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Resonance Raman Spectra of Lobster Shell Carotenoproteins and a Model Astaxanthin Aggregate. A Possible Photobiological Function for the Yellow Protein†

V. R. Salares,* N. M. Young, H. J. Bernstein, and P. R. Carey

ABSTRACT: A yellow protein, isolated from lobster shells and containing ~20 astaxanthin molecules per unit of protein, has absorption and resonance Raman properties identical with those of aggregates of astaxanthin. The astaxanthin electronic absorption maximum shifts from ~480 to 410 nm upon aggregation or binding to the yellow protein. Taken with the resonance Raman data on the frequency of the C=C stretching vibration, this indicates that in both systems a large perturbation of the electronic excited state takes place while only a minimal perturbation of the ground state occurs. Low temperature (−95 °C) absorption measurements of the yellow protein and the astaxanthin aggregate show no vibronic structure and suggest that the electronic absorption is solely due to the 0-0 or vibrationless transition. The molecular exciton model satisfactorily explains the spectral data in terms of interactions of chromophore molecules in the excited state. The altered absorption spectrum of the yellow protein compared to that of free astaxanthin is thus attributed to chromophore-chromophore (exciton coupling) rather than protein-chromophore interactions. Resonance Raman experiments on live lobsters and reflectance measurements on shell fragments reveal a preponderance of the yellow protein at the surface of the shell. The pigment distribution in the lobster shell together with several known physiological observations and the energy-transfer capability of excitons suggest a possible photobiological function for the yellow protein, viz. that the yellow proteins act as bulk light harvesters for photons in the deep blue spectral region.

Two types of proteins, both containing astaxanthin as the chromophore, are responsible for the shell color of the lobster *Homarus americanus*. These are the three blue crustacyanins which absorb in the 600-nm region (Wald et al., 1958; Jencks and Buten, 1964; Cheeseman et al., 1966, 1967; Buchwald and Jencks, 1968b) and a yellow protein of λ_{max} 410 nm (Buchwald and Jencks, 1968a). Since free astaxanthin, in organic solvents, absorbs at 480 nm, binding in both types of protein causes large shifts in absorption (Figure 1). The mechanism of these shifts is of interest in its own right and in relation to studies on the visual pigments (Ebrey and Honig, 1975; Sulkes et al., 1976) and enzyme-substrate reactions (Carey et al., 1976). On the basis of mainly absorption and circular dichroic (CD) data, different and often conflicting mechanisms for the spectral shifts have been proposed for the astaxanthin-containing proteins. One problem with absorption and CD data is that both techniques are sensitive to changes in the ground and the excited electronic states. In contrast, Raman and resonance Raman peak positions are a property solely of the ground state. The present study therefore uses resonance Raman peak positions together with absorption data to discriminate between ground- and excited-state effects for astaxanthin in the yellow

† From the Divisions of Chemistry (V.R.S., H.J.B.) and Biological Sciences (N.M.Y., P.R.C.), National Research Council of Canada, Ottawa, Ontario, Canada, K1A 0R6. *Received December 3, 1976; revised manuscript received June 13, 1977.* This paper is NRCC Publication No. 16276.

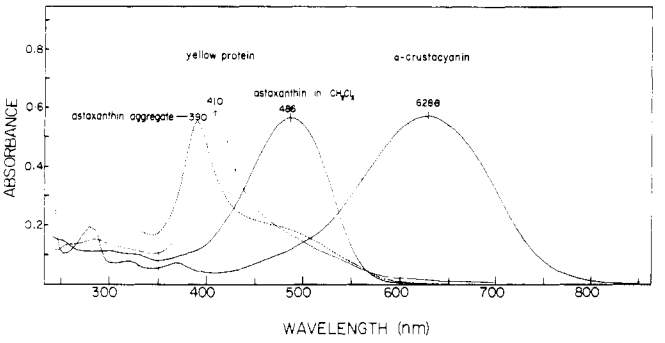

FIGURE 1: Absorption spectra of astaxanthin monomer in dichloromethane, astaxanthin aggregate in 10% acetone-water, the yellow lobster protein in 0.1 M phosphate (pH 7), and α-crustacyanin in 0.1 M phosphate (pH 7).

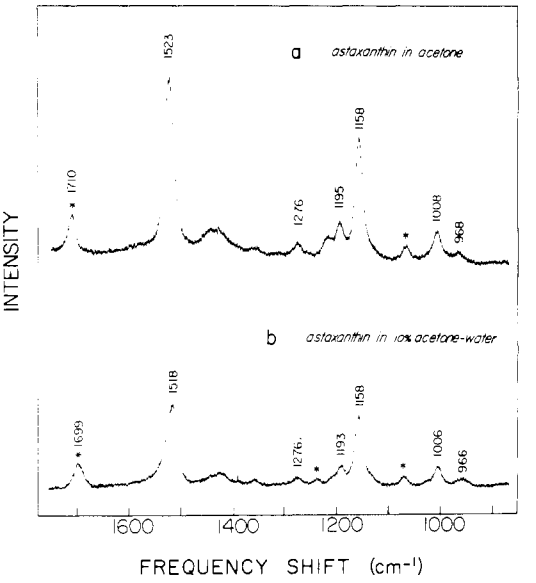

FIGURE 2: Resonance Raman spectra of astaxanthin with 457.9-nm excitation in (a) acetone (4K, 1 s, 0.5 cm^{-1}/s, 9.0-cm^{-1} spectral slits) and (b) 10% acetone-water (4K, 1 s, 0.5 cm^{-1}/s, 9.0-cm^{-1} spectral slits). Acetone bands are marked with an asterisk.

protein. A model system consisting of aggregated astaxanthin in a mixed solvent system is used to develop spectroscopic criteria for application to this protein. Furthermore, resonance Raman measurements on the carapaces of live lobsters provide new data on the distribution of the yellow protein in situ.

Materials and Methods

Astaxanthin and the carotenoproteins were extracted from mature lobsters (*Homarus americanus*) taken from the seas off Eastern Canada in the months of April and July of 1975. For in vivo experiments, live lobsters were obtained locally.

Astaxanthin. The procedure for extracting and purifying astaxanthin has been described previously (Buchwald and Jencks, 1968a; Salares et al., 1976). The purified astaxanthin migrated as a single spot on a thin-layer chromatogram.

Carotenoproteins. Using 550 g of ground shell, the proteins were purified by the procedure of Buchwald and Jencks (1968b), viz. EDTA extraction, ammonium sulfate precipitation, and DEAE-cellulose chromatography (2.5 × 40 cm column of Whatman DE-52 developed with 0.05 M phosphate buffer (pH 7.0), followed by a gradient formed from 600 mL each of the buffer and 0.5 M KCl in the buffer). The four carotenoproteins (α-, γ-, and β-crustacyanin and yellow protein) were then individually purified by DEAE-cellulose chromatography with appropriate gradients. The yellow protein and α-crustacyanin gave visible to ultraviolet (280 nm) absorbance ratios of 5.3 and 3.0, respectively.

Absorption Spectra. The absorption measurements are similar to those described in earlier publications (Salares et al., 1976, 1977). The low-temperature absorption spectrum of the yellow protein was taken using a 0.1 M phosphate buffer-glycerol (1:1) solvent mixture. This solution was prepared by cooling the yellow protein in 0.1 M phosphate buffer (pH 7.0) in an ice bath and adding dropwise an equal volume of glycerol with stirring. The room temperature absorption spectrum of the yellow protein was unchanged by the addition of glycerol. The 50% glycerol-phosphate buffer solution froze to a clear glass at about −95 °C. Spectral measurement below this temperature was prevented by crystallization of the medium.

Reflectance Spectra. Reflectance spectra of lobster shell fragments approximately 4 cm in diameter were measured on a Zeiss DMC25 spectrophotometer.

Raman Spectra. The techniques for measuring the Raman spectra are described in previous papers (Salares et al., 1976, 1977). The red exciting line at 647.1 nm was provided by a Spectra-Physics 164 krypton ion laser. With laser powers between 4 and 15 mW there was no change in the absorption spectra or Raman spectra of the solutions before and after irradiation.

Raman scattering was detected from whole live lobsters or frozen carapace pieces with the shell surface mounted at an approximately 45° angle to the direction of incidence and scattering. Laser powers were 4 mW or less. At these low laser intensities, there was no evidence of deterioration of the surface or change in spectra with time. As a control experiment to check on the possibility of dehydration of the shell surface during irradiation, the shell was enclosed within a glass Dewar and cooled to −10 °C with a stream of cold, moist N_2 gas. No differences were observed between spectra obtained with or without cooling.

Results and Discussion

Aggregated Astaxanthin; a Model for the Yellow Protein. A resonance Raman and absorption spectroscopic study on the monomers and aggregates of six carotenoids (lutein, astaxanthin, zeaxanthin, cryptoxanthin, canthaxanthin, and echinenone) has been undertaken concurrently with the present work (Salares et al., 1977). The key results on astaxanthin are reported here since they are necessary to develop the exciton concept proposed for the yellow protein. In acetone astaxanthin exists as a monomer, while in 10% acetone-water it forms high molecular weight aggregates (Salares et al., 1977; Buchwald and Jencks, 1968a). The absorption spectra of monomeric and aggregated astaxanthin are shown in Figure 1. The marked blue shift seen upon aggregation probably represents a shift in the main π-π* transition. Singlet-triplet and n-π* transitions may be ruled out as a source of the new 390-nm aggregate feature. The resonance Raman spectrum of monomeric astaxanthin is shown in Figure 2a. The intense feature at 1523 cm^{-1} is $\nu_{C=C}$, essentially a C=C stretching frequency. Using these data and a well-established correlation between $\nu_{C=C}$ and λ_{max} (Rimai et al., 1973), it is possible to predict that the resonance Raman spectrum of aggregated astaxanthin should possess an intense band due to $\nu_{C=C}$ near 1560 cm^{-1}. It is therefore surprising that $\nu_{C=C}$ for the aggregate occurs at 1518 cm^{-1} (Figure 2b). Thus, although the correlation predicts a substantial increase in $\nu_{C=C}$ for the aggregate a decrease is actually observed.

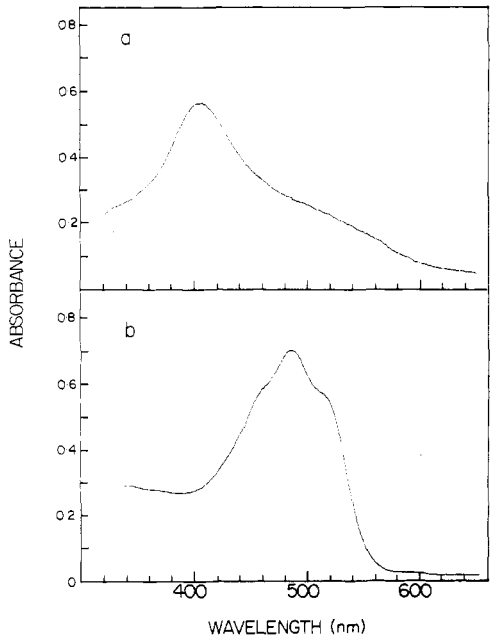

FIGURE 3: Absorption spectra of (a) astaxanthin aggregate in acetone-water-glycerol (5:45:50 by volume, also 1.8 M in NaI) at −100 °C and (b) astaxanthin monomer in diethyl ether-isopentane-ethanol (5:5:2 by volume) at −90 °C.

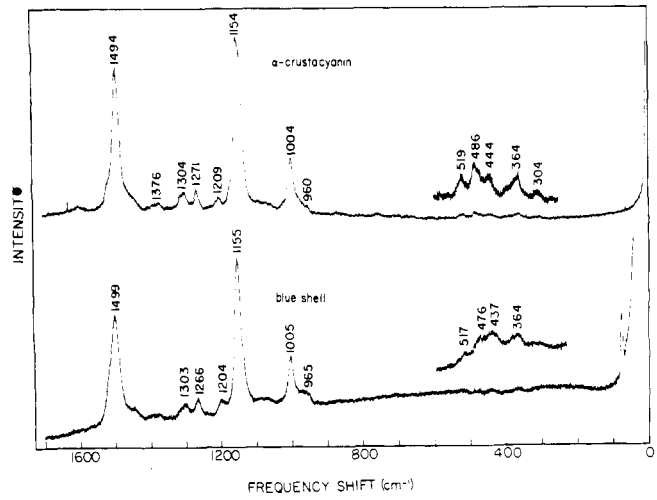

FIGURE 4: Resonance Raman spectra with 647.1-nm excitation of α-crustacyanin in 0.1 M phosphate (pH 7) (10K, 1 s, 0.5 cm^{-1}/s, 7.0-cm^{-1} spectral slits) and blue lobster shell (4K, 1 s, 0.5 cm^{-1}/s, 7.0-cm^{-1} spectral slits).

This $\nu_{C=C}$ vs. λ_{max} anomaly may be explained on the basis that λ_{max} is a function of both electronic ground- and excited-state properties while $\nu_{C=C}$ is solely a property of the electronic ground state. The small observed change in $\nu_{C=C}$ upon aggregation indicates that the electronic ground state is only minimally perturbed. However, λ_{max}, representing the difference between electronic ground- and excited-state energy levels, shifts ~90 nm upon aggregation, showing that the energy gap between the electronic states is perturbed by ~12.5 kcal. Thus, a combination of resonance Raman and absorption data indicates that the electronic excited state is greatly perturbed in the astaxanthin aggregates.

Buchwald and Jencks (1968a) proposed that the unusual absorption properties of the astaxanthin aggregate could be interpreted on the basis of the exciton model. The resonance Raman data presented here strongly support the exciton concept. The exciton model deals principally with the dipole-dipole coupling of electronic transition moments and has been developed for noncovalently bound aggregates (Kasha, 1963; McRae and Kasha, 1964; Hochstrasser and Kasha, 1964; Kasha et al., 1965). The present absorption and resonance Raman results fit the exciton model well since it predicts that the ground state is only minimally perturbed compared to the excited state. Furthermore, on the basis of the large blue shift observed in astaxanthin aggregates the exciton model predicts that the aggregated astaxanthin stacks in a card-pack manner (McRae and Kasha, 1964).

A further test for strong exciton coupling is the absence of vibrational structure in the absorption band. Structure unresolved at room temperature can sometimes by observed by cooling, e.g., the featureless absorption of monomeric astaxanthin at room temperature shows a perceptible structure at −50 °C and a well-defined structure at −162 °C (Salares et al., 1976). In contrast to the monomeric case, aggregated astaxanthin shows no sign of structure at −100 °C (Figure 3). Furthermore, resonance Raman excitation profiles (Salares et al., 1977) strongly suggest that only the 0-0 or vibrationless transition is observed at the low temperature. This is in good agreement with the exciton model (Kasha, 1963).

The Absorption and Resonance Raman Spectra of the Blue Crustacyanins. Several crustacyanins can be isolated from the lobster carapace and separated chromatographically. They are all characterized by an intense absorption band near 600 nm, e.g. α-crustacyanin (Figure 1) absorbs at 628.8 nm with relatively weak features at 280, 320, and 370 nm.

The resonance Raman spectra of α-crustacyanin (Figure 4) as well as γ-crustacyanin and β-crustacyanin (V. R. Salares et al., unpublished results) show the three intense bands in the 1500-, 1150-, and 1000-cm^{-1} regions which are characteristic of carotenoids (Rimai et al., 1973; Salares et al., 1976). The C=C stretching frequency of the crustacyanins ranges between 1494 and 1500 cm^{-1}. Thus, for these molecules the $\nu_{C=C}$ vs. $1/\lambda_{max}$ correlation (Rimai et al., 1973; Oseroff and Callender, 1974; Salares et al., 1977) holds since on the basis of λ_{max} near 600 nm, $\nu_{C=C}$ is predicted to be near 1500 cm^{-1}. The 25-cm^{-1} shift of $\nu_{C=C}$ in the crustacyanins shows that large changes in the ground electronic state take place in the protein binding sites and rules out an exciton mechanism as a principal cause of the shift in λ_{max}.

The Absorbance and Resonance Raman Properties of the Yellow Protein. Buchwald and Jencks (1968a), on the basis of the similarity of the absorption spectrum of the yellow lobster protein to that of the aggregate of astaxanthin in aqueous solution (Figure 1), suggested that in these two systems the same mechanism causes the shift in λ_{max} to near 400 nm from about 480 nm for free astaxanthin in organic solvents. This analogy receives strong support from the resonance Raman spectrum of the yellow protein and from the behavior of its absorption spectra at high and low temperatures.

The resonance Raman spectrum of a solution of the yellow protein using 441.6-nm excitation is shown in Figure 5a. In contrast to the blue crustacyanins the observed C=C stretching frequency (1518 cm^{-1}) deviates from the value predicted (~1545 cm^{-1}) on the basis of the correlation of $\nu_{C=C}$ vs. $1/\lambda_{max}$. However, the C=C stretching frequencies of the yellow protein and the yellow aggregate of astaxanthin are identical. The small shift between $\nu_{C=C}$ in the yellow protein (1518 cm^{-1}) and in free astaxanthin (1523 cm^{-1}) effectively eliminates charge effects in the protein binding sites as a cause of the blue shift in the absorption spectrum. If charge effects in the protein environment were important these would be

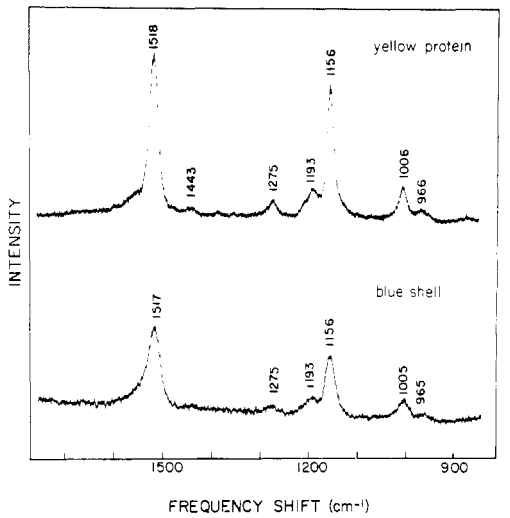

FIGURE 5: Resonance Raman spectra with 441.6-nm excitation of (a, top) yellow protein in 0.1 M phosphate (pH 7) (4K, 1 s, 1.0 cm^{-1}/s, 10-cm^{-1} spectral slits) and (b, bottom) blue lobster shell (4K, 1 s, 1.0 cm^{-1}/s, 10-cm^{-1} spectral slits).

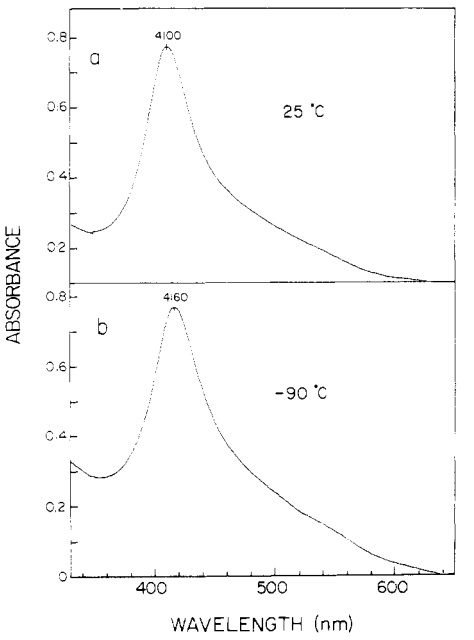

FIGURE 6: Absorption spectra of the yellow protein in glycerol-0.1 M phosphate (pH 7) (1:1 by volume) at (a) 25 °C and (b) −90 °C.

reflected in changes in the ground electronic state and hence in large shifts of $\nu_{C=C}$.

The effect of raising temperature on the absorption and resonance Raman spectra of the yellow protein parallels that seen for aggregates of astaxanthin and lutein (Salares et al., 1977). For the protein, as the solution is warmed, absorption at 410 nm decreases while absorption at longer wavelengths increases. At 60 °C the spectrum broadens and the maximum is at 450 nm, but upon cooling absorption near 410 nm increases again. The original intensity near 410 nm, however, is not recovered completely probably due to some denaturation of the protein. In the resonance Raman, $\nu_{C=C}$ shifts from 1518 cm^{-1} at 23 °C to 1521 cm^{-1} at 60 °C. This frequency change is reproducible and upon decreasing temperature it is reversible.

The absorption spectra of the yellow protein at 25 °C and −90 °C in 50% glycerol-water are shown in Figure 6. At room temperature glycerol does not appear to perturb the absorption of the protein. At −90 °C, the absorption spectrum of the protein is unchanged except for a red shift of ~6 nm. The shift of the absorption spectrum to longer wavelengths is known to occur for other polyenes upon cooling (Jurkowitz, 1959; Loeb et al., 1959; Wald, 1959; Ke et al., 1970). The important result is that even at −95 °C, the onset of vibronic structure is not observed, a result which parallels the findings for aggregated astaxanthin in aqueous solution but contrasts those for monomeric astaxanthin in organic solvents (Salares et al., 1976). The absence of structure at low temperature together with resonance Raman excitation profile measurements (Salares et al., 1977) strongly suggest that only the 0-0 electronic transition is allowed.

It is clear that the resonance Raman and absorption spectral characteristics at room and low temperature of the yellow protein mimic those of astaxanthin aggregates. As in the aggregate model, the shift of the absorption maximum of the yellow protein compared to that of unbound astaxanthin is attributed to chromophore-chromophore excited-state interactions. The binding of the protein to astaxanthin, though not directly responsible for the spectral shift, is probably necessary to induce aggregation of the astaxanthin molecules and to impart stability to this conformation.

Raman Scattering from the Carapaces of Live Lobsters. The large separation between λ_{max} for the yellow protein (410 nm) and λ_{max} for the crustacyanins (600 nm) allows selective resonance Raman enhancement of one chromophore from a mixture containing both. Thus, in a mixture of yellow protein and α-crustacyanin which have equal absorbance at 410 and 628.8 nm, only the α-crustacyanin is seen using 647.1 nm while only the yellow protein spectrum is observed using 441.6-nm excitation.

Lobster shells appear to the eye to be predominantly blue, yellow, green, or mottled depending on the amount and distribution of yellow and blue proteins. The experiments about to be described were carried out on bright yellow or deep inky blue portions of the intact carapace of a live lobster. The physical requirements of a Raman experiment, grazing a laser beam of ~2 mm diameter on the shell surface and collecting the scattered light, meant that small areas of shell could be sampled and care was not needed to select particularly smooth areas.

Raman Spectra of Yellow-Colored Shell. 441.6-nm Excitation. The Raman spectrum from a yellow-pigmented shell with blue laser excitation (441.6 nm) (Figure 7a) is very similar to the spectrum of the purified yellow protein. The C=C stretching frequencies are identical within experimental error indicating both that the resonance-enhanced modes are those of the yellow protein component in the shell, and that the astaxanthin environment in the native yellow protein has not been perturbed by the extraction process. Although the relative Raman intensities are essentially the same from the yellow shell and from the isolated yellow protein, line widths from the shell are clearly broader. Table I compares the bandwidths at half-height for the entire 1518- and 1156-cm^{-1} bands. Both are broader in the shell; a possible cause for this is the existence of heterogeneous environments for the protein at the surface of the carapace. This could lead to a distribution of chromophore conformation with resultant line broadening (Salares et al., 1976).

647.1-nm Excitation. With red laser excitation (647.1 nm) on exactly the same portion of the shell (Figure 7b), the C=C stretching region shows a weak band at 1497 cm^{-1} and a shoulder near 1518 cm^{-1}. The intensity at 1497 cm^{-1} is clearly due to blue protein component(s) of the shell while the shoulder

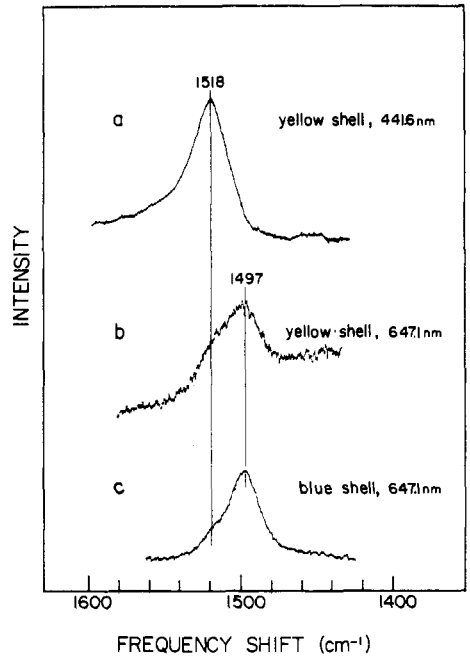

FIGURE 7: Raman spectra in the C=C stretching region of (a) yellow shell with 441.6-nm excitation, (b) the same shell as in a with 647.1-nm excitation, and (c) blue shell with 647.1-nm excitation.

near 1518 cm^{-1} is due to the yellow protein component. Although blue coloration is not visible to the eye, the Raman result clearly indicates blue crustacyanin in the yellow shell. Relative intensities at 1518 and 1497 cm^{-1} similar to those in Figure 7c are obtained from an approximately 80:1 mixture of yellow protein and α-crustacyanin with 647.1-nm excitation. This indicates the relative amounts of yellow protein and blue crustacyanin sampled by the laser beam at or near the surface.

Raman Spectra of a Deeply Blue-Colored Shell. 647.1-nm Excitation. The Raman spectrum of an intensely blue pigmented shell with 647.1-nm excitation is shown in Figure 4 (along with the spectrum of purified α-crustacyanin using the same excitation) and also in Figure 7c. The spectrum of the shell closely resembles that of α-crustacyanin, the major blue protein extracted from the shell. The spectra of other crustacyanins show slight (4–6 cm^{-1}) shifts in $\nu_{C=C}$, compared to the α form, and this probably accounts for the broadened and slightly shifted $\nu_{C=C}$ from the shell. In contrast to the yellow shell (above) only $\nu_{C=C}$ is broadened in the shell; the 1154-cm^{-1} band has the same width at half-height (Table I). This indicates that the yellow protein probably experiences a greater heterogeneity in its native environment.

441.6-nm Excitation. The Raman spectrum of the blue shell with 441.6 nm shown in Figure 5b was completely unexpected. Since there was no trace of visible yellow coloration in the shell the spectrum with 441.6 nm was expected to be a weakly enhanced spectrum of the blue protein, i.e. $\nu_{C=C}$ near 1497 cm^{-1}. However, the observed C=C stretching frequency and relative intensity pattern are those of the yellow protein. Thus, the Raman spectrum shows that the yellow protein co-exists with the blue proteins even in blue pigmented shells. Since Raman scattering from a hard medium such as the shell occurs at or near the surface, the detection of a yellow protein spectrum from an intensely blue pigmented shell demonstrates that the yellow protein is at or near the surface of the shell. This film of yellow protein in the outer layers of the shell may be a further cause for the broadened $\nu_{C=C}$ of the blue shell using 647.1-nm excitation (above).

TABLE I: Half-Widths of $\nu_{C=C}$ and ν_{C-C} of the Yellow Protein and α-Crustacyanin in Solution and in the Shell.

	cm^{-1}	
	$\nu_{C=C}$	ν_{C-C}
441.6-nm excitation	1518 cm^{-1}	1156 cm^{-1}
Purified yellow protein	22	17
Blue shell	31	25
Yellow shell	34	22
647.1-nm excitation	1494 cm^{-1}	1154 cm^{-1}
α-Crustacyanin	22	24
Blue shell	32	22

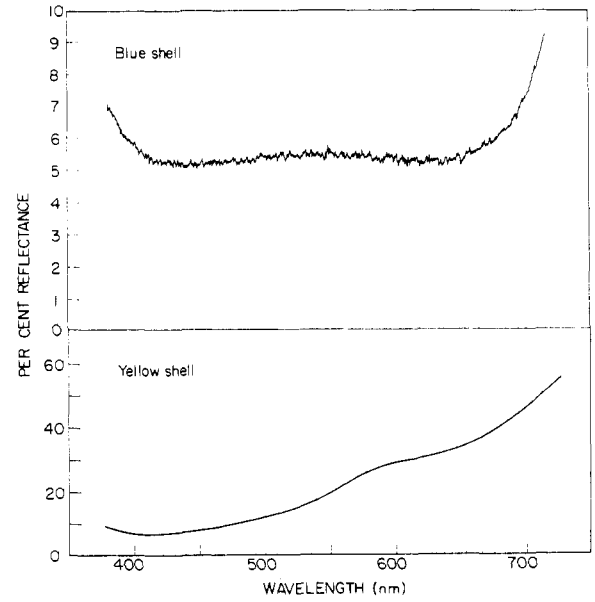

FIGURE 8: Reflectance spectra of blue and yellow pigmented shells. Note the 0–10% reflectance scale in the spectrum of the blue shell.

Two further experiments support the existence of an outer yellow layer extending over the surface of the carapace.

Absorption Spectra. Yellow material gently scraped from the surface of blue pieces of carapace and then dissolved in 0.5 M EDTA solution gave absorption spectra (λ_{max} at 410 nm) and Raman frequencies ($\nu_{C=C}$ at 1518 cm^{-1}) similar to the purified yellow protein. The absorption spectrum of the solution did not show any peak near 600 nm.

Reflectance Spectra. The reflectance spectra of blue and yellow pieces of carapace are shown in Figure 8. The data are presented as percent reflectance (% R) against wavelength, where R is the ratio of the intensity of reflected radiation to the intensity of incident radiation. The lower reflectance of the yellow shell at 410 nm than at 620 nm corresponds to higher absorbance at 410 nm. With a blue shell, the reflectance at 410 nm is comparable to that at 620 nm, indicating a high concentration of yellow protein at or near the surface.

Possible Function for the Yellow Protein. Resonance Raman data on the yellow protein have produced two advances. From among the several possible mechanisms for moving λ_{max} to 410 nm, the Raman measurements provide compelling support for strong exciton coupling. Secondly, the resonance Raman data on the carapaces of live lobsters show, unexpectedly, that the yellow protein exists in significant concentrations at or near the surface of the shell. Moreover, supported by reflectance measurements, the Raman data also show that this film extends over the entire shell, although it is often obscured to the eye by intense blue pigmentation.

The exciton properties and surface distribution of the yellow protein suggest a possible photobiological function. This follows from the ability of exciton states to effect energy transfer in molecular aggregates (Kasha, 1963; McRae and Kasha, 1964). If the excitons are the lowest energy excited state they can act as photon conductors and transmit a photon across a number of coupled chromophores (Witt, 1974; Govindjee, 1975). Thus, the yellow protein has the capability of harvesting photons in the 410-nm spectral region and transmitting them to photochemically active reaction centers. The "skin" of yellow protein could thereby provide a dermal light sensitivity and indeed such a function is known to occur in crustacea (Waterman, 1961; Viaud, 1948, 1951) and other animals, particularly aquatic species (Steven, 1963).

Many animals, blinded or eyeless, which lack an obvious receptor structure have shown a general body surface sensitivity to light (Harris and Mason, 1956; Vevers and Millott, 1957; Millott, 1957; Steven, 1963; Singer et al., 1963). Lobsters are found at great depths (Herrick, 1909) where light levels are extremely low (Waterman et al., 1939). The distribution of the yellow protein over the entire carapace can provide an efficient light gathering apparatus for these low light levels. Moreover, the transmitted light at great depths is mainly blue (Jerlov, 1951; Tyler and Smith, 1970), in the same spectral region as the yellow protein absorption. Finally, lobsters taken from shallow waters are blue while those from great depths are yellow (D. Aiken, private communication; Herrick, 1909). At such great depths the low light levels strongly mitigate against the use of the yellow protein as a protective pigment. However, the skin of yellow protein can provide a very sensitive collector for low light levels. On this basis, it is possible to speculate that a dermal light sensitivity in lobsters may result from the gathering of photons by the yellow protein as the first event. This could be used to stimulate hormonal activity leading to known physiological changes such as seasonal migration, carapace molting, or simply color changes to provide camouflage.

It must be emphasized that this association between the presence of yellow protein at or near the surface of the lobster shell, possessing exciton-like properties and the known dermal light sensitivity in crustacea, is only circumstantial. The photochemical changes following the absorption and transmission of photons in the 410-nm region, if they occur, are not known. It is hoped, however, that the present results will act to focus attention in this area.

Acknowledgments

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