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Apocrustacyanin A1 from the lobster carotenoprotein α-crustacyanin: crystallization and initial X-ray analysis involving softer X-rays

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The A1 subunit of the carotenoprotein α-crustacyanin, isolated from lobster carapace, has been crystallized using the vapour-diffusion method. The crystals, grown in solutions of ammonium sulfate containing methylpentanediol (MPD), diffracted to 2.0 Å. The crystals are stable to radiation. The space group of the crystals is $P2_12_12_1$. The unit-cell parameters are $a = 41.9$, $b = 80.7$, $c = 110.8$ Å. 'Standard structure determination' has been unsuccessful within this crustacyanin family. Instead, an approach based on the S atoms is being undertaken involving softer X-rays at the SRS, Daresbury.

1. Introduction and biological relevance

The blue colouration of the lobster carapace is provided by the astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) binding protein α-crustacyanin. The absorption spectrum of the carotenoid in the carotenoprotein is bathochromically shifted by some 150 nm. The mechanism for this large spectral shift, evident when lobsters are boiled, is of interest in relation to the visual pigments, in which the spectral shift for retinal, a C_{15} polyene (half-size carotenoid), is of a similar magnitude (Wald *et al.*, 1948). The quaternary structure of α-crustacyanin is complex. It is an aggregate of 16 apoprotein subunits of about 20 kDa, with one astaxanthin molecule being bound per apoprotein monomer. The apoproteins consist of five electrophoretically distinct components which fall into two main types: C1, C2 and A1 (called CRTC) and A2 and A3 (called CRTA), with C1 and A2 predominating (Quarmby *et al.*, 1977). The CRTC subset are similar in size, amino-acid composition and N-terminal sequence, and in peptide mapping; the A2 and A3 subset are likewise closely related to each other. The differences between apoproteins within the subsets (CRTC and CRTA) may result from post-translational modification, since only a single gene has so far been isolated for CRTA and for CRTC (A. Cox and J. B. C. Findlay, unpublished work). The amino-acid sequences of CRTA (A2) and CRTC (C1) (Keen *et al.*, 1991*a*,*b*) show the two consensus sequences characteristic of the lipocalin superfamily of proteins responsible for binding small lipophilic ligands (North, 1989), typified by serum retinol-binding protein and β-lactoglobulin.

2. Preparation of protein

Crustacyanin was extracted and purified from lobster carapace and apocrustacyanin A1 was prepared as previously described for the A2 subunit (Keen *et al.*, 1991*a*), but using ethyl-acetate/ether [3:1(*v*/*v*)] in place of acetone/ether to remove the carotenoid; less aggregated protein is formed using the former organic solvent. The apoprotein (20 mg ml^{-1}) was prepared for crystallization in 0.1 *M* Tris–HCl, 1 m*M* EDTA pH 7, as described in Chayen *et al.* (1996).

3. Crystallization

Trials containing the A1 subunit of lobster crustacyanin were initially set up in hanging drops using the conditions for the crystallization of the C1 and C2 apoproteins (as reported by Wright *et al.*, 1992; Chayen *et al.*, 1996). No crystals were obtained under these conditions. However, when a higher concen-

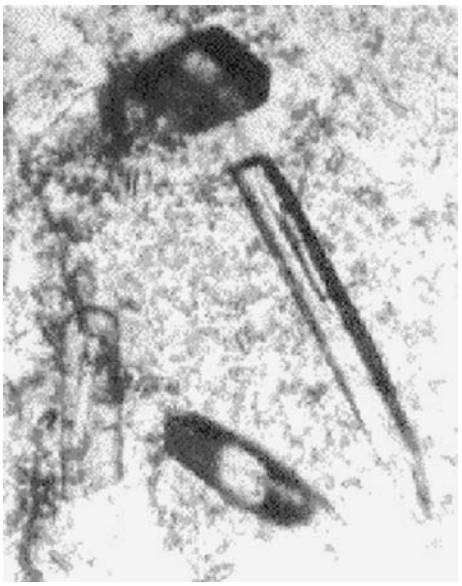

Figure 1
Crystals of apocrustacyanin A1. The width of the long needle (on the right) is 0.1 mm and serves as a scale.

tration of ammonium sulfate was used in wells, rod-shaped crystals similar in shape to the apoprotein C1 and C2 crystals but larger in all dimensions appeared within 3 d. Some crystals continued growing over a period of two weeks to reach dimensions of up to 1.2 × 0.4 × 0.15 mm (Fig. 1). The best shaped crystals grew at 291 K over reservoirs containing 0.8 ml of 0.1 *M* Tris–HCl pH 9.0, 5% MPD (2-methyl 2,4-pentanediol), 1 m*M* EDTA and 1.9 *M* ammonium sulfate. The ammonium sulfate concentration thus considerably exceeds that (1.4 *M*) found sufficient for the growth of crystals of the C1 and C2 apoproteins. The conditions are very similar to the crystallization conditions for the C1 subunit of the astaxanthin protein, V600, of the chondrophore *Velella velella* (Chayen *et al.*, 1999). The final volume of the hanging drops was 2 or 3 µl, made up by mixing equal volumes of the protein solution [containing 20 mg ml^{-1} A1 subunit (in 0.1 *M* Tris–HCl pH 7.0 and 1 m*M* EDTA)] and of the reservoir solution.

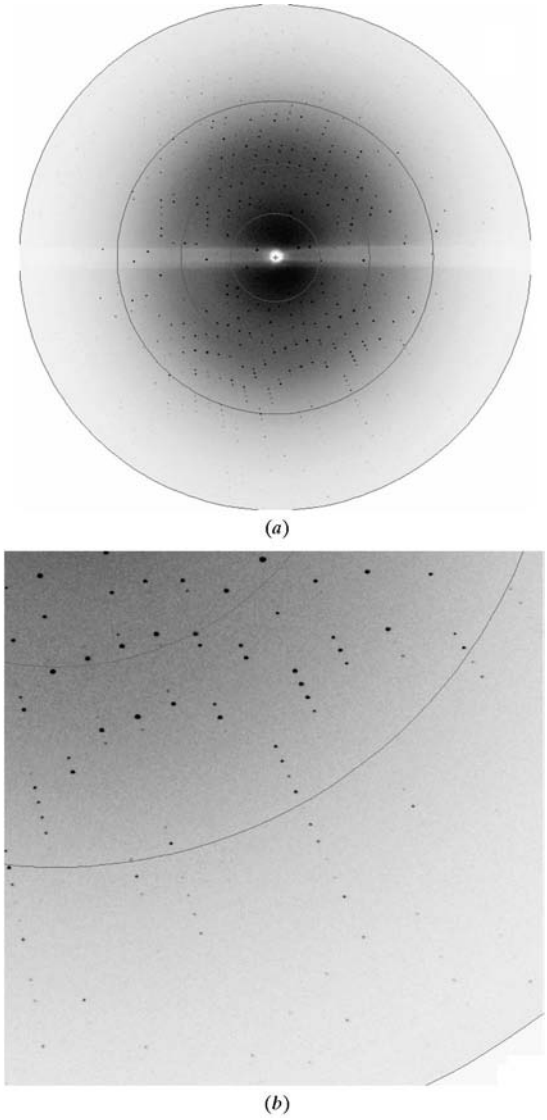

Figure 2
Apocrustacyanin A1 data recorded on SRS station 7.2 at 2.0 Å wavelength: (*a*) whole image and (*b*) zoomed image. The outer two diffraction resolution rings are at 3.0 and 2.3 Å (for quantitative details of processed data, see Table 2).

4. Data collection and analysis

Crystals were mounted in capillary tubes and exposed to X-rays at station 7.2 (Helliwell *et al.*, 1982) of the SRS, where a Ge(111) single-crystal monochromator was set to reflect X-rays at λ = 1.488 Å. The experiment was conducted at room temperature (293 K) and a MAR 345 image-plate detector system was used. A total of 90 frames were recorded and processed, each covering an oscillation range of 1.0°, from two parts of one crystal.

MOSFLM (Leslie, 1992) was used to deduce the unit-cell parameters and symmetry and to reduce the reflection data. The unit cell was orthorhombic, space group $P2_12_12_1$ (No. 19), with unit-cell parameters $a = 41.8$, $b = 81.0$, $c = 110.6$ Å. Assuming two apoprotein molecules (∼20 kDa each) were present in the asymmetric unit, the calculated value of V_m (Matthews, 1968) would be around 2.5 Å^3 Da^{-1}, which is within the expected range for most globular proteins. The volume fraction occupied by solvent would then be around 51%. These unit-cell parameters are similar to those of apocrustacyanin C1 ($a = 42.0$, $b = 80.9$, $c = 110.7$ Å, also $P2_12_12_1$). The c unit-cell parameter can vary between crystals of apocrustacyanin C1 (with $c = 105$ Å also observed once; Boggon, 1998). The molecular basis of that variation is also then of keen interest.

The *CCP*4 package (Collaborative Computational Project, Number 4, 1994) was used for scaling and merging the final data set (*SORTMTZ*, *SCALA*, *TRUNCATE*). Relevant statistics are shown in Table 1.

5. Structure-determination approach

For structure determination of apocrustacyanin C1, the three different (*i.e.* 'standard') avenues of molecular replacement with a lipocalin protein model, heavy-atom derivative search and xenon pressure have not been successful (for lobster, Gordon, personal communication; for lobster and *V. vellela*, Boggon, 1998). For heavy-atom derivatization in particular, this is a problem arising from the high pH of the crystals, which appears to disfavour derivatization as revealed *via* a database search. New avenues for structure solution must be sought. The amino-acid sequence for apocrustacyanin C1 is known and contains no methionine residues. Apo A1 is believed to be similarly without methionine (Zagalsky, personal communication). An avenue being investigated is the use of anomalous differences from the three putative disulfides per monomer (six per asymmetric unit from 12 cysteines) so as to then use the 12 S atoms in conjunction with atomic resolution data (for apocrustacyanin C1, 0.95 Å cryocrystallography data have now been recorded at the ESRF; Cianci *et al.*, 1999). The expected anomalous differences (on *F*) at 1.488 and

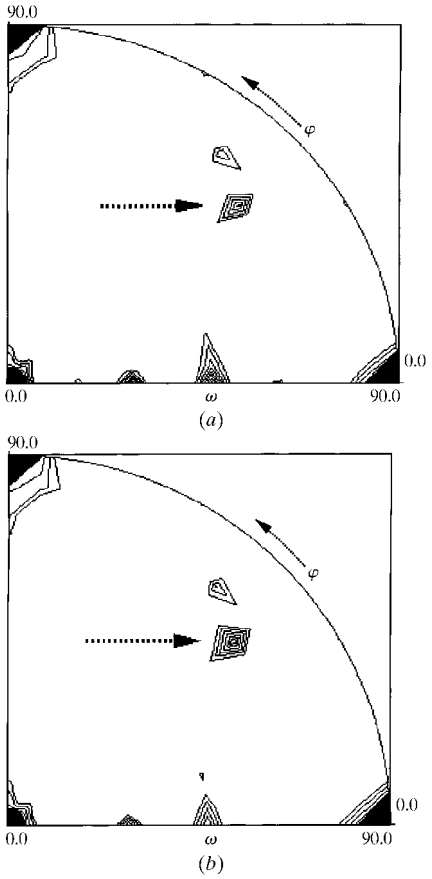

Figure 3
Self-rotation $\kappa = 180°$ section for (*a*) the data in Table 1 (1.488 Å wavelength) and (*b*) the data in Table 2 (2.0 Å wavelength) for apocrustacyanin A1. The general peak marked by the arrow is 5σ in (*a*) and 5.5σ in (*b*).

Table 1
Merging statistics of the 1.488 Å wavelength data set of apocrustacyanin A1.

Resolution range	R_{merge}	$I/\sigma(I)$	N_{meas}	N_{unique}	Completeness	Multiplicity
6.32	0.064	8.7	3199	914	97.6	3.3
4.47	0.066	8.2	6119	1668	100.4	3.6
3.65	0.070	8.0	7816	2099	99.4	3.7
3.16	0.076	7.2	9422	2495	100.5	3.7
2.83	0.098	6.3	10707	2778	99.4	3.8
2.58	0.124	4.9	12071	3075	100.3	3.9
2.39	0.154	4.0	13138	3287	99.6	4.0
2.24	0.192	3.2	14128	3503	99.7	4.0
2.11	0.247	2.4	15196	3738	100.4	4.0
2.00	0.335	1.7	16037	3869	99.5	4.1
Overall	0.097	4.8	107833	27426	99.8	3.9

Table 2
Merging statistics of the 2.0 Å wavelength data set of apocrustacyanin A1.

Resolution range	R_{merge}	$I/\sigma(I)$	N_{meas}	N_{unique}	Completeness (%)	Multiplicity
7.27	0.039	11.8	2113	580	98.0	3.6
5.14	0.045	12.4	3752	994	98.4	3.8
4.20	0.051	11.3	4808	1301	99.1	3.8
3.64	0.057	10.2	5555	1532	99.4	3.7
3.25	0.066	8.9	6245	1723	99.5	3.7
2.97	0.073	8.3	6806	1886	99.7	3.7
2.75	0.085	7.1	7221	1993	99.8	3.7
2.57	0.099	6.0	7657	2087	99.8	3.7
2.42	0.113	5.2	7969	2174	99.9	3.7
2.30	0.122	5.1	8055	2194	99.7	3.6
Overall	0.064	7.9	60181	16464	99.7	3.7

2.0 Å wavelength on this basis are 1.4 and 2.4%, respectively, if the two S atoms in each disulfide reinforce each other's signal, as is largely true at low to medium resolution. The feasibility of recording softer X-ray wavelength data on station 7.2 has been demonstrated at a wavelength of 1.860 Å (Einspahr *et al.*, 1985) and even up to 2.6 Å wavelength (Helliwell, unpublished work).

6. Experimental results with softer X-rays on apocrustacyanin A1

Diffraction data have been recorded at 2.0 Å wavelength on station 7.2. Fig. 2 shows the diffraction pattern and Table 2 the data-processing statistics from *DENZO*/*SCALEPACK* (Otwinowski & Minor, 1997). These show that the 2.0 Å softer X-rays can easily penetrate the sample glass capillary, the air path to the detector and the MAR 345 window itself. As Fig. 2 and Table 2 show, the storage phosphor yields very good quality data at this wavelength. Moreover, another indicator of the high data quality and completeness obtained is the self-rotation $\kappa = 180^\circ$ section for both the 1.488 Å wavelength (Table 1) data set and the 2.0 Å wavelength (Table 2) data set (Figs. 3*a* and 3*b*). Absorption in these crystals (approximate cross section 0.2 × 0.1 mm) is quite tolerable, *i.e.* T_{max}/T_{min} is 1.2 at 2 Å wavelength compared with 1.09 at 1.488 Å wavelength. Radiation damage was not especially severe at the longer wavelength, as the data statistics (Table 2) show; indeed, the 2 Å wavelength data has better statistics, especially at higher resolution, thus demonstrating the effectiveness of a longer wavelength for data collection from smaller crystals (for a discussion, see Helliwell, 1993). These two data sets scale well ($\Delta F/F$ is 13% to 2.3 Å and 7% on the strongest *F*s) and have a correlation coefficient of 0.97. The average $\Delta_{anom}/\sigma(\Delta_{anom})$ also shows an improvement at 2 Å wavelength compared with 1.488 Å wavelength, with an average of 0.77 compared with 0.56, respectively (compared with 2.3 Å). However, anomalous difference Patterson Harker sections have not yielded convincing triplets of vector peaks. Hence, future experiments will be targeted at increased multiplicity of data (the result shown here using 2.0 Å wavelength has a multiplicity of 'only' 3.7; Table 2). Working with a single crystal to achieve high data multiplicity will require cryoprotection of the crystal, the feasibility of which has been demonstrated with apocrustacyanin C1 (Cianci *et al.*, 1999). The weak anomalous signal of sulfur has been harnessed *via* a high data multiplicity (20-fold) approach by Dauter *et al.* (1999) for lysozyme as a test case using 1.54 Å wavelength at NSLS for 1.5 Å resolution data recorded at cryotemperature. Weak isomorphous signals and a high data multiplicity (30-fold) were also successfully used to solve the crystal structure of cubic concanavalin A, with 50 kDa in the asymmetric unit (Harrop *et al.*, 1996). Moreover, weak anomalous differences were used successfully with *MULTAN* to locate the Mn cofactors in pea lectin with 50 kDa in the asymmetric unit (Mukherjee *et al.*, 1989).

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