Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

β-Crustacyanin, the blue–purple carotenoprotein of lobster carapace: consideration of the bathochromic shift of the protein-bound astaxanthin

P. F. Zagalsky

Department of Molecular Biology and Biochemistry, Royal Holloway College, University of London, Egham, Surrey TW20 OEX, England

Correspondence e-mail: p.zagalsky@rhul.ac.uk

The crystal structure of a β-crustacyanin allows an analysis of the various proposals for the mechanism of the bathochromic shift from orange to purple–blue of astaxanthin in this lobster carotenoprotein. Structural and previous chemical and biophysical studies suggest that extension of conjugation by coplanarization of the β-ionone rings with the polyene chain and polarization resulting from hydrogen bonding at the C(4) and C(4′) keto groups may be mainly responsible for the bathochromic shift. Additional contributions may arise from medium effects and possibly from bowing of the polyene chain on binding. Previous biophysical data revealing a somewhat symmetrical polarization of astaxanthin in crustacyanin are thereby also accounted for. A puzzling feature remains unexplained: the bathochromic shifts, larger than that of astaxanthin, shown by some cyclopentenedione carotenoids in reconstituted carotenoproteins. This mini review enlarges on the original analysis and conclusions of Cianci *et al.* [(2002), *Proc. Natl Acad. Sci. USA*, **99**, 9795–9800].

Received 22 April 2003
Accepted 16 June 2003

© 2003 International Union of Crystallography
Printed in Denmark – all rights reserved

1. Introduction

The mechanism for the 150 nm spectral shift undergone by the orange-coloured carotenoid astaxanthin (ASX; I in Fig. 1; λ_{max} = 472 nm in hexane) in the slate-blue oligomeric (16-mer) carotenoprotein α-crustacyanin (α-CR; λ_{max} = 632 nm) of lobster carapace has been the subject of several detailed reviews and of much speculation (Buchwald & Jencks, 1968; Salares *et al.*, 1979; Britton *et al.*, 1997). The recently published crystal structure of a β-crustacyanin (β-CR; λ_{max} = 580 nm), a dimer of two lipocalin subunits with two bound ASX molecules, revealing at high resolution the nature of the ASX-binding sites and the configuration of the protein-bound ASX, enables an assessment to be made of several plausible proposals for the mechanism of the spectral shift (Cianci *et al.*, 2002). β-CR occurs in extracts of lobster carapace and is formed irreversibly by dissociation of the predominant carapace pigment α-CR.

2. Discussion

In the crystal structure of β-CR, one end of each ASX lies within the calyx of its respective lipocalin partner, with the C(4)-keto group linked to a protein-bound water molecule by a short hydrogen bond within the hydrophobic cavity. The opposite end of each ASX is connected to the neighbouring lipocalin subunit, thereby cementing the dimer with the C(4′) keto group close to the NE2 of a His residue in each case. Although the latter sites are mainly hydrophobic, one is more accessible to water than the other. The C(20) and C(20′) methyl groups, essential features of the carotenoid structure for protein binding (Britton *et al.*, 1997), are firmly embedded in a jigsaw-like manner in the protein structure. In the combination, all four β-ionone rings are essentially coplanar with the main polyene chain, with marked and similar bowing of each ASX; the ASX approach within 7 Å at their centres.

In the unbound state, the β-ionone rings of ASX are out of the plane of the polyene chain, with dihedral angles of ∼−43° owing to steric hinderance between the C(5) methyl and C(8) hydrogen. The ring keto groups and C(5)=C(6) and C(5′)=C(6′) are consequently only partially conjugated with the polyene chain. Carotenoids with isopentenone rings, which are more planar with the polyene chain, have λ_{max} at longer wavelengths (actinioerythrin II, λ_{max} = 497 nm in hexane; violoerythrin III, λ_{max} = 562 nm in hexane; Britton, 1995). The effect on λ_{max} of coplanarization of the end rings of ASX may be evaluated following the guidelines given by Karrer & Jucker (1950) and Britton (1995): removal of a C(5)=C(6) from a β-ionone ring, −9–11 nm; addition of a C=C to the conjugated chain, +20–22 nm. Each end ring C=C therefore adds 11 nm to the spectral shift on coplanarization. Comparing β,β-carotene (λ_{max} = 450 nm) and ASX (λ_{max} = 472 nm) in unbound form, each ring keto group contributes +11 nm

to the λ_{max} of ASX. However, if a keto group is fully conjugated it contributes 40–45 nm (Karrer & Jucker, 1950) or 25–30 nm (Britton, 1995) to the λ_{max}. Thus, coplanarization of the ASX rings would bathochromically shift λ_{max} by at least 50 nm and possibly by as much as 90 nm (11 nm for the C═C and 14–34 nm for the keto group of each β-ionone ring).

The λ_{max} of carotenoids is a function of the refractive index (polarizability) of the solvent. In benzene, λ_{max} values are shifted bathochromically by 18–24 nm compared with measurements in hexane (Britton, 1995). In β-CR the ASX are closely packed with Phe and Tyr residues so that the environment of the carotenoids is closer in nature to that of benzene than hexane. The polarizability of the ASX-binding sites should therefore be considered. The λ_{max} of ASX in benzene is at 485 nm (Britton, 1995), a shift of 13 nm from that in hexane.

As well as resulting in electron delocalization, flattening of the rings of ASX allows the electrophilic effects of hydrogen bonding at the C(4) and C(4′) keto groups to be fully transmitted along the polyene chain. Perturbation of the electronic distribution of ASX in α-CR has been substantiated in ^{13}C MAS NMR (Weesie *et al.*, 1997) and Stark spectroscopy (Krawczyk & Britton, 2001) studies. MAS NMR measurements on α-CR reconstituted with various ^{13}C-ASX (4,4′-, 12,12′-, 14,14′-, 15,15′- and 20,20′-$^{13}C_2$) have so far only explored the central portion of ASX. These studies have shown a bisymmetrical polarization about the centre with alternating changes in electron charge density and small quantitative differences in the two halves of ASX. There is a decrease in fractional charge densities at the 12,12′ and 14,14′ positions estimated at 0.03–0.05 and very small net increases in electron density for the 13,13′ and 15,15′ positions. The difference between NMR signals for free and complexed forms of 4,4′-$^{13}C_2$ and 20,20′-$^{13}C_2$ ASX are small. A change in polarizability of the bound ASX, also modest, is seen in Stark spectroscopy (13 D for α-CR compared with 7.4 D for ASX).

Resonance Raman (RR) spectroscopy has provided information on the ground state of ASX in carotenoproteins (Salares *et al.*, 1979; Clark *et al.*, 1980). The strong band in the RR spectrum at 1521 cm^{-1} in unbound ASX is predominantly the C═C stretching frequency (ν_1) and is a measure of the stiffness of the double bond. An empirical relationship has been found between ν_1, a ground-state property, and λ_{max}, which depends on both the ground and excited states, for a series of carotenoids of different length. A plot of ν_1 against $1/\lambda_{max}$ gives a smooth curve, ν_1 decreasing with increasing λ_{max} (Rimai *et al.*, 1973). A similar relationship is shown by astaxanthin proteins of varying λ_{max}, where the absorbing entity is the same (Clark *et al.*, 1980). In α-CR and β-CR ν_1 is lowered to 1498 and 1494 cm^{-1}, respectively, inferring greater electron delocalization in the ground state of ASX (Salares *et al.*, 1979). The use of ^{13}C-labelled ASX (13,13′-, 14,14′- and 15,15′-$^{13}C_2$) allows ν_1 at central positions of ASX to be seen as isolated (lower ν_1) vibrations from the remaining higher vibrations (Britton *et al.*, 1997). In reconstituted α-CR the isolated ν_1 show a greater shift than the higher-frequency component. It is concluded that ν_1 of C(13)═C(14) and C(13′)═C(14′) are more perturbed than the outer C═C [C(11)═C(12) and C(11′)═C(12′)], in keeping with the MAS NMR studies.

Astaxanthin is converted to a blue product in strong acid or alkali in the absence of oxygen. Protonation or deprotonation of ASX have both been considered, but not favourably, in early reviews (Buchwald & Jencks, 1968; Salares *et al.*, 1979) as candidates responsible for the bathochromic shift in α-CR and β-CR. The analogous

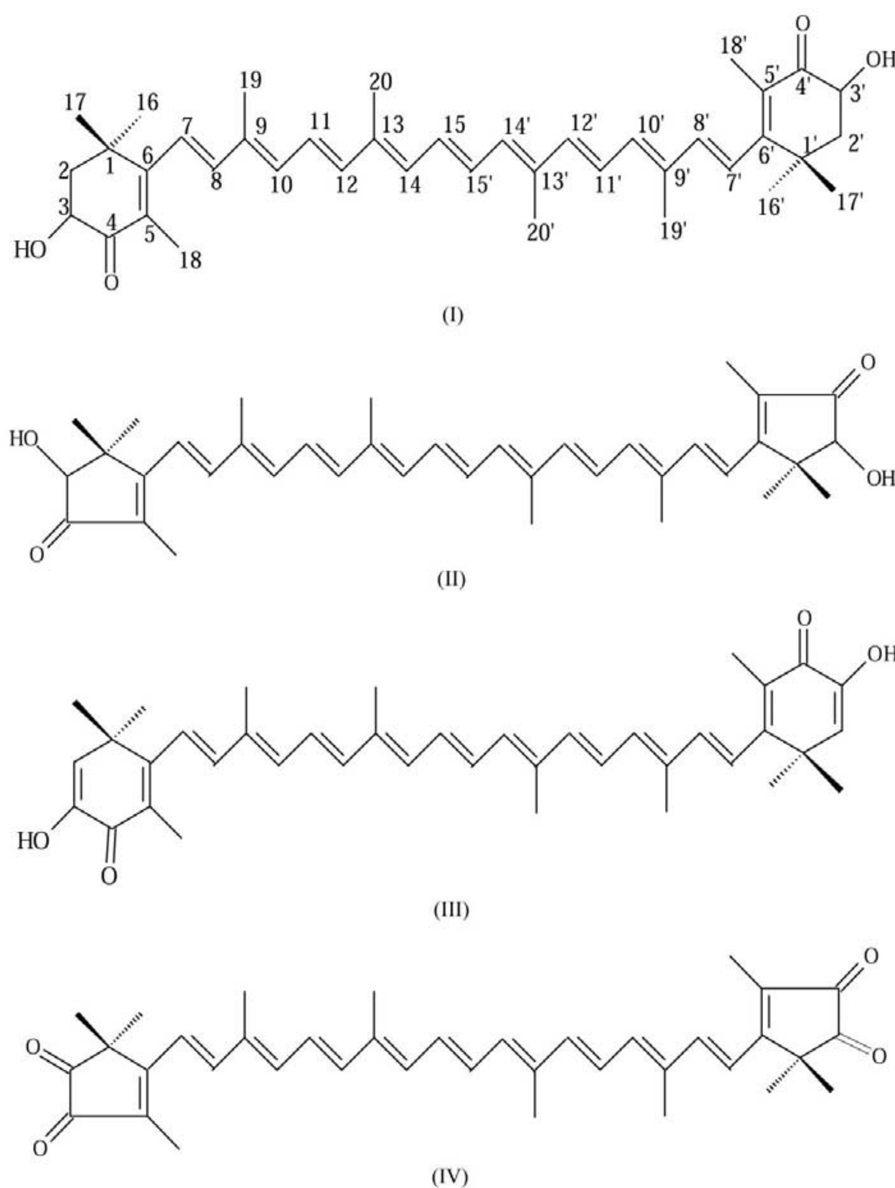

Figure 1
Carotenoid structures. (I), astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione). (II), actinioerythrin (2,2′-dinor-β,β-carotene-3,3′-dihydroxy-4,4′-dione). (III), astacene (β,β-carotene-3,3′,4,4′-tetraone). (IV), violoerythrin (2,2′-dinor-β,β-carotene-3,3′,4,4′-tetraone).

spectral shift for retinal in the rhodopsins, where the protonated Schiff-base linkage of retinal interacts with protein counterions, is attractive to apply to α-CR and β-CR. The biophysical data detailed earlier, verifying electron delocalization in the ground state of ASX in α-CR, has been attributed to protonation at both the C(4) and C(4′) keto groups. Semiempirical quantum-chemical calculations, taking into account the RR data, show that protonation at both keto groups of ASX can account adequately for the spectrum of α-CR (Weesie *et al.*, 1999). Protonation is not, however, supported by the β-CR crystal structure since there are no charged groups (counterions) in the vicinity of the ASX rings or polyene chain. A charged His or H_3O^+ at the ring binding sites (Weesie *et al.*, 1999) therefore seems unlikely. α-CR and β-CR keep their colour at pH 8.6 where His would normally be uncharged; a higher pK for His in a hydrophobic environment may be considered but, as noted earlier, one of the His-binding sites is fairly exposed. Also, unlike the case of rhodopsin, the keto groups of ASX in α-CR and β-CR are readily reduced with sodium borohydride and are therefore accessible to water (Buchwald & Jencks, 1968).

Both the biophysical data and the bathochromic shift of ASX in β-CR are adequately accounted for by the electron delocalization resulting from the conformational change of ASX on binding together with electronic pull from hydrogen bonding at the ring keto groups. The bowing of ASX helps to relieve the steric hinderance consequent on coplanarization of the rings. It seems unlikely that bowing has no effect on the C=C bond order and polyene-chain electron distribution and this factor needs to be assessed. The use of quantum-chemical methods together with molecular-dynamics simulation, taking into account the amino-acid surroundings (Siddarth & Marcus, 1993; Stuchebrukhov & Marcus, 1995), would lead to a greater understanding of the electron transition of the protein-bound ASX.

ASX shows strong optical activity in α-CR and β-CR, evident in the circular dichroism spectra, with negative and positive Cotton effects above and below the position of the λ_{max}, respectively (Buchwald & Jencks, 1968). The splitting of the excitation, as proposed previously, must result from exciton interaction between the two carotenoids in β-CR, which approach within 7 Å at their centres. On heating to 333 K α-CR undergoes a reversible colour change to λ_{max} = 460 nm (loss of strained ASX conformation), but still retains these optical properties under the new λ_{max} (Britton *et al.*, 1997). Of the possible origins of the optical activity discussed by Young & Williams (1983), extrinsic interaction with protein chromophores therefore seems more probable than C(6)—C(7) bond twisting or polarization.

An anomaly to the above account requires explanation. Carotenoids with isopentenedione (but not isopentenone) rings, which are more nearly planar with the polyene chain, give reconstituted carotenoproteins with a λ_{max} more strongly shifted to the red than ASX (Zagalsky & Herring, 1977; Britton *et al.*, 1997). Thus, actinioerythrin (II in Fig. 1) and violoerythrin (IV in Fig. 1) give crustacyanin complexes with λ_{max} at 620 and 837 nm, respectively (Britton *et al.*, 1982, 1997); the λ_{max} of the complex with astacene (III in Fig. 1; λ_{max} = 473 nm in hexane) is at 630 nm (Lee & Zagalsky, 1996). It is suprising that violoerythrin and astacene, with the same conjugated system in the planar form, have such differing λ_{max} values. Clearly, another mechanism applies in the violoerythrin case. If protonation is suggested for both violoerythrin and astacene in these complexes, the λ_{max} would be expected to be similar. The crystal structure of a violoerythrin complex is needed to resolve this dilemma and this is being undertaken.

An understanding of the further spectral shift to 632 nm in α-CR that accompanies the octomerization of the dimeric β-CR, representing ~28% of the energy change of the ASX/β-CR comparison, must also await determination of the crystal structure.

The author thanks the Leverhulme Trust for financial support.

References

Britton, G. (1995). *Carotenoids*, Vol. IB: *Spectroscopy*, edited by G. Britton, S. Liaaen-Jensen & H. Pfander, pp. 13–62. Berlin: Birkhauser Verlag.

Britton, G., Armitt, G. M., Lau, S. Y. M., Patel, A. K. & Shone, C. C. (1982). In *Carotenoid Chemistry and Biochemistry*, edited by G. Britton & T. W. Goodwin, pp. 237–251. Oxford: IUPAC/Pergamon.

Britton, G., Weesie, R. J., Askin, D., Warburton, J. D., Gallardo-Guerrero, L., Jansen, F. J., de Groot, H. J. M., Lugtenburg, J., Cornard, J.-P. & Merlin, J.-C. (1997). *Pure Appl. Chem.* **69**, 2075–2084.

Buchwald, M. & Jencks, W. P. (1968). *Biochemistry*, **7**, 844–859.

Cianci, M., Rizkallah, P. J., Olczak, A., Raftery, J., Chayen, N. E., Zagalsky, P. F. & Helliwell, J. R. (2002). *Proc. Natl Acad. Sci. USA*, **99**, 9795–9800.

Clark, R. J. H., D'Urso, N. R. & Zagalsky, P. F. (1980). *J. Am. Chem. Soc.* **102**, 6693–6698.

Karrer, P. & Jucker, E. (1950). *Carotenoids*, ch. VII, pp. 52–65. Amsterdam: Elsevier Publishing Co. Inc.

Krawczyk, S. & Britton, G. (2001). *Biochim. Biopys. Acta*, **1544**, 301–310.

Lee, W. L. & Zagalsky, P. F. (1996). *Biochem. J.* **101**, 9c–11c.

Rimai, L., Heyde, M. E. & Gill, D. (1973). *J. Am. Chem. Soc.* **95**, 4493–4501.

Salares, V. R., Young, N. M., Bernstein, H. J. & Carey, P. R. (1979). *Biochim. Biophys. Acta*, **576**, 176–191.

Siddarth, P. & Marcus, R. A. (1993). *J. Phys. Chem.* **97**, 13078–13082.

Stuchebrukhov, A. A. & Marcus, R. A. (1995). *J. Phys. Chem.* **99**, 7581–7590.

Weesie, R. J., Merlin, J. C., De Groot, H. J. M., Britton, G., Lugtenburg, J., Jansen, F. J. H. M. & Cornard, J. P. (1999). *Biospectroscopy*, **5**, 358–370.

Weesie, R. J., Verel, R., Jansen, F. J. H. M., Britton, G., Lugtenburg, J. & de Groot, H. J. M. (1997). *Pure Appl. Chem.* **69**, 2085–2090.

Young, N. M. & Williams, R. E. (1983). *Can. J. Biochem. Cell Biol.* **61**, 1018–1024.

Zagalsky, P. F. & Herring, P. J. (1977). *Philos. Trans. R. Soc. London Ser. B*, **279**, 289–326.