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On the photophysics and photochemical properties of carotenoids and their role as light-harvesting pigments in photosynthesis

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Abstract: In photosynthetic organisms, carotenoids have been implicated in several diverse roles. Yet, owing to profound technical difficulties encountered in attempting to examine the electronic state energies and dynamics of carotenoids both *in vitro* and *in vivo*, several questions remain, and much of the data and interpretations of the results are controversial. This paper will discuss some of these questions and controversies, the resolution of which is important in unraveling the manner in which carotenoids function in photosynthetic systems.

INTRODUCTION

Carotenoids have been implicated in at least five different roles in photosynthesis (ref. 1). These are: (i) light-harvesting; (ii) photoprotection; (iii) singlet oxygen scavenging; (iv) excess energy dissipation; and (v) structure stabilization and assembly. Understanding how carotenoids are capable of this diversity of function is a major objective of the research on these molecules. Most of these roles are related to the structures the molecules adopt *in vivo* and the general complexion of their energy states. In comparison with most other organic π-electron systems, carotenoids are unique in that they do not possess the systematic gerade/ungerade symmetry alternation of their molecular orbitals and excited states. Owing to substantial configuration interaction between electronic states of like symmetry, the lowest excited state, S_1, of carotenoids has the same symmetry, A_g in the point group C_{2h}, as the ground state, S_0. (See Fig. 1). This renders the $S_0 \rightarrow S_1$ ($1^1A_g \rightarrow 2^1A_g$) absorption transition symmetry forbidden. Transitions from the ground state to the S_2 state are strongly allowed, however, because S_2 possesses B_u symmetry. These $S_0 \rightarrow S_2$ ($1^1A_g \rightarrow 1^1B_u$) transitions are responsible for the strong absorption in the visible region that characterizes all carotenoids and gives them their coloration. Whereas the $S_0 \rightarrow S_2$ ($1^1A_g \rightarrow 1^1B_u$) transition energies can readily be determined from absorption spectra, because the $S_0 \rightarrow S_1$ ($1^1A_g \rightarrow 2^1A_g$) transition is symmetry forbidden the S_1 state energies are not as easily discerned, and are the topic of considerable debate.

CAROTENOID FLUORESCENCE

Energy level determinations

Despite several fundamentally sound, early reports of fluorescence from carotenoids (refs. 2-7), until recently it was largely believed that carotenoids were non-fluorescent. Indeed, many reports in the literature of carotenoid fluorescence can be attributed to fluorescent impurities in the samples. With the advent of improved high pressure liquid chromatography (HPLC) systems for the purification of carotenoids, samples devoid of fluorescent impurities can now be prepared. When these samples are analysed by sensitive fluorescence spectrometers, emission from carotenoids which typically have quantum yields on the order of 10^{-4} to 10^{-5} can be observed (refs. 8-10).

The first reports of fluorescence from β-carotene showed a small Stokes shift between the strongly allowed $S_0 \rightarrow S_2$ ($1^1A_g \rightarrow 1^1B_u$) absorption and the emission, suggesting that the fluorescence originated

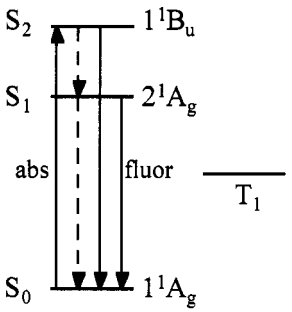

Fig. 1. Energy level scheme of carotenoids. S_0, S_1 and S_2 are singlet states, T_1 is the lowest lying triplet state.

from the $S_2 \rightarrow S_0$ ($1^1B_u \rightarrow 1^1A_g$) transition (refs. 2-7). Later work of Gillbro and Cogdell (ref. 8) and Cosgrove *et al.* (ref. 9) supported this assignment. Most carotenoids exhibit $S_2 \rightarrow S_0$ ($1^1B_u \rightarrow 1^1A_g$) emission. Fluorescence associated with the $S_1 \rightarrow S_0$ ($2^1A_g \rightarrow 1^1A_g$) transition is more rare. The only naturally occurring carotenoids exhibiting fluorescence that can be reliably assigned as S_1 emission are fucoxanthin (ref. 11), 3,4-dihydrospheroidene (ref. 10) and β-carotene (ref. 12). Other reports of $S_1 \rightarrow S_0$ emission have appeared in the literature (refs. 13, 14), but independent confirmation of the data is needed (see below). The relatively short chromophore synthetic carotenoids, 3,4,7,8-tetrahydrospheroidene, 3,4,5,6-tetrahydrospheroidene, 1,1'-(3,8-dimethyl-1,3,5,7,9-decapentaene-1,10-diyl)bis[2,6,6-trimethylcyclohexene] (denoted mini-7-β-carotene) and 1,1'-(3,4-dimethyl-1,3,5-hexatriene-1,6-diyl)bis[2,6,6-trimethylcyclohexene] (denoted mini-5-β-carotene) exhibit S_1 emission, and these observations have been important in determining, by extrapolation, the energies of the S_1 states of longer chromophore carotenoids (refs. 10, 12).

Extrapolation of the S_1 energies of the shorter chromophore carotenoids to the longer chromophore molecules has been carried out by several authors using a variety of approaches (refs. 9, 10, 15-17). In general, the energies of both the S_1 and S_2 states of carotenoids decrease as the extent of conjugation increases, and the energies approach constant values in the limit of infinite conjugated chain length. This convergence has been observed in model polyenes and a simple description of the effect using Hückel theory with configuration interaction was advanced by Kohler (ref. 15). Andersson and Gillbro (ref. 17) applied this model to extrapolate the S_1 and S_2 energies of several β-carotene analogs to the long-chain limit. We have also applied this model to a series of spheroidene analog molecules (Fig. 2).

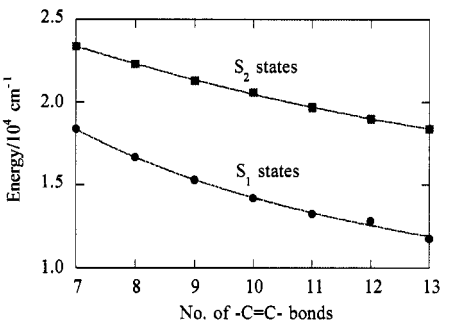

Fig. 2. The data for the S_1 and S_2 state energies of a series of spheroidene analog molecules having extents of π-electron conjugation from 7 to 13 carbon-carbon double bonds are fit by an A + B/(N+C) expression following Kohler (ref. 15). The parameters were A = 3,802 cm^{-1}, B = 1.1 × 10^5 cm^{-1} and C = 0.500 for the series of S_1 state energies, and A = 10,955 cm^{-1}, B = 1.1 × 10^5 cm^{-1} and C = 2.020 for the S_2 state energies.

Another method of determining the S_1 energies of carotenoids is to use the dynamics of their S_1 excited states in conjunction with the energy gap law for radiationless transitions set forth by Englman and Jortner (ref. 18). The appropriate form of the energy gap law is:

$$k_{ic} = \frac{C^2(2\pi)^{1/2}}{\hbar(\Delta E \hbar\omega_M)^{1/2}} \exp\left(-\gamma \frac{\Delta E}{\hbar\omega_M}\right) \quad (1)$$

where $\gamma = \ln(2\Delta E / d\Delta_M^2 \hbar\omega_M) - 1$. Because of the complexity of this expression, the $S_1 \rightarrow S_0$ ($2^1A_g \rightarrow 1^1A_g$) transition energy, *i.e.* ΔE, for a particular carotenoid cannot be obtained analytically from a measurement of k_{ic}. A phenomenological approach is to measure the dynamics of the $S_1 \rightarrow S_0$ ($2^1A_g \rightarrow$

1^1A_g) transitions for carotenoids whose energies are known from fluorescence studies (ref. 19). A fit of the energy gap law to these data then yields a curve (Fig. 3) from which the S_1 energies of other, longer carotenoids, that do not exhibit fluorescence from their S_1 states, may be determined. Table 1 lists all the S_1 energies of carotenoids, derived from this work. The values of the energies have several implications.

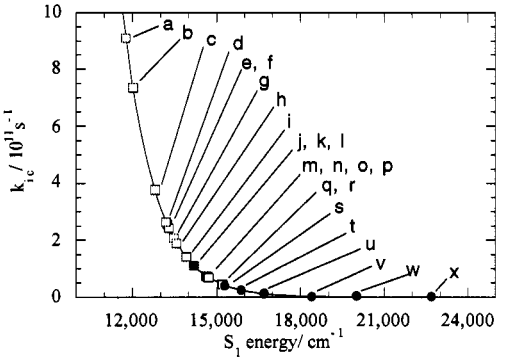

Fig. 3. A fit of the energy gap law (Eq. 1) to the S_1 dynamics and energies of three spheroidene analogs (s, u, v), fucoxanthin (t) β-carotene (k) and two of its analogs mini-5-β-carotene (x) and mini-7-β-carotene (w) (solid circles). From the curve (solid line) the energies of the S_1 states of other carotenoids (open squares) were deduced. The key to the letters and the values of the energies are given in Table 1.

1. The S_1 energies of all naturally occurring carotenoids having up to 13 conjugated carbon-carbon double bonds are higher than the S_1 energy of the 850 nm (11,765 cm^{-1}) absorbing bacteriochlorophyll found in the B800-850 pigment-protein complex of photosynthetic bacteria. This suggests that energy transfer from the S_1 state of carotenoids to bacteriochlorophyll is energetically feasible in all B800-850 light-harvesting complexes.

2. The S_1 energies of β-carotene (14,200 cm^{-1}), and zeaxanthin (14,188 cm^{-1}) which is isoelectronic with β-carotene, are lower than that of chlorophyll a absorbing at 680 nm (14,705 cm^{-1}). This suggests that these molecules cannot act as efficient light-harvesting pigments by transferring energy via their S_1 states. They may, however, quench chlorophyll excited states, providing photoprotection by dissipating excess excitation energy. They may also regulate the flow of energy among the antenna pigments (ref. 20).

3. Whereas zeaxanthin has an S_1 energy lower than chlorophyll a, violaxanthin, which is the epoxidized derivative of zeaxanthin, is found to have an S_1 energy higher than chlorophyll a. Violaxanthin and zeaxanthin are known to be involved in the enzymatic epoxidation/de-epoxidation cycle known as the xanthophyll cycle (ref. 21). This cycle has been implicated widely in the literature in the roles of excess energy dissipation and energy flow regulation in photosynthetic antennae (ref. 20). The probability that a carotenoid either transfers energy forward to chlorophyll or quenches a chlorophyll excited singlet state is probably determined, among other factors, by the positions of their S_1 energy levels. Diatoxanthin and diadinoxanthin, two molecules involved in a similar enzymatic cycle in diatoms, show an identical relationship to the S_1 energy of chlorophyll a as violaxanthin and zeaxanthin, suggesting that this is a general feature of xanthophyll cycle carotenoids regardless of species of origin (ref. 22).

4. The S_1 energies of lutein (14,724 cm^{-1}) and peridinin (14,680 cm^{-1}) are roughly isoenergetic with that of chlorophyll a, consistent with the fact that these molecules transfer energy very efficiently to chlorophyll a in photosynthetic antenna systems (ref. 23, 24).

5. The plot of the energies of the S_1 and S_2 excited states in Fig. 2 shows their different dependence on conjugated chain length. This provides a qualitative rationale for the trends in the fluorescence behavior of carotenoids with different extents of π-electron conjugation. Carotenoids having chain lengths longer than nine conjugated double bonds tend not to fluorescence from their S_1 states owing to the facts that the S_1 and S_2 states lie at relatively low energies, and that the S_2 - S_1 energy gap is reasonably large. The large energy gap between S_2 and S_1 reduces the probability of internal conversion between these states and enhances radiative decay from S_2. Carotenoids having chain lengths shorter than eight conjugated double bonds have dominant fluorescence from S_1 at least partly because the S_2 - S_1 energy gap is small enough to promote efficient internal conversion between S_2 and S_1.

TABLE 1. S_1 lifetimes and energies of carotenoids either measured or deduced from fluorescence data or Eq. 1.

Carotenoid	Key to Fig. 3	τ_{S_1} (ps)	S_1 Energy (cm^{-1})	Ref.
1',2'-dihydro-3',4',7',8'-tetradehydrospheroidene	a	1.10	11,775	25
spirilloxanthin	b	1.36	12,026	25
7',8'-didehydrospheroidene	c	2.66	12,805	25
5',6'-dihydro-7',8'-didehydrospheroidene	d	3.91	13,245	25
lycopene	e	3.82	13,215	25
rhodopin glucoside	f	4.12	13,305	25
astaxanthin	g	4.79	13,479	25
canthaxanthin	h	5.26	13,584	26
locked-15,15'- cis-spheroidene	i	7.09	13,922	25
zeaxanthin	j	9.00	14,188	27
β-carotene	k	9.09	14,200	12
spheroidene	l	9.09	14,200	28
diatoxanthin	m	13.3	14,620	25
peridinin	n	14.04	14,680	25
antheraxanthin	o	14.4	14,709	27
lutein	p	14.6	14,724	25
diadinoxanthin	q	22.8	15,214	25
violaxanthin	r	22.3	15,190	27
3,4-dihydrospheroidene	s	25.4	15,300	28
fucoxanthin	t	41.0	15,873	29
3,4,5,6-tetrahydrospheroidene	u	84.7	16,700	28
3,4,7,8-tetrahydrospheroidene	v	406	18,400	28
mini-7-β-carotene	w	345	20,000	30
mini-5-β-carotene	x	2,000	22,700	30

Fluorescence bandshapes

Absorption and fluorescence are the most direct methods for determining the energies of the excited states of carotenoids. Yet, even when these techniques succeed, the assignment of the spectral origin associated with the transition can be difficult. This is particularly true for the $S_1 \rightarrow S_0$ ($2^1A_g \rightarrow 1^1A_g$) transitions of carotenoids whose bandshapes yield little in the way of vibronic structure. This was carefully noted by Cosgrove et al. (ref. 9) who contrasted the vibronic structure in the fluorescence and fluorescence excitation spectra of all-*trans*-2,4,6,8,10,12,14-hexadecaheptaene with that observed from all-*trans*-β-apo-12'-carotenol. The hexadecaheptaene molecule displayed sharp vibronic features whereas the carotenol did not. A lack of vibronic structure is typical of spectra from the more highly substituted carotenoid systems. The work further illustrated that the Franck-Condon maximum of the fluorescence can be substantially removed in energy from the spectral origin. Hence, even if fluorescence from carotenoids is observed, the electronic state energy, *i.e.* the spectral origin, may be uncertain and open to varied interpretation depending on how one assigns the poorly resolved vibronic features.

Most reports of the fluorescence from the S_1 states of carotenoids indicate that the spectral profiles are very broad, spanning over 200 nm (roughly 4,000 cm^{-1} in the vicinity of 700 nm) and typically without much vibronic structure. The reports of the fluorescence from spheroidene presented by Watanabe and Koyama, *et al.* (refs. 13, 14), however, stand in stark contrast to the other reports. These authors present S_1 emission spectra from spheroidene that appear at ~673 nm, are relatively sharp (bandwidth ~ 30 nm, roughly 600 cm^{-1}) with almost all of their intensity in a single vibronic band, and with comparable intensity to the pronounced $S_2 \rightarrow S_0$ ($1^1B_u \rightarrow 1^1A_g$) emission typically observed from carotenoids having more than 9 carbon-carbon double bonds. Notwithstanding the fact that the energy of the S_1 state of spheroidene assigned from these measurements is very close to that assigned by the extrapolations discussed above, the spectra are unusual not only in their intensity and lack of extended vibronic development, but also in their excitation spectra. This led other workers to speculate that the emission is more reminiscent of that from a porphyrin derivative than from a carotenoid (ref. 31).

A proper assignment of the S_1 emission of spheroidene is important for several reasons: (i) There is only one other carotenoid, β-carotene, having greater than 9 conjugated carbon-carbon double bonds whose S_1 emission has been reported; (ii) The electronic state energy is important in attempting to understand the mechanism of singlet energy transfer in the B800-850 complex from *Rb. sphaeroides* wild type where spheroidene is the major carotenoid; (iii) The bandshape of the emission will determine quantitatively the contribution of spectral overlap (see below) to the rate and efficiency of energy transfer between spheroidene and bacteriochlorophyll in the antenna complexes of the photosynthetic bacteria; (iv) The excitation spectra of the emission observed by Watanabe and Koyama, *et al.* (refs. 13, 14) were used to assign the positions of electronic states other than S_1 and S_2. These assignments need verification; (v) If indeed the S_1 emission from spheroidene and other long chromophore carotenoids can be observed readily by steady state fluorescence spectroscopic methods, a discussion of the validity of the extrapolation methods described above is rendered moot.

For these reasons, we attempted to reproduce the narrow-line emission Watanabe and Koyama, *et al.* (refs. 13, 14) assigned to spheroidene. We prepared two types of spheroidene samples. The first was from spheroidene that had been extracted from the cells of the bacterium, *Rb. sphaeroides* wild type. Another sample was prepared from spheroidene synthesized in the laboratory of Johan Lugtenburg at the University of Leiden. Figures 4a and b show the fluorescence and excitation spectrum of the two preparations. The synthetic spheroidene lacks the narrow, red-shifted emission, whereas this emission is quite prominent in the sample prepared from the extracted cells.

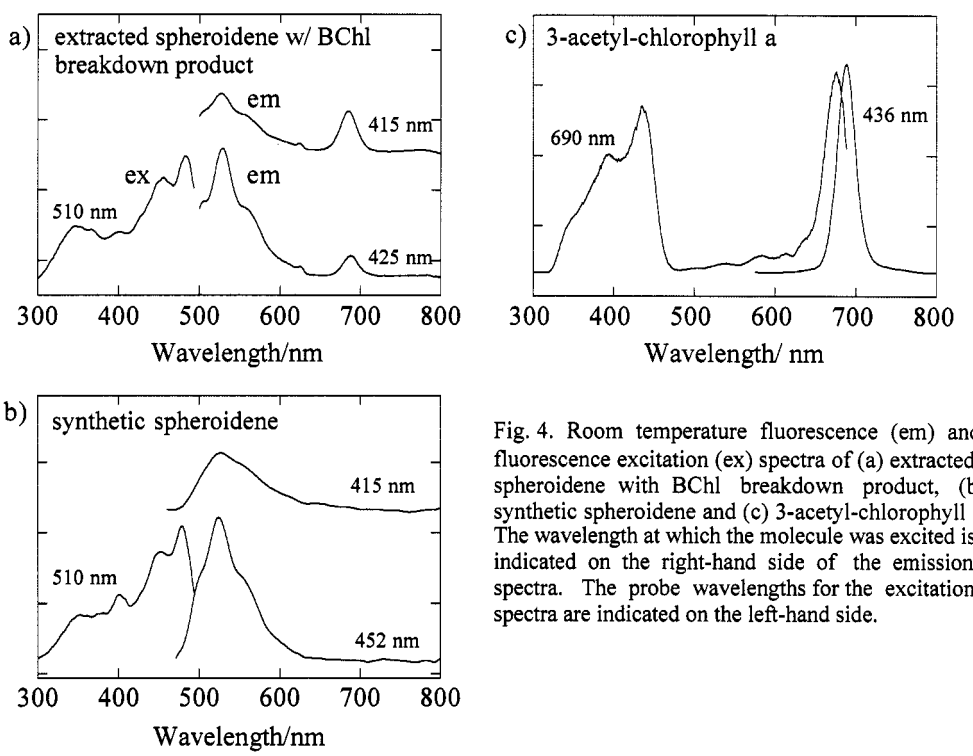

Fig. 4. Room temperature fluorescence (em) and fluorescence excitation (ex) spectra of (a) extracted spheroidene with BChl breakdown product, (b) synthetic spheroidene and (c) 3-acetyl-chlorophyll a. The wavelength at which the molecule was excited is indicated on the right-hand side of the emission spectra. The probe wavelengths for the excitation spectra are indicated on the left-hand side.

The origin of the narrow emission becomes evident if one analyses an air-saturated sample of bacteriochlorophyll that had been illuminated with white light (Fig. 4c). The fluorescence observed from the bacteriochlorophyll sample after this treatment is indistinguishable in the region around 675 nm in both its emission and excitation profiles from that seen in the extracted carotenoid solution (Fig. 4a). The narrow emission around 675 nm very likely does not arise from spheroidene, but rather can be assigned to 3-acetyl-chlorophyll a (also known as 2-desvinyl-2-acetylchlorophyll a) which is a major oxidation product formed during the isolation of bacteriochlorophyll (ref. 32). 3-Acetyl-chlorophyll a is highly fluorescent. Consequently, a trace amount of this impurity in the carotenoid sample, which could easily go

undetected by absorption spectroscopy, could give rise to an emission intensity comparable to the very weak (quantum yields ~10^{-4} to 10^{-5}) emission seen from the carotenoids.

ENERGY TRANSFER

The energies of the excited states of carotenoids are not the only factor important in determining the rate and efficiency of singlet energy transfer between carotenoids and chlorophyll in photosynthetic systems. The nature of the excited state wavefunctions involved in the transfer, the orientation of the carotenoids relative to the chlorophyll, and the extent of spectral overlap between the donor emission and the acceptor absorption are also important. These factors are expressed in the formulations describing energy transfer processes. These include: (i) The Förster induced dipole-dipole resonance transfer mechanism (ref. 33); (ii) the Coulomb mechanism with strong coupling between closely associated transition dipoles (ref. 34); (iii) the Coulomb mechanism involving multipolar interactions (ref. 35); and (iv) the Dexter or electron exchange mechanism (ref. 36). The low quantum yields of carotenoid fluorescence argue against the Förster resonance energy mechanism being operative in carotenoid-to-chlorophyll energy transfer. However, the Coulomb mechanism involving either dipolar or higher order multipolar coupling or electron exchange may indeed be important. We examine here the relevant formalisms of the Coulomb mechanism involving strongly interacting transition dipoles and the electron exchange mechanism.

Coulomb dipole mechanism

The fundamental equation describing the Coulomb interaction energy between transition dipoles is given by

$$E_{12} = \frac{C\mu_1\mu_2\kappa}{r^3} \tag{2}$$

where C is a proportionality constant, μ_1 and μ_2 are the transition dipoles of the donor (carotenoid) and acceptor (chlorophyll or bacteriochlorophyll) molecules, κ describes the relative orientations of the dipoles, and r is the intermolecular separation (ref. 34). The dependence of the rate of energy transfer on this interaction energy will be affected by the strength of the molecular coupling compared to the bandwidths of the molecular electronic transitions (ref. 37). This mechanism is most probable if the energy transfer occurs from states into which absorption has a significant oscillator strength, as is the case for the $S_0 \rightarrow S_2$ ($1^1A_g \rightarrow 1^1B_u$) carotenoid transition. Energy transfer originating from the S_2 states of carotenoids may be attributed to this mechanism. This is not the case for energy transfer originating from the S_1 states of carotenoids. Because the $S_0 \rightarrow S_1$ ($1^1A_g \rightarrow 2^1A_g$) transition is symmetry forbidden, this renders transfer from the S_1 state via a dipole mechanism very unlikely.

Exchange mechanism

The fundamental equation describing the dependence of the rate of energy transfer on distance in this mechanism is

$$k_{Dexter} = ZJ_{Dexter} \tag{4}$$

where Z is a function of the donor-acceptor distance, r, as

$$Z = K\exp\left(\frac{-2r}{L}\right) \tag{5}$$

K is related to the nature of the orbitals involved and L represents the van der Waals radius (ref. 38). The overlap integral in this mechanism is given by

$$J_{Dexter} = \frac{\int_0^\infty F_d(\nu)\varepsilon_a(\nu)\nu^{-4}d\nu}{\int_0^\infty F_d(\nu)\varepsilon_a(\nu)d\nu} \tag{6}$$

where $F_d(\nu)$ represents the fluorescence spectrum of the donor and ε_a corresponds to the absorption of the acceptor on a frequency (ν) scale. This mechanism is probable if the energy transfer occurs from states into which absorption does not have a significant oscillator strength. This is true for triplet states and for the $S_0 \rightarrow S_1$ ($1^1A_g \rightarrow 2^1A_g$) carotenoid transition. Energy transfer originating from the S_1 states of

carotenoids is usually attributed to this mechanism, although a mechanism based on higher order multipole interactions may also operate in this case (ref. 35).

An issue fundamental to all mechanisms is that the rate of energy transfer depends on spectral overlap between the fluorescence of the donor and the absorption of the acceptor. Because the rate of energy transfer depends on spectral overlap, the efficiency will also. The efficiency of energy transfer, ε_{ET}, can be written as

$$\varepsilon_{ET} = \frac{k_{ET}}{k_{ET} + \sum_i k_i} \tag{7}$$

where k_{ET} is the rate constant for energy transfer described by any of the above-mentioned mechanisms, and k_i is the rate constant for any process, *e.g.* internal conversion or intersystem crossing, that competes with energy transfer. Of particular importance is the position of the Franck-Condon maximum for the transitions relative to the spectral origin. If there is a significant energy difference between the Franck-Condon maximum of the fluorescence band relative to the spectral origin, as is typical of the S_1 emission from carotenoids, then the S_1 energy of the donor (carotenoid) molecule can be higher than the S_1 energy of the acceptor (chlorophyll) molecule but the spectral overlap could be very poor (Fig. 5) leading to a low efficiency of energy transfer. This is probably at least one of the reasons why carotenoids having greater than 10 conjugated carbon-carbon double bonds in the antenna complexes of photosynthetic bacteria have lower energy transfer efficiencies than those having 9 or 10 carbon-carbon double bonds (ref. 1). In no case is the S_1 state energy of the carotenoid lower than that of bacteriochlorophyll, yet the energy transfer efficiencies for rhodopin (~50%) (refs. 39, 40) which has 11 carbon-carbon double bonds and spirilloxanthin (~30%) (ref. 41) which has 13 conjugated carbon-carbon double bonds are low compared to that observed for spheroidene (~90%).

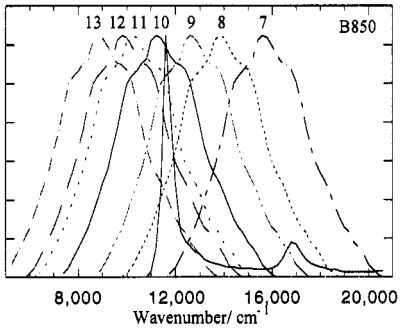

Fig. 5. Spectral overlap between the B850 light-harvesting complex from *Rb. sphaeroides* R-26.1 and hypothetical fluorescence traces from spheroidene analogs containing 7-13 carbon double bonds. The hypothetical traces were derived from the actual fluorescence of 3,4,7,8-tetrahydrospheroidene, shifted to correspond to the spectral origins of the other analogs. Note that the spectral origin of the longest chromophore molecule in the series is higher in energy than B850, but the overlap is low.

It was previously suggested (ref. 42) that, compared to the shorter chromophore (9 and 10 carbon-carbon double bonds) carotenoids, the faster rates of internal conversion from the S_1 (2^1A_g) states for the longer chromophore (11-13 carbon-carbon double bonds) carotenoids would compete more effectively with energy transfer to bacteriochlorophyll thereby lowering the overall efficiency of energy transfer. Until recently, it has generally been accepted that $S_2 \rightarrow S_1$ ($1^1B_u \rightarrow 2^1A_g$) internal conversion is so rapid that there would be little possibility of transfer to the bacteriochlorophyll originating from the S_2 states of carotenoids. However, Shreve, *et al.* (ref. 43) using femtosecond time resolved optical spectroscopy have demonstrated that, after excitation of the carotenoid, excited states of bacteriochlorophyll are formed in the same (~200 fs) time domain as the S_2 state of the carotenoid decays. This is convincing evidence that transfer from the S_2 state of the carotenoid to bacteriochlorophyll occurs. Also, Andersson *et al.* (ref. 44) provided data from fast transient optical studies on *Chromatium purpuratum* that were consistent with energy transfer occurring directly from the B_u^+ state of the carotenoid, okenone, to the Q_x state of BChl. Precisely how much energy absorbed by the carotenoid is partitioned to bacteriochlorophyll via its S_2 state and how much is transferred via S_1 is not completely understood, but undoubtedly it depends on the specific carotenoid, the position and nature of its energy states, the orientation of the transition dipoles (ref. 45), spectral overlap, and the dynamics of its excited states. However, despite the likely prospect of energy transfer occurring from the S_2 states of carotenoids, presumably via the Coulomb mechanism, the S_2 route does not appear to completely compensate for the loss of efficiency in the long chromophore carotenoids brought about by the lowering of the energy states with extending conjugation.

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