Studies on the reconstitution of *P. clarkii* blue carotenoprotein from its isolated subunits

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Summary. A blue carotenoid-protein complex (λ_{max} 635 nm) was extracted and purified from the carapace of the crayfish *Procambarus clarkii*. The complex was further liberated from astaxanthin, its prostetic group, causing dissociation into apoprotein subunits. Reconstitution of the complex from the various sub-units (isolated by chromatofocusing) plus astaxanthin was attempted. Apoprotein-size pigments of rose-purple color (λ_{max} 545 nm) were obtained. It was found that both monomers are required in order to reconstitute a blue complex fairly similar in structure ot the native one. However, the native conformation was not completely recovered, as indicated by some differences in the UV spectrum.
Key words. Carotenoproteins; protein reconstitution; astaxanthin.

Carotenoid pigments frequently occur in nature, especially in crustaceans, specifically bound to proteins, constituting the so-called carotenoid-protein complexes or carotenoproteins. The mechanism of interaction between protein and carotenoid that accounts for the change in color of the latter, from orange when it is free to blue when bound to the protein, is the main subject of investigation in most studies of carotenoproteins at present.

The approach to the elucidation of such a mechanism can be made by means of reconstitution experiments. In a previous report[1], we described some structural requirements of the carotenoid for its binding to the protein. Now, the reconstitution of the complex is studied from the protein side.

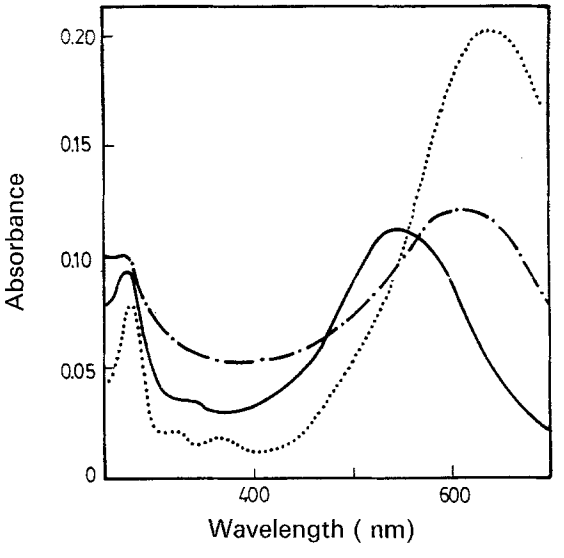
Materials and methods. The blue carotenoprotein from the carapace of the crayfish *Procambarus clarkii* was extracted using the method of Quarmby et al.[2] and purified as described in Gárate et al.[6]. Once the carotenoprotein had been purified, its prosthetic group astaxanthin was liberated from the protein by adding N, N-dimethyl-formamide (DMF); this resulted in the dissociation of the complex into subunits[3]. The apoprotein subunits so obtained were separated by chromatofocusing in a column (25 × 1.0 cm) of Polybuffer Exchanger PBE™ 94 (Pharmacia) by the method of Zagalsky[3]. Two protein fractions were collected; the first one eluted at pH 6.2 and the second at pH 5.7. These fractions were identified by 6 M urea polyacrylamide slab gel electrophoresis, performed by the method of Laemmli and Favre[5], and called P_1 and P_2 respectively, according to the nomenclature reported by Gárate et al.[6].

Previously it has been shown[1] that keto groups at positions 4 and 4′ and hydroxyl groups at positions 3 and 3′, or at spatially analogue positions, as well as an unmodified polyenic chain, are required in the carotenoid structure for binding to the protein. Later, by using the chromatofocusing technique, we obtained a sufficient amount of isolated apoprotein subunits to undertake reconstitution experiments in order to study separately the interaction of the native carotenoid (astaxanthin) with each of the subunits and with both.

Reconstitution was achieved by the Britton et al. procedure[4]. The following combinations were tried: subunit P_1 plus astaxanthin, subunit P_2 plus astaxanthin and subunits P_1+P_2 plus astaxanthin. The molecular weights of the reconstituted pigments were determined in a column (30 × 1.0 cm) of Sephadex G-100 SF (Pharmacia). Absorption spectra of these pigments were recorded on a Beckman UV-5260 spectrophotometer.

When the reconstitution was attempted with separated P_1 or P_2 (plus astaxanthin), apoprotein-size pigments (mol.wts circa 21,400 and 19,200) were obtained, with identical absorption spectra exhibiting a λ_{max} at 545 nm (fig.). In a further experiment with both subunits (plus astaxanthin), the blue complex was reconstituted, its absorption spectrum showing a λ_{max} at 605 nm (fig.).

Results and discussion. It is known from previous studies[6] that the blue carotenoprotein from *P. clarkii* in its native form, designated the α-form, (mol.wt circa 246,000) has a λ_{max} at 635 nm. Under adverse conditions (such as low ionic strength, high temperature, extreme pH, etc.) the blue complex dissociates into purple dimers designated as β-forms (mol.wt circa 41,600) which have a λ_{max} at 585 nm and consist of two kinds of apoprotein subunits: P_1 (mol.wt circa 19,200) and P_2 (mol.wt circa 21,400). The prosthetic group of the complex, astaxanthin, is bound to the native α-complex in a 6 to 1 ratio and to the β-dimer in a 1 to 1 ratio. The λ_{max} of the complex in any preparation depends on the relative proportion of α to β species (produced by adverse conditions of the medium) present in solution; this dependence has been accurately correlated with the percentage of each species present. It could be determined that the complex reconstituted from both subunits plus astaxanthin, exhibiting a λ_{max} at 605 nm, consisted of 56% native α-complexes (containing 6 astaxanthin molecules per complex molecule) and 44% β-dimers (containing 1 astaxanthin molecule per dimeric molecule).



Comparative absorption spectra of the native blue carotenoprotein (...) and the reconstituted pigments from separated subunits P_1 or P_2 (–) and from both subunits together (–.–).

The results showed that separated subunits P_1 or P_2 were able to bind to a single molecule of astaxanthin, giving rise to a change in its spectral properties, shifting λ_{max} from 482 (astaxanthin in DMF solution) to 545 nm, thus revealing the interaction of the subunit with the carotenoid. However, dimers or polymeric structures could not be formed.

When reconstitution was achieved with both subunits P_1+P_2, the astaxanthin λ_{max} shift was much higher (482–605 nm), providing evidence that a stronger interaction between carotenoid and protein took place. That is, the simultaneous presence of P_1 and P_2 subunits was necessary for the complete interaction with the carotenoid, indicating that each subunit contained one half of the carotenoid binding site, what is in agreement with the previously determined stoichiometry of 1 astaxanthin molecule per β-dimer. It is concluded that the presence of both subunits is

required to reconstitute the polymeric blue carotenoprotein. However, some differences in the UV region of the absorbance spectrum of the reconstituted complex were found when it was compared with the native one (fig.). This indicates that although the structure of the reconstituted complex is fairly similar to that of the original one, since the interaction with the carotenoid occurs, the native conformation of the protein has not been fully recovered (probably the exposure of aromatic aminoacids has varied); nevertheless, the changes do not affect the carotenoid binding site to any marked extent.

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Hymenin, a novel α-adrenoceptor blocking agent from the Okinawan marine sponge *Hymeniacidon* sp.

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Summary. A novel bromine-containing alkaloid, hymenin, has been isolated from the Okinawan marine sponge *Hymeniacidon* sp. as a potent α-adrenoceptor blocking agent and its structure determined to be **1** on the basis of the spectral data.
Key words. Sponge; hymenin; *Hymeniacidon* sp.; alkaloid; α-blocker.

α-Adrenoceptor blocking agents have been employed in basic research and in therapy under a wide variety of conditions. During our investigation on bioactive substances from marine invertebrates[1-4], we have examined the α-adrenoceptor blocking activity of 70% ethanolic extracts of various marine sponges collected at Okinawa, using isolated vascular smooth muscle. As a result, an orange marine sponge *Hymeniacidon* sp. has been found to possess a remarkable α-adrenoceptor blocking activity on the isolated rabbit aorta. In this communication, we report the isolation and structure elucidation of hymenin **1**, a novel α-adrenoceptor blocking constituent of *Hymeniacidon* sp.

Male albino rabbits (2–3 kg) were used. The procedure for preparing the isolated rabbit aorta and the technique of measurement of contractions were as previously described[5]. *Hymeniacidon* sp. was collected at Ishigaki Island, Okinawa, in June 1984. The methanol-toluene (3:1) extract of the sponge was partitioned between toluene and water. The aqueous phase was then extracted with chloroform, ethyl acetate and n-butanol. The butanol soluble material was passed through a silica gel column with chloroform-n-butanol-acetic acid-water (3:12:2:2) to afford an active fraction. This fraction was chromatographed on a Develosil ODS column with methanol-water (4:6) containing 0.05M acetic acid to yield hymenin **1** (0.005% wet wt) as an amorphous colorless solid.

The UV spectrum $\{\lambda_{max}^{MeOH}$ 274 nm (ε 9200)$\}$ and a positive color test of **1** with Echtrotsalz B suggest the presence of a substituted pyrrole chromophore[6,7]. The IR spectrum (KBr) showed an amide carbonyl band at 1680 cm^{-1}. The EI mass spectrum exhibited intense molecular ions at m/e 387, 389 and 391 (1:2:1), indicating that **1** is a dibrominated compound. The molecular formula $C_{11}H_{11}N_5OBr_2$ was established by high resolution FAB mass spectrometry (△ 1.2 mmu). The ^1H and ^{13}C NMR spectra of **1** were compared with those of oroidin **2** containing dibromopyrrole and guanidino moieties, which had been previously isolated from marine sponges of the genus *Agelas*[8-10].

The detailed analyses of the ^1H and ^{13}C NMR spectra (table) revealed the structure (fig.) which was considered to result from an intramolecular cyclization at C–4 and C–10 of **2**. Thus, the ^{13}C chemical shifts of the pyrrole (C–2, δ 106.2; C–3, 100.7; C–5, 130.1) and amino imidazole (C–11, δ 124.5; C–15, 110.7; C–13, 147.6) of **1** correlated very well with those of the corresponding carbon atoms of **2** (C–2, δ 104.4; C–3, 97.8; C–5, 128.0; C–11, 124.7; C–15, 110.8; C–13, 147.5)[10]. The remaining carbon atoms of **1** (C–4, δ 123.8; C–9, 31.0; C–8, 36.4) were assigned as compared with the corresponding ^{13}C chemical shifts (C–4, δ 121.8; C–9, 29.0; C–8, 40.0) of debromo-hymenialdisine[11,12] having the similar ring system to **1**. The assignment of C–10 (δ 34.6) was established by proton selective decoupling experiments. In agreement with the ring system of **1**, extensive spin decoupling experiments revealed the unit $-CH=C-CH-CH_2-CH_2-NH$ (H–15, H–10, H–9, H–8 and NH–7), in which observations were made of the NOE (+1%) between H–10 and H–15. The chemical shift (δ 6.07) of H–10 was compatible with that (δ 6.24) of the corresponding proton of dihydrooroidin[13]. Additional support for the structure assigned derives from the EI mass spectrum of **1**, in which fragment ion peaks produced by expulsion of CH_2CH_2NHCO from M^+ were observed. Hymenin **1** contains an asymmetric center at C–10 $\{[\alpha]_D^{25} -15°$ (C = 0.5, MeOH)$\}$. The CD spectrum exhibited a positive Cotton effect with λ_{ext}^{MeOH} 242 nm (△ε +2.07); the stereochemistry remains to be assigned.

Hymenin appears to be closely related biogenetically to bromine-containing alkaloids such as oroidin[8-10], sceptrin[14] and keramadine[15] from marine sponges of the genus *Agelas*, mono-

^1H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectral data of hymenin **1**

Position	Proton	m	J (Hz)	Carbon	m
1	12.0[a]	brs[c]			
2				106.2[b]	s[c]
3				100.7	s
4				123.8	s
5				130.1	s
6				162.0	s
7	7.78	dd	2.1, 7.3,		
8	3.01 (3.12)[b]	ddd	7.3, 7.3, 14.0	36.4	t
	3.13 (3.24)	ddd	2.1, 9.8, 14.0		
9	1.84 (1.92)	ddd	4.3, 9.8, 14.0	31.0	t
	2.25 (2.27)	ddd	4.3, 7.2, 14.0		
10	3.92 (4.18)	t	4.3	34.6	d
11				124.5	s
12	7.48	brs			
13	5.41	brs		147.6	s
15	5.74 (6.07)	s		110.7	d

[a] δ in ppm, DMSO-d_6; [b] δ in ppm, MeOH-d_4; [c] Multiplicity in off-resonance decoupled spectrum.