Biochemical Applications of Raman and Resonance Raman Spectroscopies

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V. Carotenoids

The principal structural characteristic of carotenoids is a long, conjugated hydrocarbon chain. The extensive π-electron delocalization, seen in the structures of β-carotene and astaxanthin in Fig. 5.15, gives rise to intense visible absorption bands, and much of the pigmentation in nature, from flamingos to lobsters to carrots, is attributable to carotenoids. The biological functions of many carotenoids are unknown or conjectural, although in a few cases carotenoid function is understood (e.g., their role in camouflage or in protection of organisms from the deleterious effects of singlet oxygen produced during photosynthesis). For our purposes it is convenient to divide carotenoids into two classes: those which are highly

photolabile and those which are not. The primary photolabile chromophore is retinal found in rhodopsin and bacteriorhodopsin, and these pigments are discussed in the following section. Here, the discussion is limited to those carotenoids which are relatively nonphotolabile.

Interestingly, the RR spectra of several carotenoids were reported, in 1932, by Euler and Hellstrom (1932). Coming just four years after C. V. Raman's initial report of the Raman effect, the work of Euler and Hellstrom represents the first RR study of a large truly biological molecule. During the 1950s the Raman technique was almost eclipsed by the upsurge in infrared spectroscopy; however, in Germany (see Behringer, 1967, and references therein) and Russia (see Shorygin, 1978, and references therein) some interest remained in the RR spectra of carotenoids because of the data they provided spectroscopists and theoreticians on Raman intensity enhancement. Then, in the late 1960s Rimai and coworkers in the United States began a series of investigations into the laser-excited RR spectra of carotenoids. The interest sparked by this work focused the attention of several laboratories on the potential of RR spectroscopy for elucidating the properties of biological pigments. Observations by Rimai and colleagues were stimulating because these scientists reported the RR spectra of carotenoids in intact plant tissue (Gill *et al.*, 1970) and the first studies of the visual pigments, in addition to the spectra of the purified chromophores from these materials (Rimai *et al.*, 1970c; Heyde *et al.*, 1971).

The essential RR spectral features of carotenoids are illustrated by

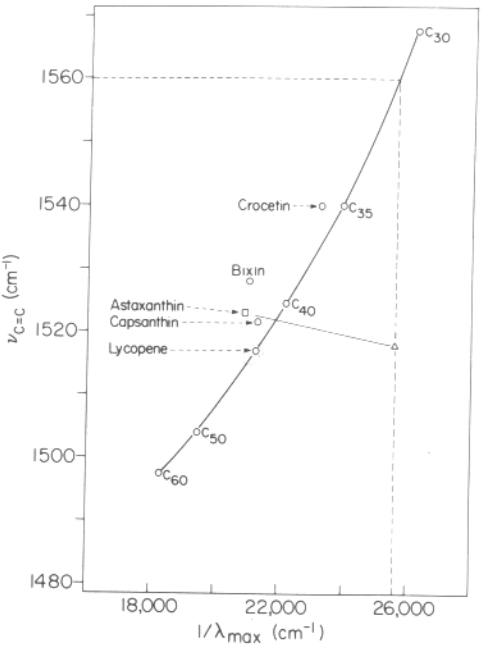

Fig. 5.18. Correlation of $\nu_{c=c}$ with $1/\lambda_{max}$. Monomeric astaxanthin (□) obeys correlation, aggregated astaxanthin (△) does not. [Adapted from Salares *et al.* (1977b).]

Astaxanthin has been used as an example in the previous discussion because of the author's interest in lobster pigmentation. The latter is entirely attributable to astaxanthin bound to various proteins. When a lobster is cooked, the astaxanthin-containing proteins denature, "unbound" astaxanthin is formed, and this "free" astaxanthin is responsible for the orange-red coloration of cooked lobsters. However, in the live crustacean protein–astaxanthin interactions bring about major absorption spectral shifts for the astaxanthin molecule. In one class of protein–astaxanthin complex a yellow protein is formed, while in a second kind of complex a blue protein results. It is a mixture of these that gives the live lobster its characteristic pigmentation. A yellow astaxanthin-bearing protein isolated from lobster shells was found, by a combination of RR and absorption techniques, to contain aggregated, excitonically coupled, astaxanthin molecules (Salares *et al.*, 1977a). Additionally, using RR *in vivo*, a film of yellow protein was found to envelop the shells of lobsters (Salares *et al.*, 1977a). Since the excitonic excited states of the aggregated astaxanthins could have the property of facile photon absorption and transmission, it was suggested that the entire lobster shell could act as a

light-collecting dish to monitor low-light levels found at the bottom of the ocean (Salares *et al.*, 1977a). Such a function could account for many of the temporal properties (e.g., seasonal migration) in the lobster life cycle. In this hypothesis, photons in the blue, green, and yellow parts of the spectrum are absorbed and passed along a chain of pigments with each successive pigment having a lower electronic energy gap than its predecessor. In other words, the photons are passed to pigments absorbing further in the red until, in the photoactive pigment, photochemistry takes place, leading to, for example, hormonal stimulation. Such a system would involve an as yet unidentified photoactive pigment absorbing further to the red of those presently identified in the lobster carapace. To begin a search for far-red absorbing pigments, the RR spectra of lobster shells were excited using infrared irradiation at 752.5 nm (Nelson and Carey, 1981). Figure 5.19 compares the spectra of lobster shell fragments taken with 647.1-, 676.4-, and 752.5-nm excitation. There are two notable features in these spectra. First, the value of $\nu_{C=C}$ at 1490.5 cm^{-1} in the 676.4-nm excited RR spectrum is the lowest value reported from an astaxanthin-like carotenoid. It is probably attributable to a pigment which has a λ_{max} to the red of 625 nm and which has not yet been isolated from the shell. The second unexpected feature is that the intensity of the 752.5-nm excited RR spectrum is higher (by a factor of 5–10) than expected on the basis of the presently known purified pigments. The unusual frequencies and intensities shown in Fig. 5.19 are inexplicable in terms of the known pigments, and further work may yield new perspectives on lobster pigment function.

The yellow protein isolated from lobster shell has its λ_{max} at 410 nm, and as mentioned previously, the shift from the value near 490 nm for free astaxanthin in organic solvents originates in astaxanthin–astaxanthin excitonic interactions. In contrast, the color of the blue proteins isolated from the shell is attributable to protein–astaxanthin interactions. The proteins appear to be blue because astaxanthin's λ_{max} is red-shifted to 610 nm. Prior to RR studies the favored model to account for the large red shift involved twisting about double bonds in the polyene chain (Zagalsky, 1976). However, the RR data eliminated this and several other mechanisms as possibilities (Salares *et al.*, 1979). Instead, the findings of the RR investigation support a charge-polarization model. In this mechanism, charged protein groups and possibly hydrogen bonds in the astaxanthin binding site set up π-electron polarization and hence a dipole moment in the astaxanthin. Changes in the placement of charges about the astaxanthin change λ_{max} and thus account for the variations in λ_{max} observed for other astaxanthin-bearing proteins. Charge polarization induced by the binding site leads to a shift to the red in λ_{max} and a concomitant drop in

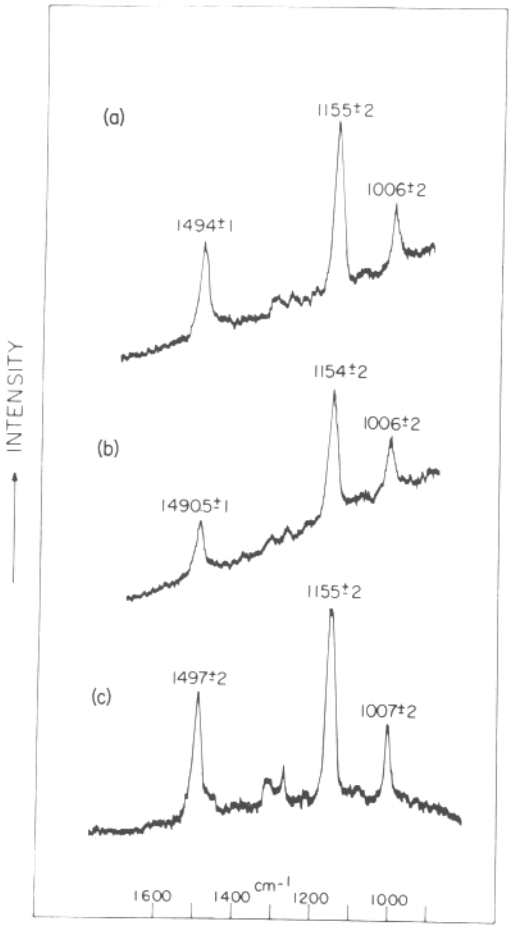

Fig. 5.19. RR spectra obtained from blue fragments of lobster shells using red and infrared excitation: (a) 647.1-nm, (b) 676.4-nm, and (c) 752.5-nm excitation. The fragments were immersed in buffer and rotated to prevent photodegradation. [From Nelson and Carey (1981).]

$\nu_{C=C}$. On this basis the $\nu_{C=C}$ observed at 1490.5 cm^{-1} in Fig. 5.19 must come from a very highly polarized astaxanthin in an, as yet, uncharacterized site. The charge-polarization model also explains how the λ_{max} of various visual pigments can be modulated (see Section VI). Furthermore, a charge-polarization model can explain the absorption and RR properties of various enzyme–substrate complexes discussed in Chapter 6, Section V.

In addition to their research concerning the chlorophylls and pheophytins in photosynthetic bacterial reaction centers, Lutz and co-workers have also studied the carotenoid which is an intrinsic component of the reaction-center assembly (Lutz *et al.*, 1978). The carotenoid spheroidene is normally found in its all-trans conformation; however, in the reaction center of *Rhodopseudomonas spheroides* the molecule binds in a cis conformation. This conclusion was reached by a detailed comparison of the RR spectrum of the carotenoid *in situ* in the reaction centers with that of 15,15′-*cis*-β-carotene. The RR spectra of the all-trans and 15,15′-cis isomers of β-carotene are very different in the number, positions, and intensities of the major bands (Fig. 5.20). However, the agreement between the cis isomer and reaction center is striking in all the regions of the RR spectrum. The presence of the cis carotenoid was a general feature of the reaction centers of all the species examined by Lutz *et al.* (1978). More recently, these investigators have been able to add all-*trans*-spheroidene to reaction centers from mutants containing no carotenoids and the RR spectrum of the reconstituted complex showed that on binding the spheroidene took on the cis conformation (Agalidis *et al.*, 1980).

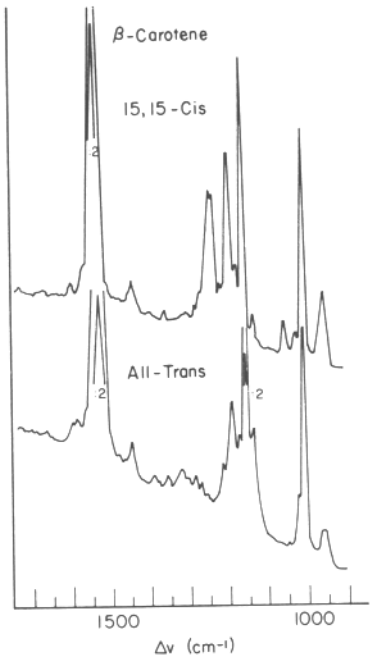

Fig. 5.20. The RR spectra of 15,15′-*cis*- and all-*trans*-β-carotene: 30°K, 496.5-nm excitation. [From Lutz *et al.* (1978).]

The RR spectra of carotenoids are among the most intense and easiest to obtain. For these reasons trace amounts sometimes yield spurious peaks in the spectra from other "purified" materials. These properties also account for the appearance of carotenoid peaks in blood plasma (Larsson and Hellgren, 1974) and provide the opportunity for carotenoid assays in phytoplankton (Hoskins and Alexander, 1977). Finally, the occurrence of carotenoids in some membranes affords an opportunity to use their RR spectra as probes of membrane properties and this topic is discussed in Chapter 8, Section II,C.

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