Carotenoproteins: advances in structure determination

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Abstract - The amino acid sequences of the two main subunits (CRTA and CRTC) of \( \alpha \)-crustacyanin, the lobster carapace carotenoprotein, have been determined. Computer graphics have been used to model the structures of the subunits, that of the dimer, \( \beta \)-crustacyanin, and the putative binding-sites for astaxanthin. Progress in determining the crystal structures and advances in sequencing carotenoproteins from other invertebrate phyla is reported.

INTRODUCTION

At the 8th International Symposium on Carotenoids in Boston (1987) the author presented (ref. 1) the partial amino acid sequence (160 consecutive residues) of one of the main 20 kDa subunits (A2; CRTA) of the lobster carapace astaxanthin-protein, crustacyanin. A significant but low sequence homology to human plasma retinol-binding protein (RBP) was shown (23% over 142 aligned residues) and several of the residues identified in contact with retinol in the crystal structure of RBP (ref. 2,3) were found to be conserved in CRTA. RBP is a member of a superfamily, the lipocalins, comprising over 20 different proteins (ref. 4,5) which bind small lipophilic ligands. The crystal structures of several other members of the superfamily are known, namely those of \( \beta \)-lactoglobulin (BLG) (ref. 6), bilin-binding protein (BBP) (ref. 7,8), insecticyanin (INS) (ref. 9) and rat urinary \( \alpha \)-2-globulin and mouse major urinary protein (ref. 10). Although residue identities between pairs of lipocalins is low, the general form of the tertiary structures is identical in the latter cases: a \( \beta \)-barrel structure consisting of 8 antiparallel \( \beta \)-strands of \( ^{+1}+1+1 \) repeated topology arranged in two orthogonal \( \beta \)-sheets with a cavity (calyx) between them where the hydrophobic ligand is bound. We were able, by combining information from different secondary structure prediction algorithms together with the sequence alignment with RBP, to define roughly the eight putative \( \beta \)-strands of CRTA.

In this article the progress made in elucidating the structure of crustacyanin and the structures of other carotenoproteins from different invertebrate phyla is reported.

CRUSTACYANIN: AMINO ACID SEQUENCES OF THE APOPROTEINS AND MOLECULAR MODELLING

The apoproteins

The complete primary structures of the two main subunits, apoprotein A, (CRTA, 174 residues) and apoprotein C, (CRTC, 181 residues), have now been assembled from the sequences of the whole proteins and peptide fragments produced by chemical and enzymic cleavage (ref. 11-13). The amino acid sequences are closely similar. The two consensus sequences of 13 and 11 residues separated by about 80 residues, characteristic of the lipocalins (ref. 14), are conserved for the apoproteins, more highly so for CRTA. CRTC has a five-residue insertion (residues 23-27) in the consensus 1 region (N terminus) and a lower conservation of consensus 2:

| Consensus 1 | Asn --- Hyd --- Acd --- Hyd --- Xaa- Bas- Hyd- Xaa- Gly- Xaa- Trp- Aro- Xaa |
| CRTA | Asn --- Phe --- Asp --- Leu- Arg- Arg- Tyr- Ala- Gly- Arg- Trp- Tyr- Gln |
| CRTC | Leu --- Trp --- Ala --- Arg- Asn- Ser- Tyr- Ala- Gly- Val- Trp- Tyr- Gln |

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Fig. 1. Amino acid sequences of crustacyanin subunits A, (CRTA) and C, (CRTC). The alignment of the sequences is that obtained using automatic and interactive computer alignment methods. Identities in the alignment are given an asterisk and conservative substitutions are indicated by a vertical line.
Consensus 2 Aro --- Xaa- Hyd- Hyd- Xaa- Thr- Asp- Tyr- Acd- Xaa- Aro
CRTA Tyr --- Glu- Val- Ile- Glu- Thr- Asp- Tyr- Glu- Thr- Tyr^12
CRTC Leu --- Val- Ile- Leu- Glu- Thr- Asp- Tyr- Ser- Asn- Tyr^12

where Xaa is any residue, Aro is Phe or Tyr, Hyd is a hydrophobic residue (Ile, Leu, Met, Phe, Tyr or Val), Acd is Glu or Asp and Bas is Arg or Lys.

Interestingly, the patterns of charged residues in the consensus sequences of both subunits are complementary in that basic and acidic residues predominate, respectively, in consensus 1 and 2, but with fewer charged residues for CRTC.

The structure of CRTA was modelled, using computer graphics, to the co-ordinates of RBP and the structure of CRTC was subsequently modelled on the inferred structure of CRTA. The sequence alignment of CRTA and CRTC based on the structural alignment of the proteins is given in Fig. 1. In this alignment 65 residues (38%) are identical and 43 conservatively substituted over 171 residues. The five cysteine residues of CRTA are conserved in CRTC but in no position are there more than six consecutive residues conserved between the proteins. Cross-reaction between the subunits and their polyclonal antibodies (CRTA/Polyclonal CRTC; CRTC/Polyclonal CRTA), between the subunits and monoclonal antibodies to RBP (CRTA/monoclonal RBP; CRTC/monoclonal RBP) and between RBP and the polyclonals (RBP/polyclonal CRTA; RBP/polyclonal CRTC) indicate that all three proteins have epitopes in common (ref. 15).

The eight β-strands in the structure of each subunit are arranged in two curved sheets with an orthogonal orientation to each other (see Fig. 3). Sheet 1 comprises β-strands A₁, B, C, D, E, and F, and sheet 2 β-strands A₂, G, H and A₁. The ligand-binding pocket between the two sheets is effectively closed at diagonally opposite corners by strands A and F which are part of both sheets. The N-terminus and C-terminus-strand I wrap around opposite sides of the barrel with the N-terminus, close to the cavity opening, fixed to sheet 2 by disulphide bridge to strand G (CRTA: 12-119; CRTC: 12-121) and with the C-terminus similarly fixed to sheet I at the beginning of strand B (CRTA: 170-46; CRTC: 172-51). This disposition of disulphide bonds is different from that in RBP but the same as that found in INS/BP. The α-helix lies against sheet 2 making contacts with residues from β-strands A₁, H and G and lies at a small angle to strand H. The locations of the N-terminal residues and of the longer C-terminal tail of CRTC, not established in the modelling against RBP, were arranged as in INS (ref. 9) with the termini near the openings of the binding pockets. Loop regions B/C, D/E and N-terminus-A₁ form an open interface area protruding from sheet 1 of the barrel perpendicular to the cavity. Loops G/H, C/D and E/F are located at the opening of the cavity; the residues of loops C/D and E/F in RBP, thought to serve as the transthyretin binding region (ref. 3), are deleted in CRTA and CRTC. Noteworthy differences between the subunits are the deletion of Asp 71-Glu 72 of CRTA in loop C/D of CRTC. the insertion of Phe 126 in loop G/H of CRTC. mutation of Glu 145 in the helix of CRTA to lys 148 in CRTC and oppositely charged residues in loops B/C.

The bottoms of the cavities are closed by the conserved Trp (strand A₁) of consensus sequence 1 and an underlying Arg residue (CRTA: Arg 135; CRTC: Arg 138), common to most members of the superfamily (ref. 4,5); consensus sequence 2 lies in the same region of the tertiary structures.

Of the residues potentially lining the cavities of CRTA (35 residues) and CRTC (33 residues), 26 are conserved or conservatively substituted (see Fig. 2). Almost half the cavity residues of CRTA and CRTC are conserved or conservatively substituted in RBP, and of 17 residues close to retinol in RBP (ref. 3) 9 residues (CRTA) and 7 residues (CRTC) are conserved or conservatively substituted in the apoproteins. These facts lend support to the putative structural models.

The astaxanthin molecule has been positioned within the cavity of each protein end-on, with the 4-keto and 4-hydroxy groups in hydrogen-bonding distance of conserved Thr and Tyr residues, respectively, of the two subunits (CRTA: Thr 63 and Tyr 51; CRTC: Thr 68 and Tyr 56), as consistent with the prerequisite for a 4-keto group in the carotenoid structure for attachment to the proteins. The carotenoids are then deep within the cavities close to the conserved Trp of consensus sequence 1, a position validated in binding experiments with hydrophobic fluorescent probes 1,8-anilinonaphthalene sulphonate and the carotenoid analogue cis-parinaric acid (ref. 16). Selective modification of Trp residues of apocrustacyanin eliminates the bathochromic shift in the absorption spectrum of the reconstituted astaxanthin-protein (ref. 17).

The positioning of the carotenoids within the cavities is somewhat subjective. In model 2 of β-crustacyanin (see later) the positions of exit of carotenoids from the cavities initially proposed (ref. 12,13) have been...
modified by minor readjustments of the protruding ends. The carotenoids are thereby in more apolar environments with better packing of aromatic residues about the carotenoid planes (ref. 18). Tyr 125 (loop G/H) of CRTA and Tyr 128 of CRTC are then close to the outer methyl groups of the polyene chains (Fig. 2A). Strand joins G/H may fold to encapsulate the carotenoids, bringing further aromatic residues into the vicinity of the carotenoid for CRTC. The cavity residues in contact with the carotenoids are essentially aromatic, aliphatic hydrocarbon and polar. Thirteen of the 15 residues in contact with astaxanthin in CRTA are conserved or conservatively substituted in CRTC; 8 of these may be close to the carotenoid. Several of the putative carotenoid contact residues are conserved at the retinol-binding site in RBP (Fig. 2B). Two charged residues, Arg 79 (strand D) and Asp 123 (loop G/H), may lie close to the polyene chain of CRTC. It is possible that Arg 79 folds to form an electrostatic linkage with Glu 98 (loop E/F). Asp 123, however, lies close to the conjugated system between methyl groups 19 and 20 of the outer half of astaxanthin.

Two of the 4 cavity Tyr residues of both CRTA and CRTC may be within 4Å of the carotenoid. Treatment of the apoproteins with tetranitromethane (TNM), a reagent specific for modification of Tyr residues, eliminates carotenoid-binding following reaction of 2 to 4 Tyr per subunit (ref. 19). Whether or not cavity Tyr are selectively modified by the hydrophobic reagent has not yet been determined, but clearly Tyr residues are of importance for structural integrity of the carotenoid-binding sites.

Residues in contact with the polar carotenoid, spheroidene, in the reaction centre of the purple bacterium *Rhodobacter sphaeroides* (ref. 20) and with retinal in bacteriorhodopsin (ref. 21) have a similar chemical composition.

Fig. 2 Residues lining the putative binding cavity in (a) CRTA (b) CRTC. Shown is a "target view" into the cavities. The concentric circles represent the "depth" of side chains within the cavity, with the outermost residues near the cavity entrance. Sheets 1 and 2 of each protein lie across the figure, above and below the conserved Trp, respectively. Residues within 4Å of the carotenoid at its proposed binding position (model 2) are boxed; those shaded are conserved or conservatively substituted positions in RBP in the 3-D superpositioned models and within 4 Å of the retinol ligand for RBP.

**β-Crustacyanin**

The structure of the dimeric β-crustacyanin was originally modelled (ref. 12,13) as a face-to-face association of CRTA and CRTC, based on the crystal structure of the dimer of BBP (ref. 8). The CRTA and CRTC subunits of β-crustacyanin and α-crustacyanin, an octamer of β-sized units, give cross-linked dimers on treatment with lysine-specific imidoesters. Consideration of the positions of lysine residues in the β-crustacyanin model (model 1) predicts that the shorter dimethyladipimidate should be more effective than dimethylpimelimidate in forming cross-linked dimers, whereas the converse is found (ref. 21). Model I may also be criticised for leaving the ends of each carotenoid protruding from the cavities and poorly shielded by protein. An alternative face-to-face orientation of CRTA and CRTC (Model 2) which addresses satisfactorily
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both these issues and preserves pseudo two-fold symmetry is shown in Fig. 3 and 4. Model 2 (ref. 18) involves the continuation of the β-sheet 1 structure across the dimer by hydrogen-bonding of strands F, (CRTA: 96-100; CRTC: 98-102). Interface interactions are more extensive than for model 1 and occur between loops A/B (CRTA: 39-41; CRTC: 44-46), between the N-termini (CRTA: 2-4; CRTC: 2-4), between loop C/D (CRTC: 70-72) and loop G/H (CRTC: 126-128) and between loop G/H (CRTC: 123-125) and loop C/D (CRTC: 74-76). In this arrangement five acidic residues are located at the subunit interface. Treatment of apocrustacyanin with diimides to convert acidic residues into amides inhibits subunit dimerisation on reconstitution with astaxanthin (ref. 22), giving some credence for the putative interface regions; a similar result is obtained on treatment of the apoproteins with TNM (ref. 18), consistent with the positioning of Tyr residues of loops A/B (CRTA: 40; CRTC: 45) at the interface.

Fig. 3. Ribbon diagram of the proposed model 2 of β-crustacyanin. CRTA is at the top of the figure.

The protruding ring of the carotenoid in CRTA is placed between sheets 1 and 2 of CRTC in the region of hydrophilic residues of loop C/D (Asp 73), loop A/B (Glu 49) and strand B (Lys 50) and covered with the N-terminus (Asp 1), and possibly C-terminus (Ser 175, Thr 177 and Gln 178), of CRTC. Likewise the protruding ring of the astaxanthin in CRTC may be in the vicinity of hydrogen-bonding residues of strand C (Asn 68), loop A/B, (Thr 44 and Arg 45), and the N-terminus (Asp 1) of CRTA. The angle between the two long axes of the carotenoids is ca 75° with ca 12.5 Å between their centres, close to the values predicted from the circular dichroism spectrum of β-crustacyanin [77° and 4 to 10 Å (Gardiner and Thomson quoted in ref. 23)].
**α-Crustacyanin**

The lipocalin superfamily shows no consistent pattern in quaternary structure (ref. 10). Several regions of possible interface contact between the dimeric units within the octameric α-crustacyanin can be considered (ref. 12), based on examples from the superfamily. Interaction involving the surface helices is found in the tetratetramers of INS (ref. 9) and BBP (ref. 7,8). The mutation of Lys 148 in the helix of CRTA to Glu 145 in CRTC is noteworthy; the helices of these subunits are amphiphilic with hydrophilic surfaces having hydrophobic grooves suitable for intermolecular interaction. The complementary charge distribution, mentioned earlier, of the consensus 1 and 2 sequences, which lie close together in the putative protein structures, make these but one of many candidates for interface interaction (ref 12).

**Crystal structures**

It must be stressed that the putative models for CRTA, CRTC, the carotenoid-binding sites and β-crustacyanin may well contain inaccuracies. They are merely a starting point for consideration and experimental testing. NMR studies with 13C-labelled astaxanthin to probe the carotenoid-binding sites are providing complementary and additional information (ref. 24).

It will be interesting to know both how near (or far) the putative protein structures are from reality and the efficacy of the predictive methods employed. Some progress towards answering these questions is being made. Crystals of CRTC (subunit C,) diffracting beyond 2.2 Å have been obtained and a preliminary 2.4 Å data set collected (ref. 25). Data sets have also been collected from crystals of subunits A, and C, and from crystals of subunits C, and C, with bound astaxanthin (ref. 26); subunits A, and C, are variants of subunit C,. Large, variously shaped crystals of α-crustacyanin have been obtained under many different conditions but have failed to show a diffraction pattern (ref. 26). There has been better success with β-crustacyanin and it is hoped to improve on the quality of the crystals so far obtained, which diffract to 6 Å (ref. 27).

Molecular replacement using the lipocalins as search models has failed to assist in interpreting the X-ray data and the search is now on for suitable heavy-atom derivatives so that the structures may be solved by the isomorphous replacement method.

**OTHER CAROTENOPROTEINS**

A single type of carotenoid-binding site is unlikely to account for the diverse range of variously coloured carotenoproteins in Nature. Ovoverdin, the green astaxanthin-lipovitellin complex of lobster eggs (ref. 23) fails to cross-react with polyclonal antibodies to CRTA or CRTC, or with monoclonal antibody to RBP; the same is true for carotenoid-lipovitellins from other phyla (e.g. Cirripedia: *Lepas* sp.) and for ovorubin (ref. 23), the egg astaxanthin-glycoprotein of the gastropod, *Pomacea canaliculata* (ref. 15). The blue carotenoproteins of the chondrophores *Vellella* and *Porpita* (Phylum: Coelenterata) (ref. 23), on the other hand,
cross-react with the CRTA/CRTC antibodies (ref. 15). Preliminary N-terminal amino acid sequences of two closely related subunits of the Velette carotenoprotein have confirmed their similarity to CRTA/CRTC (ref. 1, 28).

Studies on the amino acid sequences of the skin carotenoproteins, linckiacyanin and asteriarubin, of the primitive starfish (phylum: Echinodermata) Linckia laeviswega (calculated) and Asterias rubens (non-calculated), respectively, have revealed a new carotenoid-binding protein family. The two 8 kDa glycoprotein subunits (apolinc. 1, 2) of linckiacyanin have not been separated but are, fortunately, highly homologous in sequence. The combined sequence (63 residues, Fig. 5), almost complete, reveals that mutations are limited to 7 positions, of which 3 are conservative substitutions; the glycosylation site is at Asp 19 (ref. 28). Two subunits of asteriarubin (apoast.1 and apoast.2), of similar size to those of linckiacyanin, have been separated and partially sequenced (41 and 36 residues, respectively) (ref. 29). They are more closely related to the linckiacyanin subunits than to each other (apoast.1/apoast.2: 5 identities over 36 residues; apoast.1/apolinc.1,2: 11 identities over 41 residues; apoast.2/apolinc.1,2: 9 identities over 36 residues). The conservation of cysteine residues for the sequences supports the case for homology between the proteins (Fig. 5).

Apoast. 1  |  T  S  D  S  M  A  C  E  V  C  K  S  V  |
Apolinc. 1,2  |  S  G  D  Y  F  P  C  M/L  W  C  T  Q  L  |
Apoast. 2  |  D  G  Q  Q  L  A  C  T  Y  C  L  D  T  |

Apoast. 1  |  P  L  L  A  Q  Q  V  L  T  S  K  G  F  |
Apolinc. 1,2  |  V  T  L  A  Y/H  T/M  T  V  D  K  G/A  F/L  |
Apoast. 2  |  V  T  Y  L  K  S  T  D  D  I  D  T  |

Apoast. 1  |  L  D  A  V  V  K  F  L  L  I  N  C  D  L  |
Apolinc. 1,2  |  I/M  A  D  L  Q/F  R  F  L  R  Y  Y  L  C  K  Y  W  |
Apoast. 2  |  T  N  A  I  M  Q  X  M  Q  V  |

Fig 5. Partial amino acid sequences of the subunits of linckiacyanin (apolinc.1,2) and asteriarubin (apoast.1 and apoast.2). The two sequences apolinc.1 and apoast.2 are identical except at the 7 positions shown. Identities in the alignment are given an asterisk and conservative substitutions are indicated by a vertical line.

The relationships are somewhat unexpected considering the quite distinct spectral, molecular and carotenoid-binding properties of the carotenoproteins (ref. 23). A search of the protein sequence data bank has shown that the amino acid sequences of the proteins are unique. Linckiacyanin and asteriarubin thus form a distinct and novel group of carotenoid-binding proteins. Consequently, although the protein secondary structure of linckiacyanin has been predicted, modelling of the tertiary structure will be far less straightforward than for the crustacyanin subunits.

The red colours of some articulate branchiopods derive from carotenoproteins possibly involved in the shell biomineralisation. The carotenoproteins, binding canthaxanthin and 7,8-didehydroastaxanthin in one example (Terebratella sanguinea), have 6.5 kDa subunits that are highly homologous in sequence for the first 20 N-terminal residues. Again, the partial sequences appear unique. The proteins may prove to be members of a separate carotenoid-binding family (ref. 31) but there is weak similarity to residues 125-146 of CRTC.

Finally, the amino acid sequences, derived from gene nucleotide sequences, of phytoene desaturase from photosynthetic and non-photosynthetic organisms (ref. 32-34) and of a cytoplasmic membrane carotenoprotein from a cyanobacterium (ref. 35) have been published: the carotenoid-binding domains of these large proteins (450-600 residues) have not been established.
REFERENCES

15. P.F. Zagalsky, R.S. Mummery, unpublished results.
24. J. Lugtenburg, Pure Appl. Chem. this volume.
27. N. Chayen and T. Skarsinski, unpublished results.