The lobster carapace carotenoprotein, α -crustacyanin

A possible role for tryptophan in the bathochromic spectral shift of protein-bound astaxanthin

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Crustacyanin, cross-linked with dimethyl pimelimidate to stabilize the protein against denaturation, was used to test the effects of tryptophan modification with BNPS-skatole [3-bromo-3-methyl-2-(nitrophenylmercaptol)-3H-indole] on the ability of the apoprotein to recombine with astaxanthin. The cross-linked apoprotein re-forms α -crustacyanin with astaxanthin in reasonable yield following incubation of the protein under the conditions for tryptophan modification in the absence of BNPS-skatole. The BNPS-skatole-treated protein reconstitutes with astaxanthin to give a carotenoprotein with $\lambda_{\max}$ at 472 nm, that of the carotenoid in hexane, in a yield similar to that of the BNPS-skatole-untreated control. The implied involvement of tryptophan residues at the sites of astaxanthin attachment in crustacyanin and their possible roles in the binding sites of vitamin A in vitamin A-proteins are discussed in relation to the bathochromic spectral shifts of the chromophores.

INTRODUCTION

The blue coloration of the lobster carapace results from the presence of the astaxanthin-protein, α -crustacyanin ($\lambda_{\max}$ 632 nm) (Wald *et al.*, 1948). The absorption spectrum of the carotenoid ($\lambda_{\max}$ 472 nm in hexane) is red-shifted by 160 nm on combination with the apoprotein, without alteration in band shape. Particular interest in this phenomenon stems from the comparable alteration in absorption spectrum of retinal in retinal-proteins: for example, for 11-*cis*-retinal ($\lambda_{\max}$ 365 nm in hexane) in the retinal rod pigment, for rhodopsin ($\lambda_{\max}$ 498 nm) (Yoshizawa & Wald, 1963) and for all-*trans*-retinal ($\lambda_{\max}$ 370 nm in hexane) in the proton-pump pigment of *Halobacterium halobium*, bacteriorhodopsin ($\lambda_{\max}$ 568 nm) (Oesterhelt & Stoekenius, 1971).

The amino acid sequences of the two major approx. 20 kDa apoproteins (A_2 and C_1) of crustacyanin (approx. 320 kDa) show no resemblance to the sequences of the retinal-proteins; however, each apoprotein has a low level of sequence identity (approx. 25%) with human retinol-binding protein (RBP) (Findlay *et al.*, 1990; Zagalsky *et al.*, 1990a; J. N. Keen, I. Caceras, E. E. Eliopoulos, P. F. Zagalsky & J. B. C. Findlay, unpublished work). RBP has a β -barrel structure consisting of eight anti-parallel β -strands of +1+1+1 repeated topology arranged in two orthogonal β -sheets with a cavity or calyx forming the retinol-binding site between them (Newcomer *et al.*, 1984). In the latter complex the absorption spectrum of retinol is not significantly altered (Horwitz & Heller, 1974). The two consensus sequences characteristic of the RBP/ β -lactoglobulin (BLG) superfamily (North, 1989a) are largely conserved for the apocrustacyanin subunits, and their tertiary structures have been modelled using computer graphics (J. N. Keen, I. Caceras, E. E. Eliopoulos, P. F. Zagalsky & J. B. C. Findlay, unpublished work) to the co-ordinates of the crystal structure of RBP (Newcomer *et al.*, 1984).

Separately, the apoproteins bind a single astaxanthin molecule giving complexes of $\lambda_{\max}$ 555–570 nm (Warburton, 1986). The

putative binding site for astaxanthin in each apoprotein is probably situated, as for retinol in RBP (Newcomer *et al.*, 1984), within the cavity, or calyx, formed by the orthogonal β -sheet structure common to members of the RBP/BLG superfamily (J. N. Keen, I. Caceras, E. E. Eliopoulos, P. F. Zagalsky & J. B. C. Findlay, unpublished work). One end of this calyx is closed by a conserved tryptophan residue (β -strand A) present in the N-terminal consensus sequence. Studies with the isolated apoproteins using hydrophobic probes 8-anilidonaphthalene-1-sulphonic acid and the carotenoid analogue *cis*-parinaric acid show evidence for fluorescence transfer from tryptophan to the ligands and support an end-on orientation of astaxanthin in each apoprotein cavity with one β -ionone ring in close proximity to the conserved tryptophan residue (Clarke *et al.*, 1990). Apoprotein A_2 has a unique tryptophan residue (Trp-30); apoprotein C_1 has, in addition to the cavity-blocking residue of the N-terminal consensus sequence (Trp-35), a second tryptophan residue (Trp-21) internally directed but partially accessible to solvent and extraneous to the putative carotenoid-binding cavity (J. N. Keen, I. Caceras, E. E. Eliopoulos, P. F. Zagalsky & J. B. C. Findlay, unpublished work). A putative structure for β -crustacyanin ($\lambda_{\max}$ 585 nm), the dimer of the A and C subunits, in which the apoproteins are aligned face-to-face (cavity-to-cavity), so protecting the protruding ends of the carotenoid attached to each subunit, has been derived (J. N. Keen, I. Caceras, E. E. Eliopoulos, P. F. Zagalsky & J. B. C. Findlay, unpublished work).

Attempts to identify amino acid residues at the carotenoid-binding sites by chemical modification of specific residues have not met with success. One difficulty that limits the efficacy of such experiments is the instability of apocrustacyanin, which may readily lose its ability to bind carotenoid under the conditions of amino acid modification. The stability of crustacyanin to denaturation is enhanced by cross-linking the protein with dimethyl pimelimidate (DMP) (Zagalsky *et al.*, 1990b). In the present paper we report the effect of BNPS-skatole treatment on the preserved ability of the DMP-cross-linked apoprotein to interact with astaxanthin.

Abbreviations used: DMP, dimethyl pimelimidate; BNPS-skatole, 3-bromo-3-methyl-2-(nitrophenylmercaptol)-3H-indole; RBP, human serum retinol-binding protein; BLG, β -lactoglobulin; Rh₄₉₈, rhodopsin; BR₅₆₈, bacteriorhodopsin; DMP-apoprotein, apocrustacyanin prepared from DMP-cross-linked α -crustacyanin.

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MATERIALS AND METHODS

Materials

Lobsters [*Homarus gammarus* (L.)] were purchased from Nye and Son, Billingsgate Market, London, U.K. Dimethyl pimelimidate (DMP) was purchased from Pierce Chemical Co., Rockford, IL, U.S.A., and 3-bromo-3-methyl-2-(nitrophenylmercaptol)-3H-indole (BNPS-skatole) from Fluka Chemie, Buchs, Switzerland.

α -Crustacyanin was extracted and purified from lobster carapace as described in Quarmby *et al.* (1977). The protein was cross-linked with DMP as in Zagalsky *et al.* (1990b); the apoprotein was prepared by removal of the carotenoid from the cross-linked protein with acetone/diethyl ether, as detailed in the latter paper.

Tryptophan modification

Tryptophan modification was carried out in the manner of Omenn *et al.* (1970). Under the conditions of modification tryptophan, methionine and cysteine are selectively oxidized; tyrosine was included in the incubation mixture as an additional precaution to conserve protein tyrosine residues.

The cross-linked apoprotein (DMP-apoprotein) (0.7 mg/ml) was treated in 80% (v/v) acetic acid (AristaR grade, BDH Chemicals, Poole, Dorset, U.K.) with BNPS-skatole (0.13 mg/ml, 5 $\times$ molar excess over protein tryptophan + methionine residues) in the presence of L-tyrosine (0.2 mg/ml) for 25 min at 22 °C. A control sample of the DMP-apoprotein was treated similarly but with omission of BNPS-skatole. Following the reaction, both DMP-apoprotein samples (modified and control) were either (a) diluted with cold water to 10% (v/v) acetic acid, frozen in liquid N₂ and freeze-dried, or (b) passed through a column of Sephadex LH20 (Pharmacia, Uppsala, Sweden) equilibrated with 70% (v/v) acetic acid to remove reagents and then processed as in (a). In the former case, the freeze-dried protein was dissolved in cold 50 mM-Tris/HCl buffer, pH 6.9, and centrifuged to remove insoluble BNPS-skatole, and remaining reagents were removed by gel filtration on Sephadex G-25 (medium grade) (Pharmacia) equilibrated with the Tris buffer.

Reconstitution of carotenoproteins

Astaxanthin in cold acetone, recovered from the ether phase in the preparation of the apoprotein, was added dropwise to the DMP-apoprotein (BNPS-treated or control) (2 mg/ml) in 0.25 M-Tris/HCl buffer, pH 6.8, at 0 °C, until the acetone concentration of the mixture was 50%. The quantity of carotenoid added was twice the amount required for complete reconstitution of the carotenoprotein. The solution was diluted to 10% (v/v) acetone with cold Tris buffer and dialysed extensively (Cuprophane tubing; Medicell International, London, U.K.), first against distilled water (3 h) and then against 0.05 M-Tris/HCl buffer, pH 6.8 (24 h), to remove acetone. Care was taken to ensure that these operations were carried out at ≤ 4 °C. The reconstituted carotenoproteins were adsorbed on columns of DEAE-cellulose (DEAE-52) (Whatman, Maidstone, Kent), the columns washed with 0.05 M-Tris/HCl buffer, pH 6.8, and the carotenoproteins eluted with 0.05 M-Tris/HCl buffer, pH 6.8, containing 0.5 M-KCl.

PAGE

The stability of the DMP-apoprotein cross-bridges to the BNPS-skatole treatment was examined by PAGE, performed as in Zagalsky *et al.* (1990b); 5% acrylamide gels were used for non-denaturing conditions and 15% acrylamide gels for SDS-treated samples.

Fig. 1. Effect of BNPS-skatole treatment of cross-linked apocrustacyanin on the absorption spectrum of the carotenoprotein reconstituted with astaxanthin

—, Control. DMP-cross-linked apocrustacyanin was incubated with acetic acid in the absence of BNPS-skatole, passed through Sephadex LH20 and reconstituted with astaxanthin, and the carotenoprotein was purified by DEAE-cellulose chromatography (see the Materials and methods section). ----, Reconstituted carotenoprotein following BNPS-skatole treatment, as for the control.

Fig. 2. SDS/PAGE (15% acrylamide) of BNPS-skatole-treated DMP-cross-linked apocrustacyanin

β , β -Crustacyanin; (i), BNPS-skatole-treated DMP-apoprotein.

RESULTS AND DISCUSSION

The effect of BNPS-skatole treatment of DMP-cross-linked apocrustacyanin on reconstitution of the protein with astaxanthin is shown in Fig. 1. The visible-absorption maximum of the reconstituted carotenoprotein lies at 472 nm, the $\lambda_{\max}$ of the carotenoprotein in hexane. The absorption spectrum of α -crustacyanin ($\lambda_{\max}$ 632 nm) is largely recovered for the cross-linked protein subjected to the procedure but with BNPS-skatole omitted from the reaction solution. As judged from the $A_{280}/A_{\lambda_{\max}}$ ratio (0.3 for crustacyanin) the percentage reconstitution for the BNPS-skatole-treated and untreated cross-linked proteins, 33% and 27% (Fig. 1) respectively, are comparable and only slightly lower than those obtained in some reconstitutions of apocrustacyanin with astaxanthin.

The cross-links introduced into crustacyanin with DMP withstand the conditions of the BNPS-skatole treatment. In SDS/PAGE the treated protein shows a predominant band of apoprotein dimer (Fig. 2), as was obtained from the DMP-cross-linked starting material (Zagalsky *et al.*, 1990b), with no evidence of peptide bond cleavage.

The carotenoid is evidently more loosely held in the BNPS-

Fig. 3. Representation of the putative structure of (a) apocrustacyanin A_2 and (b) apocrustacyanin C_1 , viewed into the cavity and showing the positions of tryptophan, cysteine and methionine residues

skatole-treated carotenoprotein since repeated DEAE-cellulose chromatography of the reconstituted carotenoprotein results in continuous loss of carotenoid on the bed material. The similar extents of reconstitution of the BNPS-skatole-treated and untreated cross-linked proteins and the complete absence of a 472 nm-absorbing-carotenoid-binding site in the latter protein and in crustacyanin are confirmations of the native-carotenoid-binding site being occupied following the BNPS-skatole treatment. The elimination of the bathochromic spectral shift can therefore be attributed to modification of amino acid residues within the carotenoid-binding calyx of each subunit.

In addition to tryptophan residues BNPS-skatole oxidizes cysteine (to cysteic acid) and methionine (to the sulfoxide) under the conditions utilized in the treatment of cross-linked apocrustacyanin (Omenn *et al.*, 1970). Both apoproteins A_2 and C_1 have a cysteine residue not involved in disulphide bond formation, on β -strand G, in the putative tertiary structures (A_2 , Cys-115; C_1 , Cys-117); apoprotein C_1 has an additional cysteine residue as part of the putative C-terminal α -helix (Cys-150) (J. N. Keen, I. Caceras, E. E. Eliopoulos, P. F. Zagalsky & J. B. C. Findlay, unpublished work). These cysteine residues are likely to be positioned outside the carotenoid-binding cavity buried between β -strand G and the α -helix, which lies against the β -sheet structure, in the putative tertiary structures (Fig. 3). It would seem unlikely, therefore, that oxidation of the cysteine residues (to cysteic acid) could affect the structure of the carotenoid-binding cavity, especially since treatment of crustacyanin with thiol-reactive reagents (e.g. iodoacetic acid) has no effect on its absorption spectrum.

Apoprotein A_2 has a methionine residue (Met-91, β -strand E), not conserved in apoprotein C_1 , directed internally within the cavity in the putative tertiary structure (J. N. Keen, I. Caceras, E. E. Eliopoulos, P. F. Zagalsky & J. B. C. Findlay, unpublished work). This residue, although predicted to be in the vicinity of the β -ionone ring of astaxanthin at the putative carotenoid-binding site (Fig. 3), appears to be distant from the conjugated polyene system of the carotenoid.

Apoprotein C_1 has an internally directed tryptophan residue (Trp-21), partially accessible to solvent, lying close to coil D/E (Fig. 3). Trp-21 looks to be distant from the carotenoid and

shielded from it by residues lining the cavity. Although oxidation of this residue could result in structural perturbation that would allow greater water penetration in this region, this part of the protein structure is remote from the carotenoid-binding cavity (Fig. 3). Our present understanding of the tertiary structures would suggest, moreover, that it is modification of the conserved Trp-30 (apoprotein A_2) and Trp-35 (apoprotein C_1) lying close to the β -ionone ring of astaxanthin at the putative carotenoid-binding sites (J. N. Keen, I. Caceras, E. E. Eliopoulos, P. F. Zagalsky & J. B. C. Findlay, unpublished work), rather than other residues, that primarily results in the loss of the bathochromic spectral shift following BNPS-skatole treatment.

The fine structure and bathochromic shift (approx. 1530 cm^{-1}) of the spectrum of all-*trans*-retinol (λ_{max} , 325 nm in ethanol) bound to another member of the protein superfamily, β -lactoglobulin [λ_{max} , (317), 330, 342 and (369) nm], has been attributed to interaction of the conserved tryptophan residue of the first consensus sequence (Gly-Xaa-Trp) with the β -ionone ring of the vitamin (Fugate & Song, 1980). The fluorescence excitation spectrum of BLG shows excitation resonance transfer from tryptophan to retinol; modification of a single tryptophan residue per BLG monomer, with 2-hydroxy-5-nitrobenzyl bromide, results in loss of the spectral shift and of the fine structure of the absorption spectrum without impairment of ligand binding (Fugate & Song, 1980). The retinol must lie deeper in the cavity of BLG (Papiz *et al.*, 1986), in closer proximity to the conserved tryptophan residue of the superfamily, than for RBP, in which retinol is bound without significant spectral alteration (Horwitz & Heller, 1974).

The bathochromic spectral shift for astaxanthin in crustacyanin (approx. 5300 cm^{-1}) is more closely similar to that for bound retinal in the retinal-proteins rhodopsin (Rh_{498}) (approx. 7300 cm^{-1}) and bacteriorhodopsin (BR_{568}) (approx. 9400 cm^{-1}), proteins dissimilar in structure from members of the RBP/BLG superfamily. The external point-charge model (Honig *et al.*, 1979) or variations thereof has generally been accepted as providing an adequate explanation for the spectral characteristics of these retinal-proteins. According to this theory, the shift in the λ_{max} of retinal is due to (a) interaction of a charged amino acid counterion with the protonated Schiff-base retinal-lysine

linkage (primary mechanism for BR₅₆₈ and Rh₄₉₈; Palings *et al.*, 1987) together with (b) a negative point-charge near the polyene chain (Rh₄₉₈; Honig *et al.*, 1979; Baasov *et al.*, 1987) or a negative point-charge (BR₅₆₈; Nakanishi *et al.*, 1980) or ion-pair (BR₅₆₈; Spudich *et al.*, 1986) near the β -ionone ring. In rhodopsin, Asp-83 and Glu-113, positioned in modelling studies in the cavity of the putative seven-helical bundle structure close to one of the putative positions of retinal, are candidates for the point-charge and Schiff-base counterion respectively (Findlay, 1986; Findlay & Pappin, 1986; Findlay *et al.*, 1989). Recent high-resolution electron crystallography of BR₅₆₈ has enabled a complete atomic model of the seven-helical structure (residues 8–225) to be built and the location of most of the residues that form the retinal-binding site; Asp-85 and Asp-212, within 0.4 nm (4 Å) of the Schiff base, form a complex counterion, and Asp-115 is situated near the β -ionone ring (Henderson *et al.*, 1990).

Mutagenesis studies, however, have cast doubt on the point-charge model. Replacement of Asp-83 (by Asn) or of Glu-134 (by Asp) has only a marginal effect on the spectrum of bovine rhodopsin (Janssen *et al.*, 1990). Also, single and double mutants of rhodopsin, with Asp-83, Met-86, Glu-122 or His-211 substituted, have similar $\lambda_{\max}$ values (Nathans, 1990). The conservation of Asp-83 in most G-protein-associated receptors (Findlay & Eliopoulos, 1990) also suggests that this residue fulfils a role other than that of wavelength regulation in the rhodopsins. There is conflicting experimental evidence as to whether replacement of Glu-113 (by Gln) abolishes the bathochromic spectral shift (Sakmar *et al.*, 1989) or leaves it unaffected (Janssen *et al.*, 1990). The absence of Glu-113, or any apparent equivalent residue, to act as the counterion on other putative helices in the octopus rhodopsin (Ovchinnikov *et al.*, 1988) and squid rhodopsin sequences (Hall *et al.*, 1991) adds a further element of uncertainty. In addition, results with retinal analogues have questioned the role of the chromophore–protein linkage region in the red-shift for Rh₄₉₈ (Van der Steen *et al.*, 1989). It is noteworthy in this respect that resonance-Raman studies suggest that the retinal Schiff-base linkage of insect u.v.-absorbing rhodopsin ($\lambda_{\max}$ 345 nm) is protonated (Pande *et al.*, 1987). Mutagenesis of the charged residues lining the retinal pocket of BR₅₆₈, identified in high-resolution electron microscopy (Henderson *et al.*, 1990), e.g. Asp-212, Asp-115 or Asp-85 to Asn (Mogi *et al.*, 1988; Stern *et al.*, 1989), rules out these residues as acting singly as primary modulators of the $\lambda_{\max}$; the influence of the complex counterion (Asp-85 and Asp-212) is not fully addressed with single mutants.

Recent attention has focused on the importance of tryptophan residues for the structural integrity and spectral characteristics of retinal–proteins. In the case of BR₅₆₈ Trp-182 (in the sequence Trp-Ser) and Trp-189 (helix 6) have been implicated in the bathochromic spectral shift; replacement of either of these residues with phenylalanine causes a large hypsochromic shift in the $\lambda_{\max}$ of the pigment (Hackett *et al.*, 1987). The retinal makes contact with four tryptophan residues and probably two tyrosine residues in the binding pocket; Trp-182 and Trp-189, together with Trp-86, serve as a sandwich for the retinal (Henderson *et al.*, 1990). There is re-orientation of a single tryptophan residue on bleaching Rh₄₉₈ (Chabre & Breton, 1979; see Findlay & Pappin, 1986). Trp-126 (in the sequence Trp-Ser) (helix 3) of Rh₄₉₈ is unlabelled with hydrophobic probes and may be positioned close to the β -ionone ring of retinal in one of its putative positions (Findlay *et al.*, 1989). The sequence Trp-Ser (helix 3) is conserved in the three human cone pigments, human, bovine, sheep, mouse and chicken rhodopsins and two *Drosophila* pigments (NinaE and Rh2 opsins) (Pak, 1986; Findlay & Pappin, 1986; Baehr *et al.*, 1988; Takao *et al.*, 1988), but not in octopus (Ovchinnikov *et al.*, 1988), squid (Hall *et al.*, 1991) or the u.v.-absorbing

Drosophila rhodopsins Rh3 and Rh4 (Zuker *et al.*, 1987; Montell *et al.*, 1987). In the four cases the function of Trp-126 may be fulfilled by a conserved tryptophan on helix 4, corresponding to position 167 of bovine rhodopsin in sequence alignments. In molecular modelling this residue is at the same level as Trp-126 in the putative retinol-binding pocket and may similarly be positioned close to the β -ionone ring of the ligand. Interestingly, in cephalopod rhodopsins Trp-167 is present in the sequence Trp-Ser. In an alternative position for the retinal in the rhodopsin structure the β -ionone ring is placed close to Trp-175 (Findlay *et al.*, 1989). This residue is conserved not only in all vertebrate and invertebrate opsins but also in G-protein-associated receptors (Findlay & Eliopoulos, 1990), and may therefore have an, as yet undefined, important common function. However, although a tryptophan residue may be part of the ligand-binding site, it should be noted that a single tryptophan residue in a coupled oscillator interaction with retinal is not able, on theoretical considerations, to account for the red-shift in Rh₄₉₈ (Kakitani *et al.*, 1985).

Trp-265 (helix 6) is conserved in all vertebrate and invertebrate visual pigments save the human blue cone pigment, where it is replaced with tyrosine. It has been suggested that the conserved tryptophan residue together with conserved aromatic residues at positions 261 (Phe) and 268 (Tyr), close in the putative tertiary structure to the distortion of helix 7 at the retinal-binding site, act to seal the ligand-binding pocket in much the same way as proposed for the conserved tryptophan residue in the RBP/BLG superfamily (Findlay & Eliopoulos, 1990); the conservation of Trp-265 in other G-protein-associated receptors argues in favour of a structural role for this residue rather than of wavelength regulation. Trp-138 of BR₅₆₈ may likewise close the retinal-binding pocket (see Henderson *et al.*, 1990, Fig. 16).

Two possible mechanisms for the bathochromic spectral shift of astaxanthin in crustacyanin have been proposed, (i) polarization by charged groups and (ii) strain about the polyene

Fig. 4. Representation of the putative structure of apocrustacyanin A₂ with astaxanthin bound in the putative binding pocket

The conserved Trp-30 at the bottom of the calyx is shown space-filled together with aromatic residues close to the carotenoid β -ionone ring. Note that oxidation of Trp-30 would leave a hole in this region of the protein structure for re-orientation of the aromatic residues.

double bonds, and have been reviewed by Zagalsky *et al.* (1990a). In the putative tertiary structure of apoprotein A₂ the cavity is devoid of charged residues but packed with hydrophobic and aromatic amino acids residues that interact with the carotenoid at the putative binding site (J. N. Keen, I. Caceras, E. E. Eliopoulos, P. F. Zagalsky & J. B. C. Findlay, unpublished work). The C₁-subunit cavity composition is similar to that of the A₂ subunit (J. N. Keen, I. Caceras, E. E. Eliopoulos, P. F. Zagalsky & J. B. C. Findlay, unpublished work). The apoproteins bind astaxanthin to form monomeric derivatives with $\lambda_{\max}$ 555–570 (A₂, $\lambda_{\max}$ 555 nm, and C₁, $\lambda_{\max}$ 570 nm; Warburton, 1986), a shift of approx. 3200–3650 cm⁻¹. As previously considered (Zagalsky *et al.*, 1990a), these spectral shifts may result from distortion of the carotenoids within the binding cavities, thus giving rise to strain about double bonds of the polyenes or about single bonds given increased double-bond character (for example, by neighbouring aromatic residues) (see Kakitani *et al.*, 1985). Alternatively, they may arise directly from interaction of the carotenoids with the several cavity aromatic residues tightly packed around their conjugated systems. In this respect, it is relevant that the $\lambda_{\max}$ of octopus rhodopsin may be modified by aromatic residues, as only a single charged residue (Asp-81) is located in the proposed membrane-embedded helices (Ovchinnikov *et al.*, 1988). The further bathochromic shift in the absorption spectrum of the dimer of the apoproteins, β -crustacyanin ($\lambda_{\max}$ 585 nm), may have a contribution from charged residues present at the subunit interface in addition to that provided by the resultant encapsulation of the carotenoids (J. N. Keen, I. Caceras, E. E. Eliopoulos, P. F. Zagalsky & J. B. C. Findlay, unpublished work). Perhaps BNPS-skatole oxidation of the invariant tryptophan residues of crustacyanin may affect the packing of aromatic residues at the bottom of the calyces (Fig. 4). The loss of the bathochromic shift is then the result of the carotenoid having a less strained conformation and/or of the carotenoid occupying a different position within each cavity, thereby generating altered contacts with internal aromatic and other residues.

It may be that the origins of the spectral characteristics of crustacyanin have more relevance to the spectral shifts for retinal in retinal-proteins than would seem likely from their unique locations, sequence and structural dissimilarity and apparent unrelated evolutionary paths.

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