tryptophan fluorescence by ASX indicates that about 22% are near the carotenoid-binding site in a non-polar environment, as indicated by tryptophan resonance energy transfer to the ligand. Secondary structure ($\alpha + \beta$) was virtually not affected by cofactor removal. Holo-OR shows unusually high thermal stability. The removal of ASX does not affect the thermal or chemical stability of the quaternary structure. In conclusion, although subtle changes were observed, ASX is not essential for OR stability, unlike most invertebrate carotenoproteins. This supports the idea that OR plays an important physiological role in the storage, transport and protection of carotenoids during snail embryogenesis.

Keywords: Perivitellin; Gastropod; Mollusc; Carotenoid; Secondary structure; Thermal stability

The golden apple snail *Pomacea canaliculata* (Gastropoda: Ampullariidae) is a freshwater South American species, recently introduced in Asia where it became a pest of rice crops and taro [1]. An accessory gland of the female reproductive tract called albumen gland, synthesises and secretes the perivitellin fluid (PVF)[1] surrounding embryos in fertilised eggs that is mainly composed of polysaccharides and complex glyco-lipo-carotenoproteins called perivitellins [2,3].

Most invertebrate species present coloured eggs as a result of the formation of complexes between carotenoids non-covalently attached to either proteins, glycoproteins or glycol-lipoproteins [4,5]. In particular *P. canaliculata* egg carotenoids are bound to three perivitellins called ovorubin, PV2 and PV3 [2], which are responsible for the characteristic pink reddish pigmentation of eggs first described by Comfort [6] and Cheesman [7]. The major egg carotenoprotein is ovorubin, which represents 60% of the total PVF protein content [8]. It is an oligomer with an apparent molecular mass of 300 kDa composed of three subunits of ca. 28, 32, and 35 kDa. It is a very high-density glyco-lipo-carotenoprotein (VHDL) whose ligands are phospholipids, sterols, and carotenoid pigments and it is highly glycosilated [2,9]. Ovorubin carotenoids are astaxanthin (ASX, 3,3′-hydroxy-β-β-carotene-4,4′-dione) in its free, monoester and diester forms [10]. The cofactor ASX shows a strong antioxidant protection against oxidative damage in eggs, but only when it is not attached to ovorubin [10]. Therefore, ovorubin combines the properties of several egg proteins such as an energetic and structural

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[1] *Abbreviations used:* OR, ovorubin; ASX, astaxanthin; CD, circular dichroism; PVF, perivitellin fluid; VHDL, very high-density glyco-lipo-carotenoprotein; PAGGE, polyacrylamide gradient; GnHCl, guanidinium hydrochloride; hASA, hydrophobic accessible surface area.

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source for embryo development [11], a protease inhibitor [12], an antioxidant carrier which protects the pigment from oxidation in the PVF and protection of embryos from photoxidative damage [10]. Except for the detailed studies on crustacyanin, the lobster carapace carotenoprotein, there is little information on the structure and stability of this interesting group of carotenoid-binding proteins in invertebrates and virtually no information in molluscs [13,14]. Previous studies have shown that after complete removal of ASX, the OR particle remains soluble in the aqueous medium, maintaining the capacity for ASX transport [10]. However, it was not clear if ASX contributes to protein stabilisation in the OR oligomer, as could be expected from the studies of most invertebrate carotenoproteins [15]. In this work the carotenoid binding and the structural perturbation by temperature and chaotropic agents of holo-OR and OR without astaxanthin (apo-OR) were studied by several techniques, including fluorescence spectroscopy and circular dichroism (CD).

Materials and methods

Ovorubin isolation and purification

Just-layed eggs collected near La Plata, Argentina were homogeneized and centrifuged sequentially to obtain a 100,000*g* supernatant, as previously described [11]. Ovorubin was purified from the supernatant by ionic exchange and size exclusion HPLC as described elsewhere [8]. Purification was checked by native electrophoresis, and no contaminant proteins or peptides were detected.

Preparation of apo-OR

A solution of ovorubin (10 mg/ml) in 20 mM Tris–HCl buffer, pH 7.4 was cooled down to 0 °C and extracted with ice–cold acetone. The precipitated protein was centrifuged, dissolved in ice–cold water and reprecipitated under the same conditions, yielding an almost colourless protein. This pellet was redissolved in water and centrifuged at 10,000*g*. The integrity of the apo-OR was checked by PAGGE.

Gel electrophoresis

Total proteins were measured by the method of Bradford [16]. Non-dissociating electrophoresis was performed by a 4–20% polyacrylamide gradient (PAGGE) [17,18]. Gels were stained with Coomassie Brilliant Blue R-250 (Sigma Chemical Co, St. Louis, USA).

Fluorescence spectroscopy

Fluorescence spectra were recorded in emission scanning mode. Tryptophan emission was excited at 290 nm (5 nm slit) and recorded between 310 and 410 nm (5 nm slit) unless otherwise indicated. Emission experiments exciting at 295 and 300 nm were also performed in order to check the contribution of tyrosine fluorescence and no significant differences were observed in the results. All the experiments were performed in buffer P (100 mM phosphate, pH 7.0, 50 mM NaCl). The measurements were made in 5-mm optical-path-length quartz-cells placed in a thermostated cell holder kept at 20 °C. Equal volumes of apo-OR stock were incubated overnight with proper aliquots of ASX stock solution (79 μM in buffer P, 1.2% DMSO). Each spectrum was corrected for buffer fluorescence and averaged from, at least, two independent runs.

The stoichiometry and the K_D for the binding of ASX with apo-OR were determined following ASX induced tryptophan quenching using the modified Stern-Volmer equation [19] and fitting the fluorescence quenching data to a polynomial equation.

Equilibrium unfolding of holo-OR and apo-OR was followed by monitoring tryptophan fluorescence at different guanidinium hydrochloride (GnHCl) concentrations. A 2.1 μM OR solution was diluted to the desired GnHCl concentration by adding appropriate aliquots of sample buffer and a 6.4 M GnHCl stock solution.

Circular dichroism

Circular dichroism (CD) spectra (180–270 nm) were made either in a Jasco Inc. J-720 or J-810 spectropolarimeters using 0.2-mm cells placed in a thermostated cell holder at 15 °C. Samples were measured at a concentration of 0.2 μM in buffer P. Scanning was performed with 1 nm bandwidth, 100 nm min^{-1} scan speed and 4 s average time. Each spectrum was obtained averaging, at least, 5 individual runs and corrected for buffer optical activity. Secondary structure content was estimated by analysis of the molar ellipticities with the K2d algorithm [20]. CD melting experiments were performed by monitoring the elipticity of the absorption spectrum at 222 nm during temperature scanning at 2 °C min^{-1} using a Peltier-based heating element.

Results

Binding of ASX to apo-OR

Experiments were performed by monitoring the ASX-induced tryptophan fluorescence quenching.

The maximum of tryptophan fluorescence emission (336 nm) observed for apo-OR clearly indicates that most of the tryptophan residues are placed in hydrophobic environments. Moreover, the ASX-induced fluorescence quenching also indicates that some of the Trp residues are placed close to the ASX binding site.

From the modified Stern-Volmer plot (Fig. 1a) we were able to obtain a fractional accessibility of tryptophan (f) of $22 \pm 4\%$ indicating that only a small fraction of the tryptophan residues are placed at ASX binding sites. Moreover, we obtained a Stern-Volmer quenching constant $K_{sv} = 0.49 \pm 0.27\ \mu M^{-1}$. The quenching constant can be interpreted as the association constant or binding constant of the complexation reaction because static quenching arises from the formation of a dark complex between fluorophore and quencher.

ASX binding parameters has also been determined from fluorescence quenching data as follows:

If we assume that the binding between apo-OR (P) and ASX (L) forms the complex holo-OR (PL) that can be represented with the following reaction:

$$P + nL \leftrightarrow PL_n$$

Where K_D can be written as

$$K_D = [P][L]^n/[PL_n]$$

Moreover, the total concentration of apo-OR and ASX must obey the following closure relationships,

$$[P](\text{total}) = [P] + [PL_n] = P_T \quad \text{and}$$
$$[L](\text{total}) = [L](\text{free}) + n[PL_n] = L_T$$

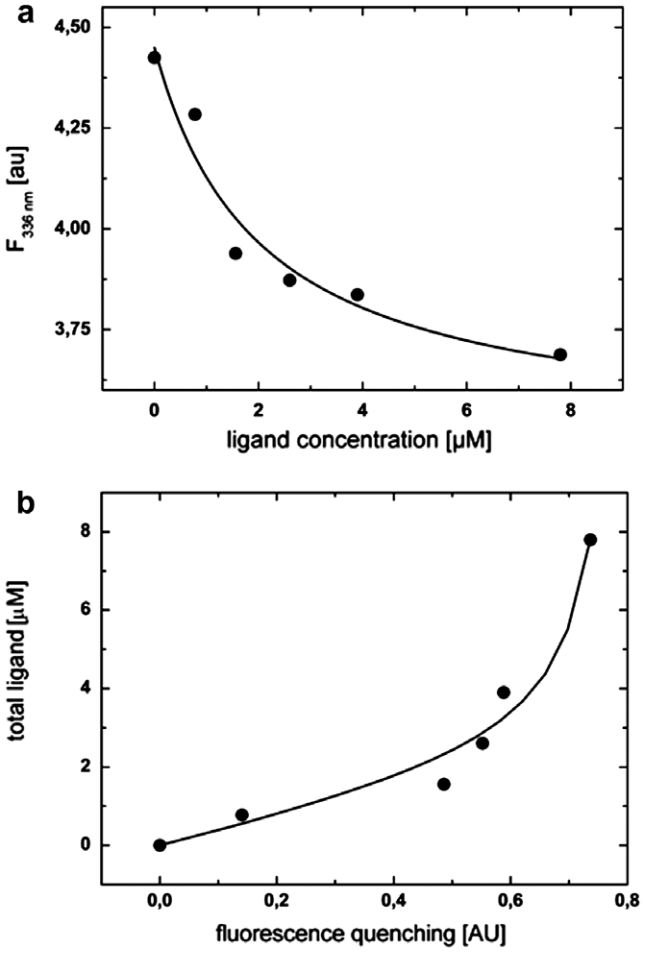

Fig. 1. Titration of apo-OR with ASX as followed by the increase in the Trp fluorescence quenching. (a) Modified Stern-Volmer plot of the apo-OR-ASX system. (b) ASX binding to apo-OR based on Trp Fluorescence quenching. Values for the dissociation constant $K_D = 0.48 \pm 0.27 \times 10^{-6}$ M.

If the amount of fluorescence quenching ($Q = F(336\text{ nm}, L_T = 0) - F(336\text{ nm}, L_T)$) is supposed to be proportional to the concentration of holo-OR

$$Q = a[PL_n]$$

we obtain the following equation relating the total concentration of ASX (L_T) to the magnitude of the tryptophan fluorescence quenching Q.

$$L_T = \frac{nQ}{a} + \left(\frac{QK_D}{(aP_T - Q)}\right)^{1/n} \quad (1)$$

The K_D value obtained using Eq. (1) was determined to be 0.48 ± 0.24 μM and the best fit in Fig. 1b was obtained for $n = 1$.

Effect of ASX removal on secondary structure and thermal stability

Far UV-CD spectra obtained for holo-OR and apo-OR are depicted in Fig. 2. Both spectra correspond to $\alpha + \beta$ structures. The estimation of the contribution of the different secondary structure types (Table 1) indicates a small increase in the contribution of random structures after ASX release (12% increase).

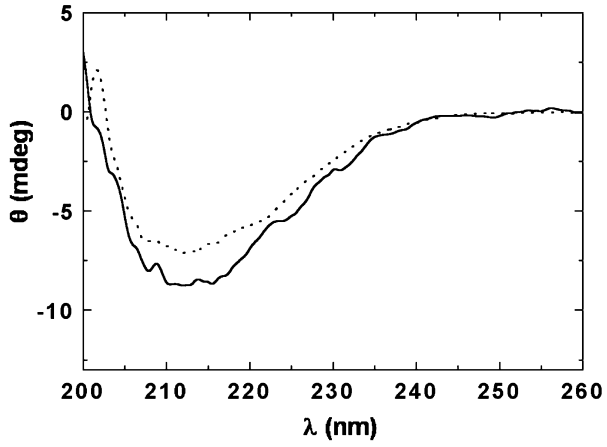

Fig. 2. Far-UV-CD spectra of snail holo-OR and apo-OR. Holo-OR (full line); apo-OR (dotted line).

Table 1
Secondary structure content of holo-OR and apo-OR from *Pomacea canaliculata*

	α-helix	β-sheet	Other
holo-OR	27	36	37
apo-OR	24	27	49

Values in percentage.

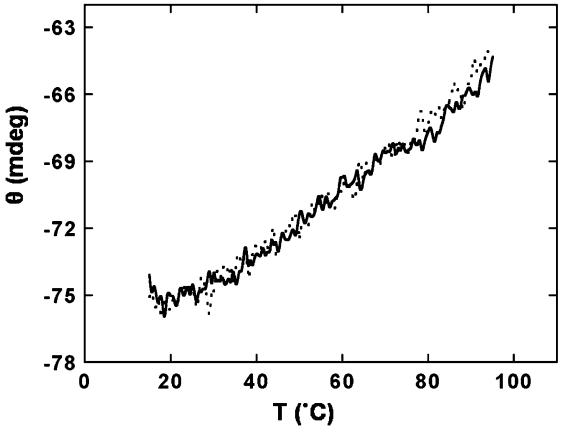

Fig. 3. Thermal unfolding of *P. canaliculata* holo and apo-OR following the elipticity of the CD spectra at 222 nm. Holo-OR (full line); apo-OR (dotted line).

Thermal stability of both OR forms was followed measuring the thermal evolution of the elipticity at 222 nm. Results indicate that both forms were very stable up to 95 °C and did not show noticeable differences in their melting curves (Fig. 3).

Chemical stability of holo-OR and apo-OR

Equilibrium unfolding experiments of holo-OR and apo-OR were performed to assay the overall chemical

stability of OR and to evaluate the influence of ASX on the stability of the OR oligomer. Fig. 4 shows the tryptophan fluorescence spectra obtained for holo-OR (Fig. 4a) and apo-OR (Fig. 4b) at different guanidinium hydrochloride concentrations. Both, an overall reduction of fluorescence intensity and a systematic red shift of the spectra were observed at increasing chaotrope concentration. Spectra showed a single well-defined isosbestic point (Figs. 4a and b), suggesting that the disassembling of the oligomer and the unfolding of the monomers occurred almost simultaneously obeying a two-state mechanism. Spectra were characterised by their mean wavelength M and the populations associated to the unfolded fraction (Fig. 4c, Table 2) were calculated from the mean wavelength as follows:

$$F = (1-\alpha)f_{\mathrm{f}} + \alpha f_{\mathrm{u}} \tag{2a}$$

$$M = \sum \lambda_{\mathrm{n}} F(\lambda_{\mathrm{n}}) / \sum F(\lambda_{\mathrm{n}}) = (1-\alpha)M_{\mathrm{f}} + \alpha M_{\mathrm{u}} \tag{2b}$$

$$\alpha = (M - M_{\mathrm{f}})/(M_{\mathrm{u}} - M_{\mathrm{f}}) \tag{2c}$$

Where M_{f} and M_{u} are the mean wavelength associated to the spectra of the folded and unfolded forms, respectively (Fig. 5a). Fig. 5b also shows the "folded" and "unfolded" components extracted for both, holo-OR and apo-OR.

Using the α values obtained from Eq. (2c), $\Delta G(\alpha)$ was obtained from the definition for the equilibrium constant $K(\alpha)$

$$K(\alpha) = \alpha/1-\alpha = \mathrm{e}^{-\Delta G(\alpha)/\mathrm{RT}} \tag{3a}$$

$$\Delta G(\alpha) = \Delta G^0 - mC_{\mathrm{GnHCl}} \tag{3b}$$

Here, ΔG^0 and m are, respectively, the standard free energy for the disassembling/unfolding process and the

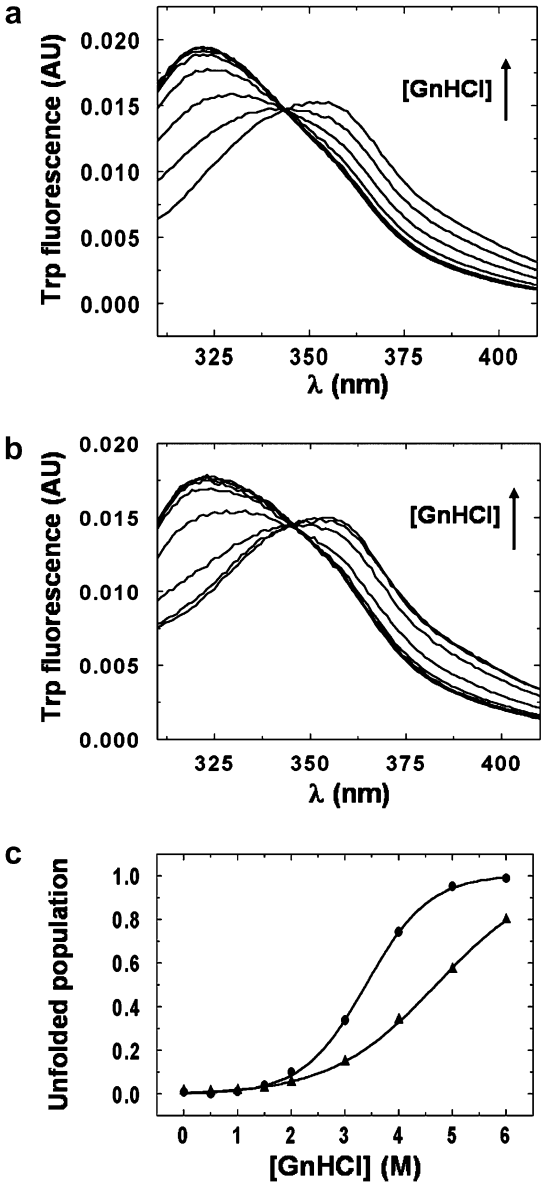

Fig. 4. apo-OR and holo-OR unfolding induced by GnHCl treatment (see Material and methods for details). GnHCl-induced unfolding of apo-OR and holo-OR. (a) holo-OR; (b) apo-OR; (c) unfolded population of holo-OR (▲) and apo-OR (●).

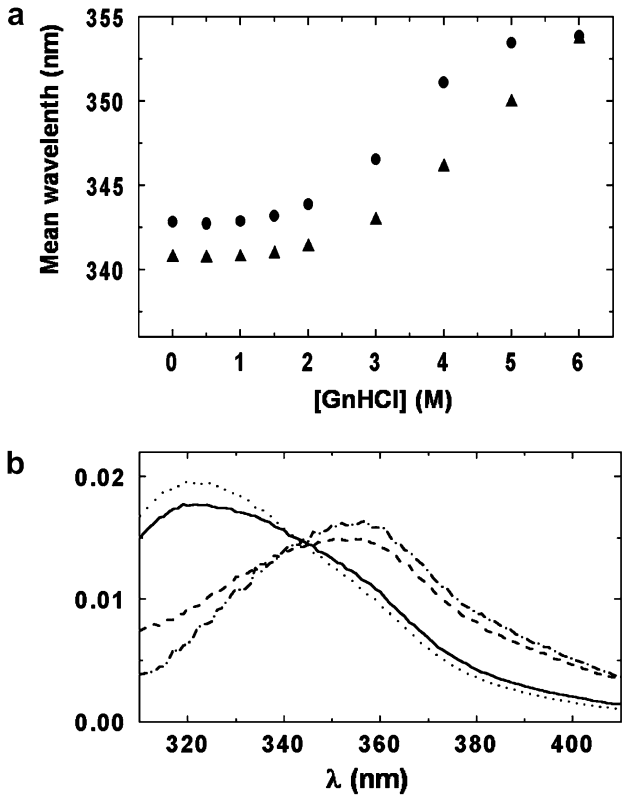

Fig. 5. apo-OR and holo-OR unfolding induced by GnHCl treatment (see Material and methods for details). (a) Mean wavelength obtained from Figs. 3a and b. holo-OR (▲) and apo-OR (●). (b) Folded and unfolded components drawn from Figs. 3a and b. Folded apo-OR (full line); Unfolded apo-OR (dashed line); Folded holo-OR (dotted line); unfoled holo-OR (dashed-dotted line).

Table 2
Thermodynamic parameters of apo-OR and holo-OR unfolding induced by GnHCl treatment

	ΔG^0 (kcal/mol)	m (kcal/mol)
holo-OR	3.48 ± 0.06	0.64 ± 0.01
apo-OR	3.00 ± 0.04	1.03 ± 0.02

corresponding "*m*-value" characterizing the influence of the chaotropoe on ΔG resulting from the association of the chaotrope molecules to the protein residues. Table 2 shows the parameters, ΔG^0 and *m*, obtained using Eqs. (3a) and (3b) for both holo-OR and apo-OR.

Discussion

Physiological and biophysical consequences of Astaxanthin binding

Astaxanthin is one of the dominant carotenoids in aquatic animals [21] showing both a strong quenching effect against singlet oxygen, and a powerful scavenging action against free radicals. These effects are considered to be part of the embryo antioxidant defense mechanisms in *P. canaliculata* and other animals [10]. We have determined that apo-OR and ASX form strong 1:1 complexes with apo-OR with a single binding site. Furthermore large excess of 8-anilinonaphthalene-1-sulphonic acid (ANS) (5-fold the OR concentration) was unable to displace ASX from the protein as determined by fluorescence experiments (data not shown). The quenching of tryptophan fluorescence emission upon ASX binding reinforces the conclusion pointing to tryptophan residues as part of the binding site. Moreover Stern-Volmer plot indicates that 22% of the total fluorescence of OR is accessible to the quencher.

Crustacyanin from the crustacean *Homarus* sp. is the best known invertebrate carotenoprotein and has been extensively studied for several decades i.e. [22–25]. Crustacyanin also contains ASX as a cofactor and shows a single high affinity site [26]. It is a carapace protein with a function quite different from the role of OR in the eggs of apple snails (see below). ASX is bound to each crustacyanin apoprotein within an internal hydrophobic pocket, or calyx containing a Trp residue [26,27]. The direct evidence of the presence of Tryptophan residues in OR located within quenching distance from bound ASX (thus associated to the binding site) are here reported for the first time for a molluscan carotenoprotein. The single ASX binding site of OR seems to have evolved to bind ASX with high affinity but more work is needed to fully understand its local structure.

The high affinity of ASX for apo-OR evidenced important physiological consequences. While it remains in the PVF, its major role would be the embryo photoprotection rather than an antioxidant, since only a few molecules of ASX would be free. This agrees with the role of OR in transferring ASX from the female to the embryo. As previously suggested [10], after OR is taken up by the embryo, the ASX molecules it carries, would be available as a lipid-phase antioxidant in membranes.

Finally, the low K_D for the carotenoid is also compatible with another eco-physiological adaptation of eggs clutches of *Pomacea* species. It has been suggested that their reddish colour is a warning signal for potential predators, as egg clutches are very unpalatable for birds and mammals including humans [28]. If ASX molecules were freed into the PVF, they would be rapidly oxidized by sunlight and the egg colour would fade bringing to an end the warning signal that assists the snails in hatching without disturbance from predators. This remarkable strategy, together with the photoprotection and antioxidant-carrying roles are an example of the fascinating multifunctional mechanisms where carotenoid pigments forming complexes with apo-OR play a central role.

Effect of astaxanthin on ovorubin structure and stability

The occlusion of 1 $Å^2$ of hydrophobic accessible surface area (hASA) contributes with 25 cal/mol to the stabilisation of the folded/assembled oligomer [29]. Thus, the hydrophobic accessible surface area exposed to the solvent after disassembling/unfolding should be $\sim$139 $Å^2$ and $\sim$120 $Å^2$ for holo-OR and apo-OR, respectively (Table 2). Calculations of the accessible hydrophobic surface area of crustacyanin (PDB ID: 1GKA) using the method outlined by Lee and Richards [30] implemented in the program Mole 1.2 (Applied Thermodynamics) indicate that the surface area difference between the holo -crustacyanin form and a putative (yet unstable) apo-crustacyanin form is 373 $Å^2$, a threefold excess compared with the figures obtained for ovorubin. Assuming that the difference on accessible hydrophobic surface area between the holo and the apo forms is the relevant parameter reflecting the thermodynamic behaviour of the system, our results suggest a plausible explanation for the remarkable difference in stability between ovorubin and crustacyanin carotenoproteins upon cofactor removal. Thus the binding site for astaxanthin in ovorubin, unlike crustacyanin, would not be located occluding hydrophobic patches in the contact interface between monomers essential for the maintenance of the quaternary structure, playing only a secondary role on quaternary structure stabilisation. This is even more remarkable considering that ASX surface area in OR represents 16% of the total exposed area of the protein (obtained from unfolding experiment of apo-OR and holo-OR) and only 8% in crustacyanin. This is in agreement with the different physiological functions described for astaxanthin in both carotenoproteins. Moreover, Norden [12] also showed that the removal of the carotenoid group from ovorubin had no effect on the ability of the protein to inhibit trypsin action.

CD spectra of apo-OR and holo-OR showed a loss of structured secondary elements which was, however, minor and probably reflects the increase in conformational flexibility gained by the aminoacid residues associated to ASX binding. Since carotenoid content of OR is low (1 ASX red chromophore per 300,000 MW protein chromophores), protein chromophores can be expected to dominate the far UV region. This is a similar situation to that of the egg lipovitelin ovoverdin from the lobster *Homarus americanus* which has 1 ASX per 326,000 Da apocarotenoprotein [31]. The present results indicate that, unlike most invertebrate carotenoproteins, astaxanthin is not essential

for ovorubin quaternary structure. This hypothesis is supported by the fact that we were able to obtain an apo-OR and a reconstituted OR with the same electrophoretic native pattern of holo-OR [10]. The quaternary structure would therefore be only marginally affected by the presence of this carotenoid. In this regard, asteriarubin and linckiacyanin from the phylum Echinoderma are the only invertebrate carotenoproteins so far reported in which carotenoids are not involved in maintaining their quaternary structures [14]. Interestingly, these carotenoproteins, like OR, are glycosilated and have no sequence homology with crustacyanin [32].

The thermal stability of OR was clearly not affected by ASX removal as indicated by the overlapping CD melting curves. This high thermal structural stability could have a physiological meaning as embryos may be exposed to temperatures as high as 50 °C especially when eggs are deposited on rock surfaces. As OR does not have disulfide bonds among subunits (results not shown) and hydrophobic interaction does not play a role in OR stability at this temperature (supported by the lack of destabilisation by ASX removal), it is tempting to speculate that the high degree of glycosilation of ovorubin (over 18% by wt, [9]) may be involved, providing for the unusually high structural stability when exposed to perturbations by temperature and chaotropic agents. This stabilizing role of oligosaccharide moieties has been observed in other glycoproteins [33] and for OR chemically deglycosilated (unpublished results of our laboratory).

To our knowledge this is the first study on the quaternary structure and stability of a molluscan carotenoprotein. Our data revealed a different interaction carotenoid-protein than in most other invertebrate carotenoprotein. Astaxanthin is tightly bound to OR and it does not make a substantial contribution to conformational stability and structure of the complex, which is likely to have significant implications with regard to ASX affinity, as well as other OR properties, in vivo. The native structure of OR oligomer is extremely resistant to thermal and chemical denaturalization. More research is needed to better understand the protein-ASX interaction and its physiological functionality.

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