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Purification, crystallization and initial X-ray analysis of the C_1 subunit of the astaxanthin protein, V_{600}, of the chondrophore *Velella velella*

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The subunit C_1 of the carotenoid-binding protein, V_{600}, of the chondrophore *Velella velella* has been purified and crystallized. The crystals, which were grown by the vapour-diffusion method from ammonium sulfate as the major precipitant, diffract beyond 3 Å and show little radiation damage over long periods (greater than 100 h) on a Cu $K\alpha$ rotating-anode X-ray source. The space group of the crystals is $P2_12_12_1$ with cell dimensions a = 42.0, b = 80.9, c = 110.6 Å.

1. Introduction

Carotenoproteins provide the varied colouration (blue, green, yellow or red) of many invertebrate species, with the carotenoid astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) commonly the ligand (Cheeseman *et al.*, 1967). The blue astaxanthin protein, crustacyanin, of the lobster carapace is a general example of a type of carotenoprotein common in animals of the Arthropoda. The 20 kDa subunits of this carotenoprotein bind a molecule of astaxanthin to form the oligomer, α-crustacyanin, which comprises 16 subunits (of two types, A and C) and 16 astaxanthin molecules. The two major subunits (apocrustacyanin A_2 and C_1) probably belong to the lipocalin superfamily of proteins, based on the possession of the two short consensus sequences characteristic of this family and on cross-reaction with monoclonal antibodies to human serum retinol-binding protein, a lipocalin-family member (Keen *et al.*, 1991*a*,*b*; Zagalsky *et al.*, 1995). The tertiary structures of the apoproteins and carotenoid-binding sites have been modelled on this assumption (Keen *et al.*, 1991*a*,*b*). Although diffracting crystals of apocrustacyanin C and β-crustacyanin, a dimer of the A and C subunits with two bound astaxanthin molecules, have been grown (Wright *et al.*, 1992; Chayen *et al.*, 1996; Snell *et al.*, 1997; Chayen, Gordon, Phillips *et al.*, 1996), attempts to solve the structures have not yet been successful.

In other invertebrate phyla, protein structures unrelated to the lipocalins have evolved with the ability to bind carotenoids. Some members of the Echinodermata, for example the starfish *Asterias rubens* and *Linckia laevigata*, possess carotenoproteins with subunits half the molecular size of the apocrustacyanins and which have unique amino-acid sequences (Britton *et al.*, 1982; Zagalsky, 1994). *V. velella*, a surface-living oceanic chondrophore (phylum Cnidaria, class Hydrozoa, order Chondrophora) is about 3 cm in diameter. It is related to the Portuguese man-of-war, *Physalia* (order Chondrophora) and is known as the 'by-the-wind-sailor' on account of the transparent sail attached to its mantle, which acts as a raft. Two astaxanthin proteins, V_{620} and V_{600}, with λ_{max} = 620 and 600 nm, respectively, both of large size (>3 × 10^5 kDa) have been isolated from the mantle. The carotenoproteins are unusual in having high-affinity anion-binding sites which influence the quaternary structures and absorption properties (both visible and CD) of the pigments (Zagalsky & Herring, 1977). The proteins are of interest in this respect since some visual pigments, such as gecko photopigment (Crescitelli, 1977) and chicken rod pigment (Knowles, 1976), have analogous anion-binding sites. The quaternary structures of the oligomers have been revealed in electron-microscopy studies (Zagalsky & Jones, 1982). The carotenoproteins are composed of two sets of subunits of similar size (~23 kDa) and behaviour in protein separations to those of crustacyanin (Zagalsky & Herring, 1977), and are denoted subunits type A and C, by analogy. Peptide mapping (Zagalsky, 1982) and cross-reaction against polyclonal antibodies to the apocrustacyanins (Zagalsky *et al.*, 1995) indicate that the apoproteins of V_{600} may be related in structure to the latter proteins.

The function of the blue mantle pigments of *V. velella* is probably that of cryptic colouration. The spectral transmission of the carotenoproteins is high over the same regions as the upward illumination of the sea; as a result, the pigments transmit ambient light with little wavelength change, so that the animal blends in with its background (Herring, 1965). The large bathochromic shift in the absorption spectrum of the protein-bound astaxanthin, which is some 120 nm for pigment V_{600}, is of similar magnitude to that for 11-cis retinal in the visual pigments. Recent MAS-NMR measurements on crustacyanin with bound ^{13}C astaxanthin support an electrostatic polarization of the conjugated polyene chain as the

mechanism for the colour shift in such blue carotenoproteins (Weesie *et al.*, 1997). A crystal structure is clearly required to reveal details of the carotenoid–amino-acid contacts at the binding site in order to validate this proposal. In this respect the *V. velella* subunit, with its attached carotenoid, is a most suitable model.

We report here the purification, crystallization and initial X-ray diffraction of the major subunit C_1 of *V. velella* pigment V_{600}.

2. Purification

2.1. Purification of *V. velella* carotenoproteins V_{600} and V_{620}

Whole animals (*V. velella*, 300 g, purchased from Pacific Bio-Marine Labs. Inc., Venice, CA, USA; transported in dry ice and stored in the deep freeze) were homogenized in a Waring Blender with 1 l of 0.1 *M* KCl and 25 m*M* Tris–HCl, pH 7, and left stirring in the cold room overnight. The mixture was centrifuged at 16000 rev min^{-1} for 30 min and the blue pigment in the supernatant precipitated between 35 and 60% ammonium sulfate saturation. The pigment was dissolved and dialysed against 0.2 *M* KCl and 0.2 *M* phosphate buffer (KH_2PO_4/Na_2HPO_4), pH 5.9.

Carotenoproteins V_{620} and V_{600} were purified and partially separated using calcium phosphate gel chromotography and gel filtration (Agarose 4B and Sephadex G75; Pharmacia Biotech, Sweden), as described previously (Zagalsky & Herring, 1977). Pigment V_{600} is not obtained free of the larger pigment V_{620} owing to spreading of the latter on Agarose 4B caused by adsorption to the bed material.

2.2. Ultrogel HA chromatography (fast protein liquid chromatography) to separate V_{600} and V_{620}

Pigment V_{600} in 0.2 *M* KCl and 0.1 *M* phosphate buffer (KH_2PO_4/Na_2HPO_4), pH 5.9 (buffer *A*) was filtered through a 0.2 µm Acrodisc filter (Gelman Sciences, Ann Arbor, MI, USA) and applied to an Ultrogel HA hydroxyapatite column (1 × 10 cm; Biosepra SA, Villeneuve La Garenne, France) at 0.3 ml min^{-1}. The column was washed with buffer *A* and the adsorbed carotenoproteins eluted at 0.2 ml min^{-1} with a gradient formed from buffer *A* and buffer *B* [0.2 *M* KCl and 0.1 *M* phosphate buffer (KH_2PO_4/Na_2HPO_4) containing 50% saturation ammonium sulfate, pH 7]. Pigment V_{600} was eluted with a gradient of 0–35% buffer *B* in 35 ml, followed by a further 10 ml 35% buffer *B*. Pigment V_{620} was eluted with a gradient of 35–100% buffer *B* in 15 ml. Fractions (0.5 ml) containing purified pigment V_{600}, with $E_{280/600} = 0.2$, were pooled and the protein stored under 60% ammonium sulfate saturation at 277 K in the dark.

2.3. Subunit C_1 preparation

The *V. velella* carotenoproteins have highly stable quaternary structures. The proteins remain oligomeric on removal of the carotenoid ligand with acetone or treatment with 6 *M* urea pH 5–9, and require 8 *M* urea and 25 m*M* HCOOH for dissociation to the apoproteins (PAGE; P. F. Zagalsky, unpublished). The following novel dissociation with ethanediol and subunit separation by chromatofocusing in this solvent was therefore developed. In this method the subunits retain most of the coloured ligand.

Pigment V_{600} (1 mg ml^{-1}) in 0.15 *M* imidazole–HCl pH 7.4, was stirred with an equal volume of ethanediol (Analar; BDH Laboratory Supplies, Poole, England) at room temperature (note that at 273 K the protein remains aggregated). The pH was readjusted to 7.4 by the addition of 0.15 *M* imidazole solution and ethanediol [1:1(*v*/*v*), pH unadjusted]. The purple sample, filtered through a 0.2 µm Acrodisk filter, was injected at 0.2 ml min^{-1} onto a PBE74 column (1 × 10 cm; Pharmacia Biotech, Sweden) equilibrated with 0.075 *M* imidazole and 50% ethanediol, adjusted to pH 7.4 with HCl, and the column was washed with this buffer. Subunit A is adsorbed onto the bed material and remains bound following the passage of polybuffer pH 4.5 [polybuffer 74 (Pharmacia Biotech, Sweden), H_2O, ethanediol, 10:40:50(*v*/*v*)] and is eluted as an aggregate with 1 *M* NaCl.

The C subunits, unadsorbed on PBE at pH 7.4, were diluted with an equal volume of water, dialysed against 0.03 *M* ethanolamine–HCl pH 9.6 and concentrated by ultrafiltration using an Amicom concentrator (UM10 diaflo membrane) (Amicom Corp., Lexington, MA, USA). The purple protein (1 mg ml^{-1}) was treated with an equal volume of ethanediol at room temperature and the pH readjusted with 0.03 *M* ethanol–ethanediol [1:1(*v*/*v*)]. The protein was filtered (0.2 µm Acrodisc) and applied (0.3 ml min^{-1}) to a column of PBE74 equilibrated with 0.015 *M* ethanolamine and 50% ethanediol, adjusted to pH 9.6 with HCl. The adsorbed purple carotenoprotein was eluted with polybuffer mixture [polybuffer 96 (Pharmacia Biotech, Sweden)/H_2O/ethanediol, 10:65:75(*v*/*v*), pH unadjusted] at 0.15 ml min^{-1}. The major purple-coloured subunit C_1 is eluted prior to a smaller coloured fraction (subunit C_2). Subunit C_1 has λ_{max} = 550 nm in 0.015 *M* ethanolamine and 50% ethanediol, pH 9.6, and λ_{max} = 590 nm, $E_{280/590}$ = 0.26 in 0.03 *M* ethanolamine–HCl pH 9.6. It irreversibly binds to PBE in the absence of ethanediol.

Following chromatography, subunit C_1 was diluted with an equal volume of water and dialysed against 0.03 *M* ethanolamine–HCl pH 9.6 to remove ethanediol. The protein was then precipitated at 90%

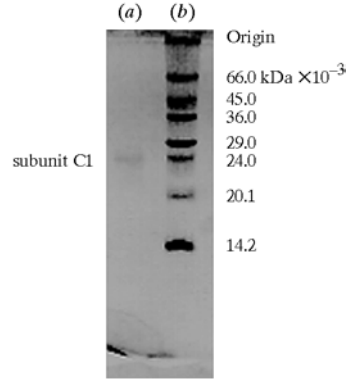

Figure 1
Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) using the discontinuous buffer system of Laemmli with 15% acrylamide separating gel, as described in Zagalsky *et al.* (1995). (*a*) Apoprotein C_1, (*b*) molecular-weight markers (Dalton Mark VII-L, Sigma–Aldrich, Poole, England).

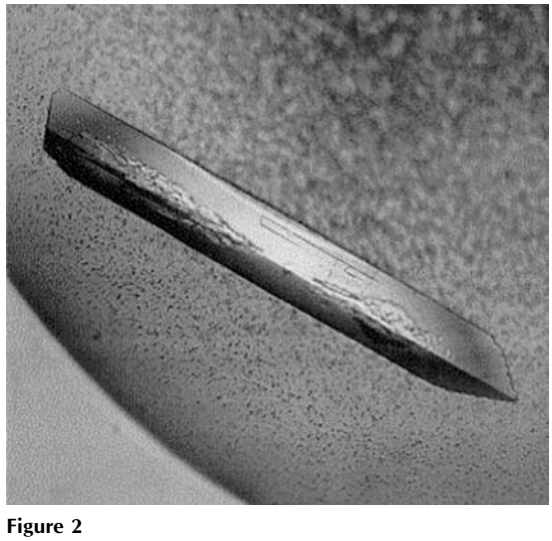

Figure 2
V. vellela C_1 crystal, dimensions 0.9 × 0.17 × 0.08 mm.

ammonium sulfate saturation and washed with ammonium sulfate solution (90% saturation) to remove polybuffer. The subunit was dissolved in 0.1 *M* Tris–HCl and 1 m*M* EDTA, pH 7, dialysed against this buffer, filtered (0.2 µm filter) and concentrated by ultrafiltration (Vivaspin 500; Vivascience Ltd, Binbrook, Lincoln, England) to ∼20 mg ml^{-1} for crystallization. The sample shows a single band in SDS–PAGE of 23.5 kDa (Fig. 1). The purple–blue colour of the sample fades on storage over several months at 277 K.

2.4. Crystallization

Trials were set up in hanging drops and the conditions for the crystallization of the apoproteins C_1 and C_2 of crustacyanin from lobster carapace (as reported by Wright *et al.*, 1992 and Chayen *et al.*, 1996) were initially applied to the protein from *V. velella*. No crystals were obtained under these conditions. However, when a significantly higher concentration of ammonium sulfate was applied, rod-shaped crystals, similar in shape to the apoproteins C_1 and C_2, appeared within 2–3 d and some continued growing over a period of 2–3 weeks to reach a size of 0.9 × 0.17 × 0.08 mm (Fig. 2). The best crystals, barely coloured to the naked eye, grew at 291 K over reservoirs containing 1 ml 0.1 *M* Tris–HCl pH 9.0, 5% MPD (2-methyl-2,4-pentanediol), 1 m*M* EDTA and 1.9–2.0 *M* ammonium sulfate (in comparison to 1.4 *M* ammonium sulfate in the case of the crustacyanin subunits). The volume of the drops was 2 µl, made up by mixing 1 µl of the protein solution containing 10 mg ml^{-1} protein (in 0.1 *M* Tris–HCl pH 7.0 and 1 m*M* EDTA) with 1 µl of the reservoir.

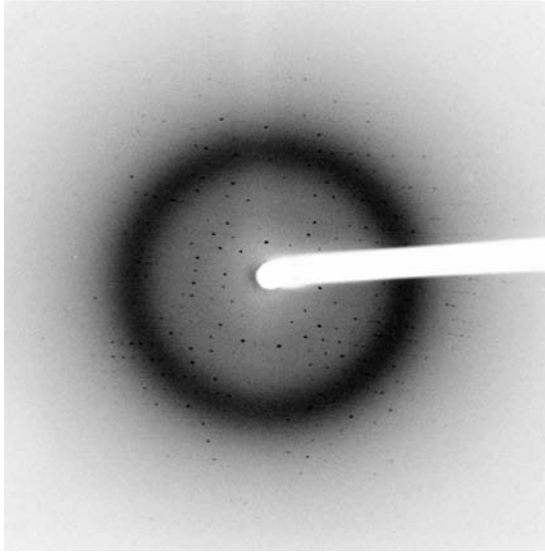

Figure 3
1° oscillation X-ray image of a crystal of the C_1 subunit of the astaxanthin protein V_{600} of *V. vellela*. Crystal-to-detector distance = 120 mm. Resolution at the (horizontal and vertical) edges of the detector is 2.27 Å.

2.5. Data collection and processing

A needle-shaped crystal (0.4 × 0.1 × 0.06 mm) was mounted in a quartz capillary at room temperature and X-ray data were collected (Fig. 3) using Cu $K\alpha$ radiation (λ = 1.5418 Å) from a Rigaku RU200 X-ray generator (50 kV, 100 mA) and an R-AXIS IIc area detector. Attempts to mount larger crystals resulted in cracking of the crystals, which are obviously fragile.

A sweep of 99° of data were collected to a resolution of 2.6 Å. There were 42 621 recorded observations, of which 11 667 were unique, this representing an overall completeness of 95.8%. Average I/σ at 2.6 Å resolution was greater than 4. $R_{\rm merge}$ exceeded 20% at 2.9 Å. The completeness at 2.6 Å was 94.2%. Overall $R_{\rm merge}$ to 2.6 Å was 11.1% with 87.2% of the data recorded at least twice. These data were integrated and processed using the *HKL* program suite (Otwinowski & Minor, 1997). The crystal showed little signs of decay after 100 h exposure.

The data at 2.4 Å had an average I/σ of greater than 3. However, these high-resolution diffraction spots displayed unreasonably high $R_{\rm merge}$ values and have therefore been omitted from this summary. Crystal cell parameters and systematic absences were consistent with the orthorhombic space group $P2_12_12_1$. Refined cell parameters were a = 42.0, b = 80.9, c = 110.6 Å. A comparison of these unit-cell parameters with those previously published for apocrustacyanin C_1 (a = 42.0, b = 81.9, c = 110.9 Å and a = 42.1, b = 81.0, c = 110.7 Å; Chayen *et al.*, 1996; Snell *et al.*, 1997) and C_2 (a = 42.0, b = 80.9, c = 110.8 Å; Wright *et al.*, 1992) show them to be very similar. All three proteins also fall into the orthorhombic space group $P2_12_12_1$. The peptide mapping (Zagalsky, 1982) and cross-reaction (Zagalsky *et al.*, 1995) show similar agreement. However, a search of the OWL protein-sequence database using four peptides, representing about half the protein, does not suggest strong sequence homology with members of the lipocalin superfamily (J. N. Keen, unpublished results). A structure analysis of this protein is now under way.

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