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Protein–chromophore interactions in α -crustacyanin, the major blue carotenoprotein from the carapace of the lobster, *Homarus gammarus*

A study by ^{13}C magic angle spinning NMR

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Abstract MAS (magic angle spinning) ^{13}C NMR has been used to study protein–chromophore interactions in α -crustacyanin, the blue astaxanthin-binding carotenoprotein of the lobster, *Homarus gammarus*, reconstituted with astaxanthins labelled with ^{13}C at the 14,14' or 15,15' positions. Two signals are seen for α -crustacyanin containing [14,14'- $^{13}\text{C}_2$]astaxanthin, shifted 6.9 and 4.0 ppm downfield from the 134.1 ppm signal of uncomplexed astaxanthin in the solid state. With α -crustacyanin containing [15,15'- $^{13}\text{C}_2$]astaxanthin, one essentially unshifted broad signal is seen. Hence binding to the protein causes a decrease in electronic charge density, providing the first experimental evidence that a charge redistribution mechanism contributes to the bathochromic shift of the astaxanthin in α -crustacyanin, in agreement with inferences based on resonance Raman data [Salares, et al. (1979) *Biochim. Biophys. Acta* 576, 176–191]. The splitting of the 14 and 14' signals provides evidence for asymmetric binding of each astaxanthin molecule by the protein.

Key words: Solid-state MAS ^{13}C NMR spectroscopy; Protein–chromophore interaction; Carotenoprotein complex; Crustacyanin; Astaxanthin

1. Introduction

Free carotenoids absorb light in the wavelength range 400–500 nm, and are responsible for many natural yellow, orange or red colours. Particularly in invertebrate animals, this colour can be modified to green, purple or blue by the formation of specific carotenoid–protein complexes. The best known example is α -crustacyanin, the carotenoprotein that is responsible for the blue coloration of the lobster carapace. It contains the carotenoid astaxanthin (3,3'-dihydroxy- β,β -carotene-4,4'-dione), which is bound in a stoichiometric but non-covalent way to the apoprotein. The α -crustacyanin complex has a molecular weight of 32×10^4 . It consists of eight β -crustacyanin units (41 kDa), each of which contains two apoprotein subunits and two astaxanthin molecules. There are five electrophoretically distinct apoprotein subunits which are termed A_1 , A_2 and A_3 , and C_1 and C_2 [1]. The apoprotein subunits A_1 , C_1 and C_2 have a molecular weight of ca. 21×10^3 while the subunits A_2 and A_3 each have a molecular weight of about 19×10^3 [1,2]. One β -crustacyanin molecule is formed by the association of one 21 kDa and one 19 kDa apoprotein in combination with

two astaxanthin molecules. All six possible combinations of apoproteins are found in the β -crustacyanin units of naturally occurring crustacyanin, but a combination of the A_2 and C_1 subunits constitutes the major species present. Reconstitution studies show that the α -crustacyanin exhibits high specificity with respect to the carotenoids that it can bind [3]. Only carotenoids containing at least the two keto groups, at C-4 and C-4', were able to form spectrally shifted α -crustacyanin sized complexes, whereas carotenoids containing one keto group were only able to form β -crustacyanin sized complexes.

Upon binding of astaxanthin to the apoprotein the absorption maximum shifts from 488 nm to 632 nm for the α -crustacyanin complex [4]. Several highly localized and specific interactions may be responsible for the large colour shift of the astaxanthin upon binding, so crustacyanin is an ideal model for studying protein–ligand interactions of the kind that play an important role in a considerable number of biochemical processes. Several mechanisms that could account for the large bathochromic shift ($\Delta E = 4675 \text{ cm}^{-1}$) have been suggested. Buchwald and Jencks [5] favoured a distortion mechanism and proposed that protein binding results in twisting of the astaxanthin chromophore by the apoprotein. Salares et al. [6], however, suggested a polarisation mechanism and opposed the distortion mechanism on the basis of their studies of the crustacyanins by resonance Raman spectroscopy.

Cross-polarisation/magic-angle-spinning (CP/MAS) ^{13}C NMR in conjunction with site-directed isotopic labelling can provide detailed information about specific interactions at the atomic level in large proteins [7]. In this work we present the results of the first application of this technique to the study of α -crustacyanin reconstituted with [14,14'- $^{13}\text{C}_2$]astaxanthin and [15,15'- $^{13}\text{C}_2$]astaxanthin (Fig. 1).

2. Materials and methods

2.1. Extraction and purification of α -crustacyanin

α -Crustacyanin was extracted from finely ground lobster carapace and subsequently purified by anion-exchange and gel-filtration chromatography essentially by the procedure of Zagalsky [8]. The extraction and purification steps were all carried out at 4°C and the pH was maintained between 6.5 and 7.5.

The purity of α -crustacyanin was measured by the ratio of absorbance at 632 nm to protein absorbance at 280 nm, and the total amount of α -crustacyanin present in the protein solution was determined from the absorbance at 632 nm. A molar absorption coefficient $\epsilon = 1.25 \times 10^5 \text{ M}^{-1}\cdot\text{cm}^{-1}$ was used [9].

2.2. Synthesis of ^{13}C -labelled astaxanthins

The synthesis of [14,14'- $^{13}\text{C}_2$]- and [15,15'- $^{13}\text{C}_2$]astaxanthin is described elsewhere [10].

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2.3. Reconstitution of α -crustacyanin

The reconstitution procedure was based on a method described by Zagalsky [8]. All stages of reconstitution were carried out at 0°C in a ground glass stoppered tube; any solvents or buffers added during the procedure were also kept at 0°C.

To α -crustacyanin in 50 mM sodium phosphate buffer, pH 7.0 (2 ml; containing up to 3 mg of protein), acetone was added quickly with much stirring, followed by diethyl ether (10 ml). The tube was inverted 3 times and the orange ether layer (containing extracted astaxanthin) was pipetted off after the two phases separated. Ether was added and pipetted off twice more until no more carotenoid could be extracted. After this any remaining ether was quickly removed under a stream of nitrogen and the protein solution was made up to 2 ml again with 50 mM sodium phosphate buffer (pH 7.0) if necessary. The acetone/ether extraction was repeated twice more, removing virtually all the astaxanthin and leaving an almost colourless apoprotein preparation. Finally all the volatile solvent remaining was removed under a nitrogen stream for 5–10 min.

To this apoprotein preparation, the labelled carotenoid (25% molar excess) in acetone (1.25 ml) was added quickly and with much stirring followed immediately by the addition of 50 mM sodium phosphate buffer, pH 7.0 (10 ml). This mixture was dialysed for 18 h against 5 l of the same buffer. The reconstituted complex was shown to be identical to the natural α -crustacyanin adjudged by the UV/Vis, resonance Raman and ^{13}C CP/MAS NMR spectra of both species.

2.4. Final sample preparations

Approximately 20 mg of α -crustacyanin reconstituted with ^{13}C -labelled astaxanthin was used to record each CP/MAS ^{13}C NMR spectrum. The samples were first concentrated by means of 20 ml 100 kDa Macrosep centrifugal concentrators (Filtron Technology Corporation) to a volume of approx. 2 ml. Then with 2.0 ml 10 kDa Eppendorf centrifugal concentrators a final volume of around 100 μl was reached. The concentration procedure yielded very dense protein solutions with a protein concentration of 200 mg/ml. UV/Vis spectra of the protein solutions, which were taken just before and after the NMR experiments, were found to be identical, and showed that no denaturation had occurred during the concentration or recording of the NMR spectrum.

2.5. Solid-state CP/MAS ^{13}C NMR

Low-temperature 100 MHz CP/MAS ^{13}C NMR experiments on the α -crustacyanin carotenoprotein complex were performed with a Bruker MSL-400 NMR spectrometer equipped with a 4 mm MAS probe (Bruker, Karlsruhe, Germany). The spinning rate around the magic angle was measured by means of an optical detection system and was kept at 6.00 ± 0.01 kHz with a spinning speed controller [11].

The spectra were accumulated in 1K channels with ^1H decoupling during acquisition. The 90° pulse lengths for the ^1H and ^{13}C were 6–7 μs , the cross-polarization time 1 ms, the recycle delay 1 s and the sweep width 50 kHz.

Chemical shifts are referenced to TMS and all spectra were recorded with a dead time of 10 μs .

All spectra were recorded at an approximate sample temperature $T = 230 \pm 5$ K which was calculated from the relationship $T \sim 0.86T_b + 50$ K where T_b is the temperature of the bearing gas which was measured with a thermocouple just outside the spinner assembly.

3. Results and discussion

Following the determination of the primary structures of the apoprotein subunits of crustacyanin, and the identification of significant homology between these sequences and those of other lipid-binding proteins, notably human plasma retinol-binding protein and β -lactoglobulin, a model of the three-dimensional structure of the dimer, β -crustacyanin, was constructed [12,13]. This model suggests that each subunit of the dimer contains 4 β -pleated sheets that form a β -barrel, and the astaxanthin molecule is positioned with one end in the apolar interior of the barrel. The other end of the astaxanthin projects out into the surrounding (aqueous) medium. When the two

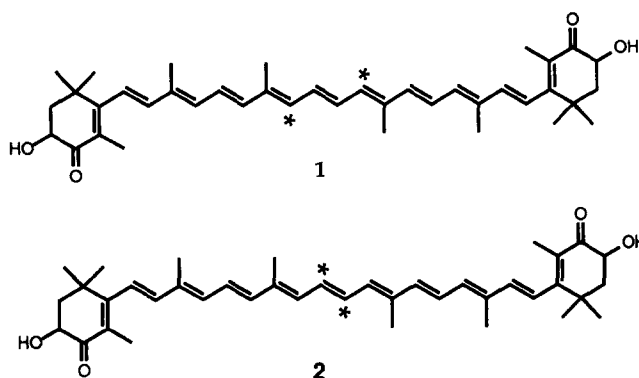


Fig. 1. $[14,14'\text{-}^{13}\text{C}_2]$ Astaxanthin (1) and $[15,15'\text{-}^{13}\text{C}_2]$ astaxanthin (2). The labelled positions are marked with an asterisk.

subunits combine, the dimer would form a capsule-like structure enclosing the two astaxanthin molecules (Fig. 2).

Although the proximity of a tyrosine residue to the end group of astaxanthin is suggested, this model tells us nothing about the specific interactions between the protein and astaxanthin, that are responsible for the large spectral shift. In order to investigate these interactions, we have adopted the strategy of synthesizing astaxanthin labelled with ^{13}C at a specific position, incorporating this into α -crustacyanin, and studying the effects of the binding on the NMR properties of the labelled carbon atoms of the astaxanthin.

Problems are encountered when solution NMR is used to study complexes such as the α -crustacyanin carotenoprotein because of spectral line-broadening due to the low tumbling rates of the large α -crustacyanin molecules. A solid-state NMR method, the CP/MAS ^{13}C NMR technique, has been used to overcome this problem [7,14]. Cross-polarization is used to take advantage of the higher gyro-magnetic ratio and faster spin-lattice relaxation of protons, but since cross-polarization is only efficient in the presence of strong dipolar interactions between the abundant ^1H spins and the less abundant ^{13}C nuclei, the protein complex is immobilized by freezing the samples. The freezing transition for the α -crustacyanin complex occurs at an apparent sample temperature of ca. 255 K and is easily detected since the ^1H tuning characteristics of the probe change as the freezing point of the sample is passed. The dipolar interactions and chemical shift anisotropy (CSA) introduced upon freezing, however, give rise to broad lines, without any fine structure. To restore a high-resolution NMR spectrum with narrow lines the causes of the line-broadening have to be eliminated. The dipolar interactions are removed by dipolar decoupling in which the ^1H system is saturated by applying a strong radiofrequency field in the mid-range of the proton spectrum. The line broadening due to the chemical shift anisotropy is removed by rapidly spinning the sample at an angle of $54^\circ 44'$ (magic angle) with respect to the external magnetic field H_0 . A CP/MAS ^{13}C NMR spectrum thus obtained consists of a sharp centre-band at the isotropic chemical shift σ_i , flanked by a set of rotational side bands spaced at integral multiples of the spinning speed ω_r relative to σ_i . The intensities of rotational side bands in the side band pattern generally decrease upon increasing ω_r .

It is virtually impossible to obtain any structural information from natural abundance CP/MAS ^{13}C NMR spectra of protein

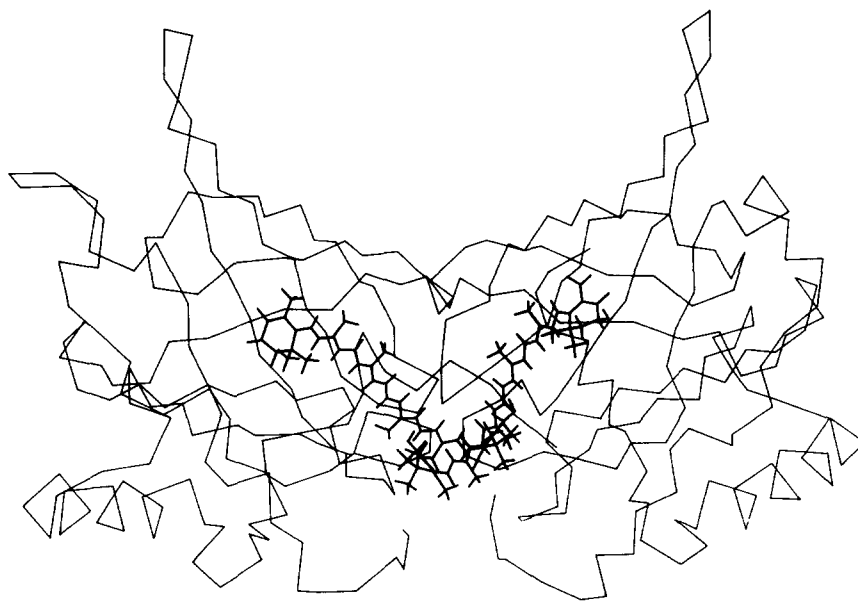


Fig. 2. Putative structure of β -crustacyanin with each astaxanthin molecule bound in an asymmetric way to the protein.

complexes but, by incorporating ^{13}C -labelled astaxanthin with 99% ^{13}C enrichment, the signals for the position(s) of enrichment become recognizable above the background natural abundance spectrum. This increase in sensitivity is demonstrated in Fig. 3 where the CP/MAS ^{13}C NMR spectra of natural abundance α -crustacyanin (Fig. 3a) and α -crustacyanin reconstituted with $[14,14'\text{-}^{13}\text{C}_2]$ astaxanthin (Fig. 3b) and $[15,15'\text{-}^{13}\text{C}_2]$ astaxanthin (Fig. 3c) are shown. The natural abundance spectrum shows a few characteristic features. Thus the $\text{C}=\text{O}$ resonance of the peptide groups gives a signal at around 172 ppm, with side bands at 232 ppm and 113 ppm. The less intense signal at ca. 128 ppm can be assigned to the aromatic and olefinic carbon atoms in the protein while the aliphatic carbons are responsible for the signals in the region between 0 and 80 ppm.

These features are also apparent in the spectrum of α -crustacyanin reconstituted with $[14,14'\text{-}^{13}\text{C}_2]$ astaxanthin, but additional resonance signals are seen at 141 ppm and 138 ppm, with side bands at 90 and 190 ppm and these signals can be attributed to the ^{13}C labels. Comparison of the CP/MAS ^{13}C NMR spectrum of the labelled protein complex with that of solid $[14,14'\text{-}^{13}\text{C}_2]$ astaxanthin shows two main differences in the $[14,14'\text{-}^{13}\text{C}_2]$ resonance signals (see Table 1). First only a single resonance signal is seen for unbound $[14,14'\text{-}^{13}\text{C}_2]$ astaxanthin, but this is split into two separate resonance signals when the astaxanthin is bound into the protein complex. From Fig. 3 and from the difference spectrum after subtraction of the natural abundance spectrum (not shown here) it is clear that the two signals have different linewidths, for which approximate values of 150 and 300 Hz have been determined. This shows that there are differences in the molecular environments of the C-14 and C-14' positions of the bound astaxanthins.

The possibility that the two different signals arise from two different populations of astaxanthin molecules in different environments must be considered. Either the two astaxanthins in each β -crustacyanin unit could be in different environments or the environment of the astaxanthin in different β -crustacyanins

could be different. If this were so, however, the difference between the interactions, required to give a splitting of 3.9 ppm, would be so great that the existence of two populations would almost certainly be detected in the visible absorption spectrum and in the resonance Raman spectrum and excitation profile. This is not the case. A further argument against the existence of 2 different populations of astaxanthin molecules is the fact that in the crustacyanin dimer model there are differences only in the exterior parts of the different subunits while the astaxanthin-binding sites are identical.

A much more likely explanation is that the two astaxanthin molecules within a β -crustacyanin dimer are bound similarly, but the two halves of each individual astaxanthin molecule experience different interactions. This is consistent with the model illustrated in Fig. 2. In the β -crustacyanin dimer, eight of which aggregate to give α -crustacyanin, two molecules of astaxanthin are bound within the envelope created by the association of the two subunits. For each molecule of astaxanthin, one end is held deep inside the cavity or calyx of one subunit and the protruding end 'capped' by the second subunit, i.e. both astaxanthins are bound by the protein in a similar manner, but each astaxanthin is bound asymmetrically to the protein binding sites. This model would account for the splitting of the NMR signal. Although the astaxanthin molecule is labelled symmetrically at the 14 and 14' positions, it is associated with the protein in an asymmetrical way, so that the 14 and 14' positions become non-equivalent and give two signals. One of the labelled atoms sits more deeply inside the β -barrel structure than the other.

The second major feature of the spectrum is that the signal for the free astaxanthin is at 134.1 ppm, but those for the complex are at 138.1 and 141.0 ppm, corresponding to downfield shifts of 4.0 and 6.9 ppm, respectively. This is consistent with an electronic perturbation of the astaxanthin chromophore caused by binding, leading to a decrease in electronic charge density at both the C-14 and C-14' positions in the middle part of the polyene chain. A change in charge density

of 1 electronic equivalent corresponds to a carbon chemical shift of approximately 155 ppm in aromatic and conjugated systems [15–17]. The 4.0 and 6.9 ppm downfield shifts of the $[14,14'\text{-}^{13}\text{C}]$ resonances after binding to the α -crustacyanin complex can therefore be translated into +0.03 and +0.05 electronic equivalent of charge, respectively.

These shifts of 4.0 and 6.9 ppm are quite significant, but the crustacyanin dimer model of Keen et al. [12,13] places no charged residues in the direct vicinity of the astaxanthin chromophore, including the 14,14' positions. If this is the case, other structural or electrostatic perturbations of astaxanthin in the α -crustacyanin complex must be responsible for these downfield shifts. A structural out-of-plane perturbation, however, is unlikely. Preliminary semi-empirical calculations indicate that twists of the order of 15° around the C(14)–C(15) and C(14')–C(15') single bonds are required to produce charge differences between +0.03 and +0.05 electronic equivalent. Such twists are expected to give rise to comparable charge effects at the 15 and 15' positions, in disagreement with our observations (see below). In addition, from resonance Raman data [6] it is clear that the central part of the astaxanthin is in a planar conformation when it is bound to the protein complex. Hence

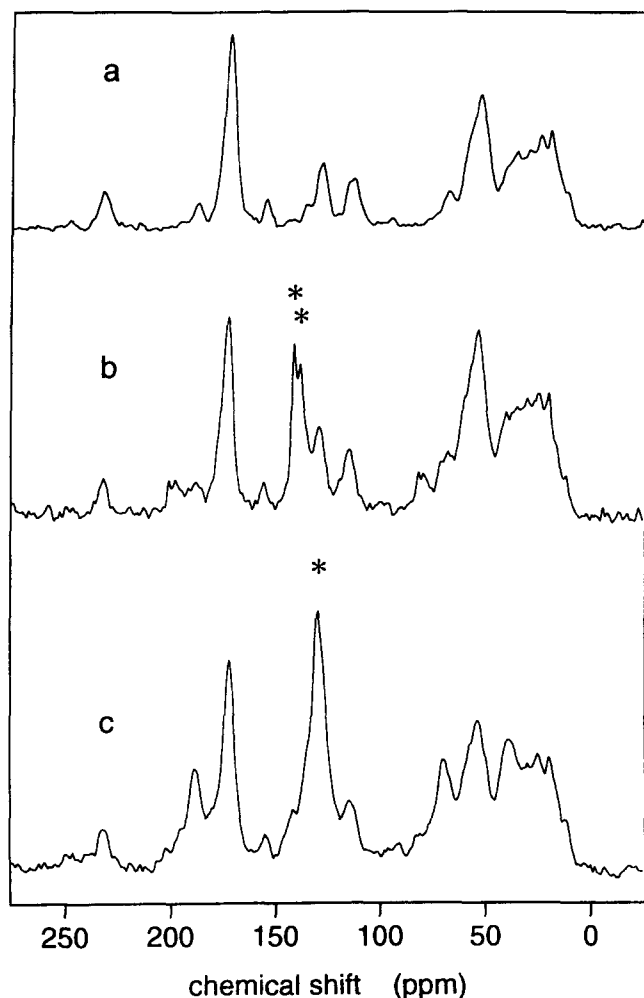


Fig. 3. CP/MAS ^{13}C NMR spectra of natural abundance α -crustacyanin (a), α -crustacyanin reconstituted with $[14,14'\text{-di } ^{13}\text{C}]$ astaxanthin (b), and $[15,15'\text{-di } ^{13}\text{C}]$ astaxanthin (c). The data were collected with a spinning speed $\omega_r/2\pi = 6.00$ kHz at $T = 230 \pm 5$ K.

Table 1

Differences ($\Delta\sigma$; ppm) between the isotropic chemical shifts (σ_i) for the complexed and uncomplexed forms of $[14,14'\text{-}^{13}\text{C}_2]$ and $[15,15'\text{-}^{13}\text{C}_2]$ astaxanthin.

	σ_i $[14,14'\text{-}^{13}\text{C}_2]$	σ_i $[15,15'\text{-}^{13}\text{C}_2]$
Astaxanthin	134.1	130.1
Reconstituted α -crustacyanin	141.0	129.5
$\Delta\sigma$	6.9	-0.6

our NMR results provide the first experimental evidence that a charge redistribution mechanism contributes to the bathochromic shift of the astaxanthin in α -crustacyanin.

In the CP/MAS ^{13}C NMR spectrum of α -crustacyanin reconstituted with $[15,15'\text{-}^{13}\text{C}_2]$ astaxanthin, only a single resonance signal at 129.5 ppm is seen for the ^{13}C labels and the shift due to binding to the protein is less than 0.6 ppm. Obviously there is no significant net electronic perturbation at the positions C-15 and C-15' due to protein binding, in contrast to the decrease in electronic charge density at the C-14 and C-14' positions of the polyene chain.

Comparison of these observations with those for rhodopsins, in which retinal is bound as a protonated Schiff base, lead us to conclude that a 'protein charge' mechanism similar to that which is used to explain chemical shift differences in bovine rhodopsin [18] is not operational in the α -crustacyanin carotenoprotein complex. With this mechanism a negative protein charge, located near a polyene chain, gives rise to shifts of the ^{13}C resonance signals of several adjacent carbon atoms along the polyene chain. This demonstrates that the perturbation of the negative charge may extend up to several bond lengths along the polyene chain [19]. This is clearly not consistent with the observation that the 15,15' resonance signals are unshifted while the 14,14' resonances both shift downfield. The protein-chromophore interactions in the central part of the astaxanthin molecule thus appear not to involve polarisation caused by a strong local protein charge, a conclusion which is in agreement with the model of Keen et al. [12,13], which shows no charged amino acid residues in the vicinity of the chromophore.

A possible mechanism that could account for the observed charge redistribution would be a complete or partial polarization of the keto groups in the ring system at both ends of the astaxanthin upon binding to the protein. The observed asymmetry would arise if the degree of polarization was not identical at both ends of the astaxanthin molecule.

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