

## Review



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# The origin of the dark $S_1$ state in carotenoids: a comprehensive model

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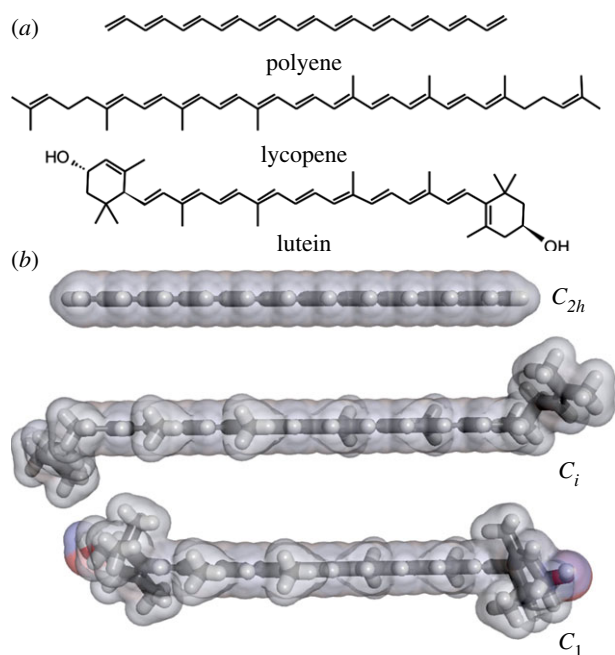
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In carotenoids, by analogy to polyenes, the symmetry of the  $\pi$ -electron system is often invoked to explain their peculiar electronic features, in particular the inactivity of the  $S_0 \rightarrow S_1$  transition in one-photon excitation. In this review, we verify whether the molecular symmetry of carotenoids and symmetry of their  $\pi$ -electron system are supported in experimental and computational studies. We focus on spectroscopic techniques which are sensitive to the electron density distribution, including the X-ray crystallography, electronic absorption, two-photon techniques, circular dichroism, nuclear magnetic resonance, Stark and vibrational spectroscopies, and on this basis we seek for the origin of inactivity of the  $S_1$  state. We come across no experimental and computational evidence for the symmetry effects and the existence of symmetry restrictions on the electronic states of carotenoids. They do not possess an inversion centre and the  $C_{2h}$  symmetry approximation of carotenoid structure is by no means justified. In effect, the application of symmetry rules (and notification) to the electronic states of carotenoids in this symmetry group may lead to a wrong interpretation of experimental data. This conclusion together with the results summarized in the review allows us to advance a consistent model that explains the inactivity of the  $S_0 \rightarrow S_1$  transition. Within this model,  $S_1$  is never accessible from  $S_0$  due to the negative synergy of (i) the contributions of double excitations of very low probability, which elevate  $S_1$  energy, and (ii) a non-verticality of the  $S_0 \rightarrow S_1$  transition, due to the breaking of Born–Oppenheimer approximation. Certainly, our simple model requires a further experimental and theoretical verification.

## 1. Introduction

Carotenoids (Crts) are a large group of natural isoprenoid pigments which play numerous important roles in living organisms. The activity of Crts ranges from light harvesting, photoprotection and stabilization of the photosynthetic complexes to being physical and chemical quenchers of singlet oxygen, antioxidants and precursors of visual pigments in animals [1–6]. The versatility of Crts and such a broad variety of processes which involve Crts obviously engage their extended  $\pi$ -electron system. The understanding of its electronic structure and properties is of vital importance and therefore for a long time these pigments have been the subject of intensive studies. Conventionally, the isoprenoid chromophores are treated as linear polyenes and on this basis their electronic structure, conformation and photophysical properties are interpreted. In particular, the lack of activity of some electronic transitions in these chromophores is rationalized in terms of linear polyene molecular symmetry [7–9]. However, our recent *ab initio* density functional theory (DFT) studies on the ground state retinal and carotenoids reveal that isoprenoid chromophores fundamentally differ from linear polyenes and the essential structural and photophysical features of the conjugated  $\pi$ -electron system of Crts and related chromophores are largely dictated by ‘innocent’ methyl substituents [10]. The methyl side groups shape the conformation and symmetry of these molecules by causing sigmoidal distortions of their skeleton and increasing its flexibility, leading to the helicality of their conjugated  $\pi$ -electron system.



**Figure 1.** The structural formulae of the representative unbranched conjugated polyene (docosa-1,3,5,7,9,11,13,15,17,19,21-undecaene) and two carotenoids, lycopene and lutein (a), and their computationally predicted conformations (b), with the assignments to the point symmetry groups indicated. The computations were carried out using Gaussian 09 [11] and the *ab initio* DFT approach, with the B3LYP potential and 6–31G(d) basis set. (Online version in colour.)

Obviously, these effects do not occur in linear polyenes. Indeed, the apparent simplicity of Crt structure, as related to that of polyenes (figure 1), is somewhat deceptive. In reality, the electronic structure of their system of alternating C–C and C=C bonds is intriguingly complicated as manifested in the peculiar photophysical properties of Crts (the reader is referred to excellent books on this topic [1,2,12,13]). As such, the understanding of Crt photophysics has long posed a challenge to both the experimental and theoretical approaches. To this end, several quite basic issues, such as the presence or the absence of some electronic levels and their energetic order, remain unresolved. In particular, the electronic transition from the  $S_0$  state to the  $S_1$  state is virtually never directly observed in Crts, in contrast to the very intense transition to the  $S_2$  state [9,14–17]. The optically inactive  $S_1$  state is nevertheless quite real, because it can be populated from the higher excited states in inter- and intramolecular processes [18] and is involved in pigment–pigment energy transfer in light harvesting antenna [3,16]. Also, a transient absorption from this state to higher excited states of Crts ( $S_N$ ) occurs and it can be indirectly detected in two-photon absorption/excitation measurements [19–22].

Formally, the molecules of most of these pigments show a high degree of asymmetry and only a few carotenoids are symmetric. In spite of this, considering the symmetry of the conjugated  $\pi$ -electron system alone, symmetry-based reasoning was carried over from polyenes to Crts in order to interpret their peculiar photophysical features.

Molecular symmetry considerations based on group theory have made a significant contribution to the development of modern chemistry. They concern molecules whose symmetry is higher than trivial  $C_1$  symmetry, i.e. have at least two elements of symmetry, in their relaxed ground state and the electronic transitions from this state to higher excited states. The beauty and strength of the symmetry-

based approach stems from the fact that a simple assignment of a molecule to a specific symmetry group precisely defines the symmetry properties of its ground state wavefunctions, from which all observables can be derived and interpreted. On the other hand, molecular symmetry imposes some restrictions on the basic physicochemical features of the molecules, such as the permanent electric dipole moment ( $\mu$ ) and chirality, both related to the presence/absence of an inversion centre. Thus, only molecules having  $C_{nv}$ ,  $C_{2v}$  or  $C_s$  symmetry possess  $\mu$  while the ones having axis of improper rotation cannot be chiral, implying that chiral molecules and the ones having  $\mu$  do not possess the inversion centre [23,24].

This attractive symmetry-based approach appears very relevant to an understanding of the photophysical properties of photosynthetic pigments, in particular Crts, and their interactions with light, which is crucial to their functioning in natural systems. Indeed, it has been extensively employed in investigations of these pigments in order to understand their properties. It is based on the assumption that strict rules originating from symmetry description of linear polyenes can be carried over on these less symmetric molecules. In this review, we aim to critically confront the symmetry-based approach with the outcomes of various experimental and computational investigations of Crts. We focus on the methods which are sensitive to the symmetry of electron density distribution and may reveal whether these molecules have any symmetry elements that would affect the activity of their optical transitions.

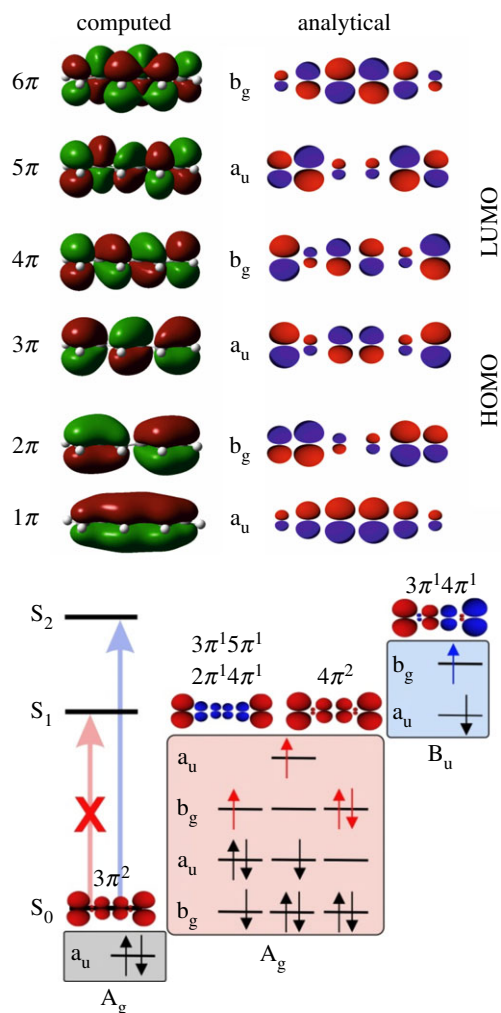
## 2. Controversies concerning the symmetry of carotenoids

In linear polyenes, which have an inversion centre and the  $C_2$  axis (i.e. the  $C_{2h}$  symmetry), the  $S_0(A_g) \rightarrow S_1(A_g)$  transition is strictly symmetry-forbidden because these two states have the same spatial parity  $g$  [7,25]. The basis of this selection rule for the simple polyene, 1,3,5-hexatriene, is depicted in scheme 1, where the symmetry changes of its frontier molecular orbitals and the two energetically lowest electronic transitions are shown. The electronic transition dipole moment  $D$  for the transitions between the ground and the two lowest excited singlet states,  $S_0$ ,  $S_1$  and  $S_2$ , is given by  $\langle \varphi(S_{i=1,2}) | \hat{D}(x,y,z) | \varphi(S_0) \rangle = D_{(S_{i=1,2} \leftarrow S_0)}(x,y,z)$ . The symmetry-based selection rules allow one to quickly determine for which transitions its value is zero, i.e. which of them are symmetry-forbidden. The symmetry of the  $A_g$  and  $B_u$  states is a direct product of the irreducible representations of the occupied  $\pi$  molecular orbitals, which in the  $C_{2h}$  group belong to the irreducible representations of  $a_u$  and  $b_g$  symmetry, as shown in equation (2.1):

$$A_g(S_1) \times \begin{bmatrix} a_u(z) \\ b_u(x; y) \end{bmatrix} \times A_g(S_0) = \begin{bmatrix} a_u \\ b_u \end{bmatrix} \quad S_1 \xleftarrow{\text{forbidden}} S_0. \quad (2.1)$$

The one-photon transition between the  $S_0$  and  $S_1$  states, which both have the  $A_g$  symmetry, is forbidden because the symmetry of the final product is improper as it does not include the  $a_g$  representation. Whereas, as seen in equation 2.2, the  $S_0 \rightarrow S_2$  transition is allowed because the final product does include such a representation:

$$B_u(S_2) \times \begin{bmatrix} a_u(z) \\ b_u(x; y) \end{bmatrix} \times A_g(S_0) = \begin{bmatrix} b_g \\ a_g \end{bmatrix} \quad S_2 \xleftarrow{\text{allowed}} S_0. \quad (2.2)$$



**Scheme 1.** Effects of  $C_{2h}$  symmetry on the shape of electronic wave functions of 1,3,5-hexatriene obtained computationally and analytically (upper panel) and on their products, and the occupancies of the  $\pi$ -orbitals in the  $S_0$  (box on left),  $S_1$  (box in the middle) and  $S_2$  (box on the right) states for one- and two-electron promotions (bottom panel). The transition to the  $S_2$  state is symmetry-allowed while either promotion to the  $S_1$  state is symmetry-forbidden, as indicated on a simplified Jablonski diagram on the left. The computed wave functions were obtained using the B3LYP/cc-pVTZ method. The analytical wave functions were generated using the method described by Scherer & Fischer [26]. (Online version in colour.)

This mathematical description has a physical origin, because these selection rules stem from the presence of an inversion centre in the chromophore molecule, which determines the properties of the transition dipole moment in such a way that it zeros for some electronic transitions. Thus, in the  $C_{2h}$  symmetry the electronic transitions are dipole-forbidden only in centrosymmetric molecules.

However, as reviewed below, many features and observables of Crts are not entirely consistent with this view. In order to account for some of these discrepancies, the pseudoparity indices (+/-) are sometimes used, based on the Pople-Pariser-Parr approximation, in which for simplicity a strict  $\pi$ - $\sigma$  separation is assumed. Because such a separation in polyenes and Crts does not exist, these indices have nothing to do with real molecular symmetry. As a matter of fact, they were introduced merely to simplify the computations [27]. In consequence, in the  $C_{2h}$  symmetry group, the genuine energetic ordering of the states must be the following:  $1A_g < 2A_g < 1B_u < 3A_g < 2B_u$ . It follows then that the one-photon

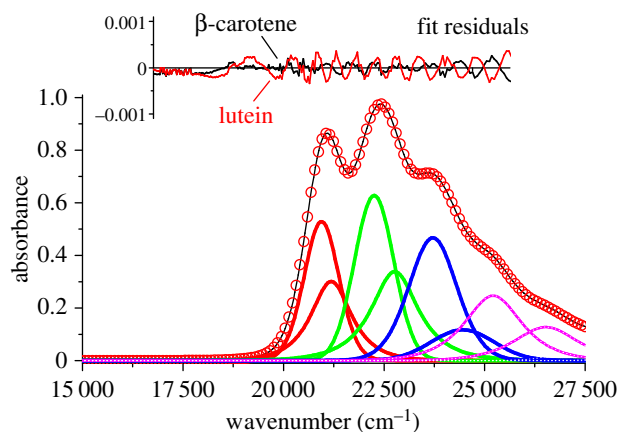
transitions  $1A_g - 2A_g$ ,  $2A_g - 3A_g$  and  $1B_u - 2B_u$  are identically strongly symmetry-forbidden in either direction. Though this straightforward conclusion does not agree with the results of the transient absorption and fluorescence emission measurements in polyenes and Crts, which show the activity of these transitions. In particular, the  $S_1 \rightarrow S_0$  transition is well seen in steady-state fluorescence emission from polyenes and carotenes [14,28,29]. These results and a growing number of other reports show that even in linear polyenes the  $C_{2h}$ -symmetry is readily broken and these molecules are rarely observed as being centrosymmetric [30,31]. Furthermore, a severe symmetry breaking of the conjugated  $\pi$ -electron system in nonlinear Crts, such as the geometric isomers of the *all-trans* forms, or asymmetrically substituted Crts, does not lead to the activation of these 'symmetry-forbidden' or 'dark' transitions [10,17,32,33]. This is in stark contrast to other 'symmetric' polyunsaturated hydrocarbons, such as benzene, in which the symmetry-forbidden electronic transitions are quite readily observed. Benzene is a textbook example of a molecule in which the Born-Oppenheimer approximation is broken and formally forbidden transitions become active due to vibronic coupling leading to the intensity borrowing from the allowed to the forbidden transitions [34]. Furthermore, in short polyenes, the  $S_0 \rightarrow S_1$  transition is one photon allowed [35,36]. It is not clear why analogous or similar mechanisms should not be active in Crts, in light of many reports evidencing vibronic couplings between various electronic states, including the  $S_0$  and  $S_1$  states, in both polyenes and Crts [30,37].

In this context, the vast majority of the numerous works devoted to Crts focus just on selected aspects of their photophysics or photochemistry (see, for instance, [38-42]), whereas only a few compare side-by-side a more complete set of relevant observables, such as electronic absorption/emission, circular dichroism (CD), the Raman and IR spectra, the Stark effect and nuclear magnetic resonance (NMR) data for these pigments [10,17,43,44]. Indeed, a closer analysis of the basic Crts observables indicates at least several inconsistencies with the prevailing symmetry-based model. Here, to validate this symmetry approach we focus on the chirality of Crts and the results obtained using CD, high-resolution NMR and vibrational spectroscopies, and X-ray crystallography, because these techniques provide the most direct information about the symmetry of the chromophore electronic wave function.

### 3. Verification of the molecular symmetry of carotenoids

Many natural Crts are chiral, implying that their molecules do lack both the inversion centre and the horizontal mirror plane [45], i.e. the very symmetry elements due to which the electronic transitions are symmetry-forbidden in the  $C_{2h}$  group. Experimentally, this chirality is best manifested in the CD spectra of Crts; for instance, peridinin (Per) and lutein (Lut) exhibit distinct CD activity in the visible range [10,46], as do many other chiral Crts [13,47]. This CD activity clearly does manifest the chirality (=asymmetry) of the ground state structures and the conjugated  $\pi$ -electron systems of these pigments. Our computational studies show that universally, due to the presence of methyl side groups, the molecules of Crts and other related chromophores, including retinals, are helically twisted and hence they show CD

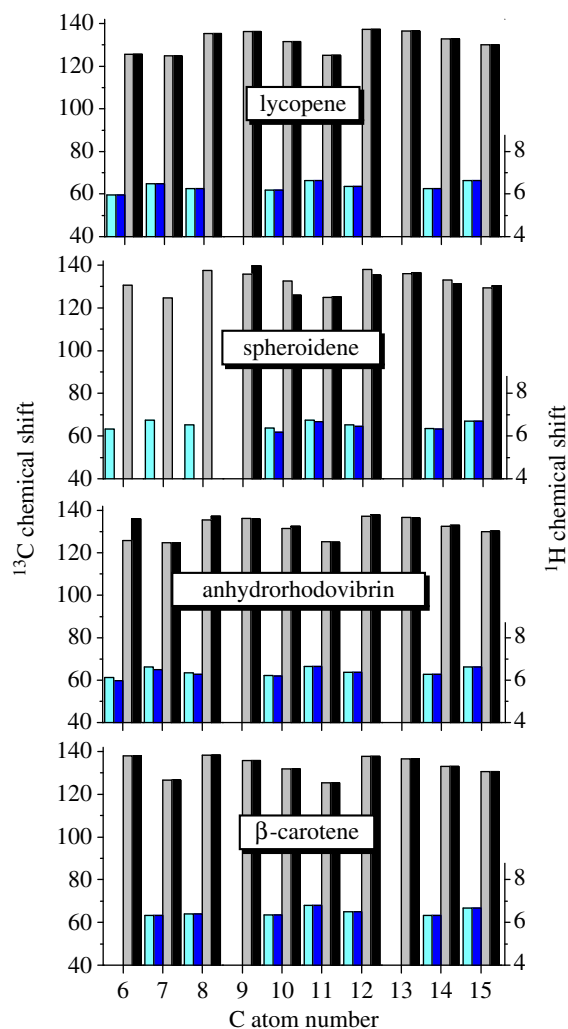




**Figure 2.** Electronic absorption spectrum of lutein in 2-propanol and its deconvolution into a Gaussian-Lorentzian product taking into consideration the composite structure of the vibronic components (three major sets (in red, green and blue) of two vibronic components corresponding to two vibrational modes in carotenoids [48]). The same method was used to analyse the absorption spectrum of  $\beta$ -carotene in the same solvent (not shown). In both cases, this approach yields the value of the coefficient of determination ( $r^2$ ) equal 1, unlike the pure Gaussian or the sum of Gaussian and Lorentzian functions. Above the spectrum, the residuals of the two fits are shown. The deconvolution was done using the PeakFit program (v. 4.12, Sea-Solve Software Inc.). (Online version in colour.)

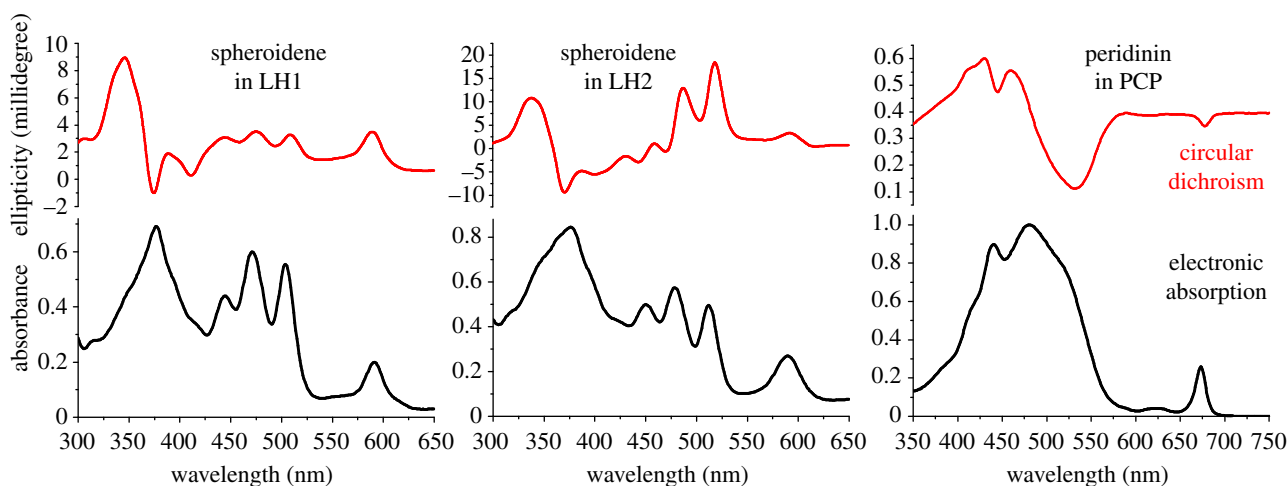
activity, which again excludes the existence of an inversion centre and symmetry plane [10]. The absence of these symmetry elements in Crts is expected to result in non-zero values of their  $\mu$ , which agrees with the results obtained with the use of Stark spectroscopy (see below). Nonetheless, the loss/lack of either of these two symmetry elements seems not to activate any detectable transition to an electronic state of energy below that of  $S_2$  [17]. To further verify this notion, we have compared the electronic absorption spectra of chiral Lut and achiral *all-trans*  $\beta$ -carotene ( $\beta$ -Car). A rigorous analysis, based on the inspection of the fit residuals obtained via spectral deconvolution of the electronic absorption profiles of  $\beta$ -Car and Lut into Gaussian-Lorentzian products, discloses no transitions below  $S_2$  level in either spectrum (figure 2). The equality of the residuals for  $\beta$ -Car and Lut, down to a level of 1/10 000, shows that their absorption profiles are virtually identical. This happens in spite of a clear CD activity of the latter pigment in the visible range and the fact that it must originate from chirality of the electronic wave function, obviously including the conjugated  $\pi$ -electron system [10].

The vibrational and NMR spectroscopies provide additional evidence for the lack of inversion centre in Crts molecules. The Raman spectra of Crts show practically no complementarity to their IR spectra [49–53]. The lack of symmetry is also evident from the uneven distribution of the values of chemical shifts ( $\delta$ ) along the  $\pi$ -electron system in the  $^1\text{H}$ -NMR and  $^{13}\text{C}$ -NMR spectra of Crts [5,32,43,54,55]. In the case of Per or cis isomers such an asymmetry is not unexpected but it is also seen in the  $^1\text{H}$ -NMR spectrum of much more ‘symmetric’ neurosporene, spheroidene (Sph), lycopene (Lyc) and  $\beta$ -Car in their *all-trans* conformation. The distribution of the  $\delta$  values along the skeletons of the *all-trans* Lyc, Sph, anhydrorhodovibrin and  $\beta$ -Car in solution is shown in figure 3. In asymmetric anhydrorhodovibrin, the  $\delta$  values on the corresponding atoms ( $C_n - C'_n$ ) in the two



**Figure 3.** The pairwise comparison of the distributions of the  $^1\text{H}$  and  $^{13}\text{C}$  chemical shifts along the halves of the skeletons of the *all-trans* lycopene, anhydrorhodovibrin and  $\beta$ -carotene molecules in solution. The cyan and grey bars denote the 6–15 carbon atoms and the blue and black bars the 6′–15′ carbon atoms. The NMR data for lycopene and anhydrorhodovibrin were taken from Qian *et al.* [43], and for spheroidene from Yue-Shun *et al.* [54] and de Groot *et al.* [56], and for  $\beta$ -carotene from Fiedor *et al.* [5]. (Online version in colour.)

halves of the molecule are uneven, whereas in Lyc and  $\beta$ -Car they are pairwise identical. This identity, though, does not necessarily reveal the real symmetry of these molecules as the measurements record the averaged values for the equilibrium mixtures of their conformers which undergo rapid interconversions (see below). The asymmetry of the electron distribution along the conjugated system is seen in the  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR spectra of Lut [57,58] or rhodopin [43]. Importantly, in all these asymmetric Crts the peripheral substituents are not directly attached to the conjugated  $\pi$ -electron system and their influence on the chemical shift values along the skeleton is rather negligible. In all these Crts, whether symmetric or not, the chart in figure 3 reveals another intriguing feature, namely the periodicity of the  $^1\text{H}$  and  $^{13}\text{C}$  chemical shifts along the skeleton, which apparently reflects their structural periodicity. Most likely, this periodic feature is related to the presence of side methyl groups which induce certain modularity in isoprenoid chromophores [10]. No such periodicity is seen in the NMR spectra of unsubstituted polyenes [59].



**Figure 4.** The electronic absorption (lower panel) and CD spectra (upper panel) of the LH1 complex (from *Rsp. rubrum*) reconstituted with spheroidene according to Fiedor *et al.* [4], the LH2 complex isolated from *Rhd. sphaeroides* according to Bandilla *et al.* [67], and the peridinin–chlorophyll–protein complex (PCP) from *Am. carterae* (purchased from Santa Cruz Biotechnology, USA). The CD activity in the 400–600 nm region results from the chirality of the protein-bound Crts molecules, which is overlapping with the signals due the intracomplex excitonic interactions [4,68–71]. (Online version in colour.)

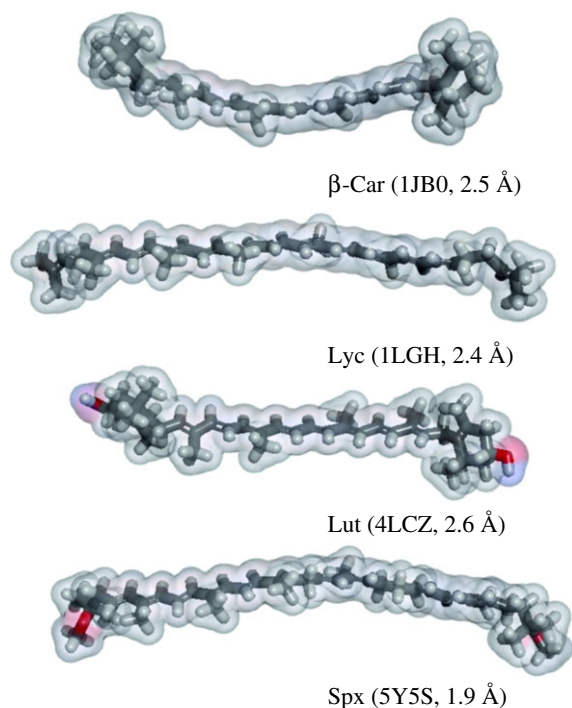
Often, the detection of two-photon absorption is considered as evidence for the  $C_{2h}$  symmetry of Crts and their derivatives [19–22,60], because it is assumed to proceed via virtual states of the symmetry inverted with respect to the symmetry of the ground state ( $\mathbf{g} \rightarrow (\text{virtual})\mathbf{u} \rightarrow \mathbf{g}$ ). However, ‘these selection rules will be relaxed for molecules deformed either statically or dynamically from  $C_{2h}$  symmetry’, as noted by several groups [31,61,62]. Furthermore, the two-photon processes may have nothing to do with  $C_{2h}$  symmetry and will also occur in molecules of lower symmetry or to the allowed states in polar(izable) molecules, and thus this technique alone is not necessarily able to discriminate, e.g. between the  $C_2$ ,  $C_s$  and  $C_{2h}$  symmetries [60,63]. Therefore, the occurrence of two-photon absorption cannot by itself be treated as proof of symmetry but only of the existence of an inactive (dark) state, which can be forbidden by any other cause and not necessarily by symmetry. Most likely, this is the reason why the two-photon excitation of Sph in LH2, Lut in LHCII and Per in Per-chlorophyll-protein (PCP) can be observed [21,64,65], despite the fact that these Crts either in solution or protein-bound are asymmetric, as evidenced by the crystallographic, CD and NMR studies. The high-resolution structure of *all-trans* Sph in LH2 is not available but the NMR data (figure 3) and Raman spectra [66] evidence the lack of the critical symmetry elements in this molecule, while Lut and Per are intrinsically chiral. The chirality of Sph and Per bound in photosynthetic antennae, evidenced by a strong CD activity in the 400–600 nm region (figure 4), entirely confirms this notion. In the light of the fact that non-centrosymmetric molecules show two-photon optical activity, the conclusions concerning the symmetry of Crts and retinals drawn from such two-photon experiments require a thorough reinterpretation, and the question arises as to the true mechanism of the two-photon absorption/excitation in these chromophores. This is further complicated by the fact that majority of the two-photon experiments with Crts is done at ambient temperatures and due to thermal activation there always be a population of molecules which are conformationally pre-prepared for the two-photon resonance to occur, leading to ‘photoselection’. On the other hand, at cryogenic conditions, even in the range of 10–20 K, in such

systems a symmetry breaking due to photoconversion may occur, similar to that in shorter polyenes [36].

Any plausible explanation of the two-photon absorption/excitation should take into account the fact that two-photon absorption may involve double excitations, as predicted theoretically [72,73]. Illustratively speaking, the two photons may transiently interact with two single electrons, each localized on two separate carbon atoms in the conjugated system. This suggests that Rydberg states are engaged in such a two-photon phenomenon, as e.g. in 1,3-butadiene [74]. It seems likely that in Crts those are the  $3p_z\pi$ - and  $3s\pi$ -Rydberg states.

#### 4. Carotenoids in their native protein environment

Further pieces of information come from analysing the available high-resolution (2.6 Å and higher) crystal structures of the photosynthetic pigment–proteins, which show virtually no Crts whose conformation would resemble that of linear polyenes. By contrast, as exemplified in figure 5, which shows the conformations of Crts from four different pigment–protein complexes, all these molecules are severely distorted, being sigmoidal and helically twisted, even if they are achiral or formally symmetric like, e.g., Lyc or  $\beta$ -Car. A deformation of the conjugated chain of the protein-bound *all-trans* Sph, in the bacterial LH2 antenna, was revealed by Raman spectroscopy [66]. Our recent *ab initio* study of the ground state geometry of Crts revealed that this type of skeletal distortion is a fundamental feature of isoprenoid pigments, which is due to the methyl side groups [10]. Similar distortions and low symmetry of Crt molecules have been predicted in several other computational studies [43,53,75–78]. For instance, the relaxed conformation of  $\beta$ -Car shows sigmoidal distortion and has  $C_i$  symmetry but it is lower in energy than its other conformers with lower symmetry by only a very narrow margin (below 0.4 kcal mol<sup>-1</sup>) [75]. This type of conformation and symmetry of  $\beta$ -Car molecule agrees with those determined in crystallographic studies (entry CCDC 253816, the Cambridge Structural Database). The  $C_i$  symmetry can be easily broken via interconversion between the conformers by virtually any interaction with the



**Figure 5.** The representative conformations of various protein-bound carotenoids:  $\beta$ -carotene ( $\beta$ -Car) in PSI antenna, *Th. elongatus*, lycopene (Lyc) in LH2, *Ph. molischianum*, lutein (Lut) in LHCII, *S. oleracea*, and spirilloxanthin (Spx) in LH1-RC, *Th. tepidum*. The structures were taken from the crystallographic structures of photosynthetic light harvesting complexes of currently the highest resolution, as deposited in the Protein Data Bank (PDB). The data files and the resolution are indicated in parentheses. (Online version in colour.)

environment or due to thermally activated motions [78]. Indeed, the interactions of Crts with protein environment in their binding pockets induce further molecular deformations as seen in the examples presented in figure 5. Owing to these distortions the conjugated  $\pi$ -electron system loses both the inversion centre and mirror plane, which means that symmetry does not impose any restrictions on the transitions between the electronic states of these cofactors, as discussed above. The absence of these critical symmetry elements in the protein-bound pigments is further confirmed by the strong CD activity of Crts in their native protein conformations, either measured experimentally (figure 4) or simulated [4,10,69–71,79].

## 5. Realistic symmetry of carotenoids

The above considerations bear very important implications for the functioning of Crts. Most importantly, there seems to be no experimental evidence for the symmetry effects and the existence of symmetry restrictions in Crts. On the contrary, the available data show that molecular symmetry is of no relevance, and many experimental and computational data reveal that Crt molecules either in solution or protein-bound are asymmetric, and thus symmetry does not impose any restrictions on their electronic states and transitions.

Even in terms of ‘local’ or ‘approximate’ symmetry of the chromophore, the conjugated  $\pi$ -electron system of Crts cannot be of the  $C_{2h}$  type, because of the severe asymmetry of their wave function, in particular of its electronic contribution. Moreover, as noted by Hudson *et al.*, the symmetry-forbidden transitions in polyenes are extremely sensitive to subtle perturbations, which break the symmetry (see above).

As mentioned already, benzene is another example of how easily the molecular symmetry is broken. The asymmetry of Crts is unequivocally evidenced by their CD activity in the visible range, the NMR data and the considerable  $\mu$  values, even in the ground state [10,80,81]. If a molecule exhibits a permanent  $\mu$  its symmetry group cannot be higher than  $C_{2v}$ ,  $C_2$ ,  $C_s$  or  $C_1$  [24]. Conversely, the centrosymmetric Crts must have null  $\mu$  and upon excitation should show nearly zero change in its value [82,83]. In other words, Crts show no features of being centrosymmetric whatsoever and consequently, there are no reasons to use the symmetry labels for the electronic states in Crts and their derivatives, including retinals, which anyway are asymmetric and symmetry rules do not apply. In particular, they cannot be used to describe their ‘approximate’ symmetry because in such a case the respective selection rules must be also considered ‘approximate’ and all the transitions between the electronic states would be partly allowed, which is clearly not the case (if they were symmetry-forbidden). Otherwise, the usage of such fictitious symmetry labels deceptively implies that there is no need to seek the genuine origin for the electronic peculiarities in Crts.

In reality, the major determinants of Crt ground state geometry, i.e. the side methyl groups located along the conjugated  $\pi$ -electron system (see their effect in the NMR spectra in figure 3) and the interactions with the chiral binding pockets in proteins seem to act so as to exclude the symmetry-related restrictions from their photophysics [10]. Moreover, considering the existence of many (hundreds) of various chiral Crts in numerous native systems, one has to take into account that in all of these organisms highly specialized biosynthetic pathways must have evolved in order to ensure their strict stereochemical control to generate stereochemically pure pigments. Obviously, the maintenance of such biosynthetic pathways bears high ‘biosynthetic’ costs.

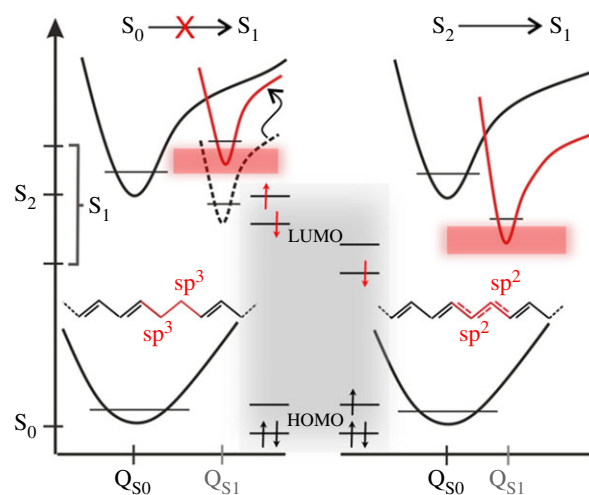
## 6. Why the $S_1$ state in carotenoids is inactive: a comprehensive model

All Crts and their derivatives, regardless of their structure and symmetry, seem to share the same peculiar property, i.e. the inability to access directly, in a one-photon process, some electronic levels, like  $S_1$ . The physical origin of this peculiarity is evidently not related to molecular symmetry. The results of the experimental and theoretical studies accumulated so far allow us to frame a unified and consistent model that accounts for the inactivity and other features of low-lying states in Crts. One hint comes from the fact that the minimum of the potential energy surface in  $S_1$  is considerably shifted from the one in  $S_0$ , which gives rise to a non-verticality of the  $S_0 \rightarrow S_1$  transition. This indicates that the Born–Oppenheimer approximation is not entirely fulfilled in this family of pigments [17,84]. The recent ultrafast X-ray spectroscopic investigations on the non-Born–Oppenheimer effects in ethylene, the common prototype of polyenes, isoprene, retinals and Crts, shed some new light on the features of  $S_1$  [85]. They show that in ethylene there is a very high energetic barrier for the  $\pi \rightarrow \pi^*$  transition which involves the core orbitals of the carbon atoms. It proceeds with a twist of the C–C bond and pyramidalization of one of the carbons (a change from the  $sp^2$  to  $sp^3$  hybridization), thus shifting the equilibrium minimum on the  $S_1$  potential energy surface away from its minimum in the  $S_0$  state. Such



a shift is required for the conical intersection of the states to take place. Similar effects very likely do occur during electronic excitation in polyenes and Crts. Indeed, there are indications from femtosecond absorption spectroscopy that with the population of the  $S_1$  state a change in hybridization of carbon atoms in the Crt conjugated system occurs [86]. This would also agree with a large increase in  $\mu$  of Crts in this state, as estimated based on the Stark and fluorescence polarization spectroscopies [65,80,81,87]. The large value of  $\mu$  in the  $S_1$  state indicates a partial loss of electronic correlation in the  $\pi$  electron system due to its asymmetry. In consequence, the  $S_0 \rightarrow S_1$  transition is non-vertical and it does not carry enough energy to overcome the high energetic barrier to change the hybridization state. In turn, the transitions to the higher excited  $\pi$  states that interact more weakly with the core orbitals, are vertical and do not involve such a drastic distortion of the C=C bonds, and thus they are strongly active.

Another hint as to the origin of the inactivity of the  $S_1$  state comes from theoretical considerations of polyenes, which indicate that this state has a doubly excited character, i.e. there is a large contribution of two-electron configurations to its electronic wave function, whereas such configurations have no share in the  $S_2$  state [61]. In line with the above discussion, the contributions from the doubly excited configurations exclude the presence of any symmetry-related restrictions in long polyenes and Crts. The quantum chemical computations confirm that the contribution of doubly excited configurations in  $S_1$  reaches as much as 50%, and that even the  $S_0$  state contains a significant admixture of such configurations [88,89]. The advanced DFT-based computations of the excited states in linear polyenes confirm all these findings. Very importantly, they also predict that due to these two-electron contributions the  $S_1$  state energy necessarily rises, near or above to the  $S_2$  level. The calculations indicate other consequences of the differences between  $S_1$  and  $S_2$  in the doubly or singly excited character. Namely, in  $S_1$  the conjugated  $\pi$ -electron system becomes somewhat more relaxed, as reflected in the expansion of the C=C bonds [90,91]. The doubly excited character of  $S_1$  implies that the two pathways for its population, one from the ground state and one from  $S_2$ , do differ both mechanistically and energetically, as illustrated schematically in figure 6. In the non-vertical two-electron transition from  $S_0$ , the energetic costs of breaking the electron correlation are exceptionally high and the energy of the  $S_1$  state is much elevated because two electrons have to be promoted simultaneously and break up the correlation. This energetic cost and the exact energy of  $S_1$  will depend on the number of  $\pi$ -electrons in the conjugated system. The second pathway to  $S_1$  opens after a promotion of one electron to the  $S_2$  level. Because now the electron correlation is partly broken and the doubly excited character of  $S_1$  is diminished, the energy of this state is lower and the probability of its population via IC is non-zero. This relaxation path is effective and extremely fast (below 200 fs) due to a strong vibronic coupling between the two states [92], and it is the most often experimentally observed pathway to populate  $S_1$ . Because these two pathways lead to different final electron distributions (with different contributions from two-electron configurations), the position of the potential energy surface minimum of for the  $S_1$  state ( $Q_{S1}$ ) will not be the same in each case (figure 6). Owing to a larger molecular distortion and a complete change from the  $sp^2$  to  $sp^3$  hybridization on one of C-C bonds, when  $S_1$  is populated from  $S_0$ ,  $Q_{S1}$  is



**Figure 6.** A diagram illustrating the major mechanisms responsible for the inactivity of the lowest energy excited singlet state ( $S_1$ ) in carotenoids. In this model, the energy and accessibility of this state vary, depending on whether it is populated from the ground state ( $S_0$ ) or from the second excited singlet state ( $S_2$ ) of the molecule. Note a large difference in the electron distributions on the frontier orbitals in the two limiting cases (centre of the diagram), resulting from two or one electron excitation. The two-electron excitation elevates the energy of the  $S_1$  state and shifts the position of its potential energy minimum, and leads to a change in hybridization type on carbon atoms in the skeleton from  $sp^2$  to  $sp^3$ . The scheme does not take into account the shifts in the ground state energies due to the distortions of carotenoid molecules in the photosynthetic pigment-proteins [10]. (Online version in colour.)

displaced further away from that of  $Q_{S0}$ . The IC to  $S_0$  competes with two other processes, an intersystem crossing to the triplet states and a *trans-cis* isomerization, each of them facilitated by structural deformation of the molecule in the  $S_1$  state. This is in line with a recent analysis of the fluorescence spectra of Crts which shows a large displacement of the  $S_1$  potential energy surface minimum [17].

Taken together, owing to the negative synergy of at least several strong effects, the intensity of the direct  $S_0 \rightarrow S_1$  transition equals zero and, instead, the vertical singly excited transition to  $S_2$  will be very strongly favoured. On top of that, by analogy to the two-photon phenomena, the probability of the two-electron transitions is expected to be extremely low. Under some circumstances, the  $S_1$  state may sort of disappear and its place will be taken over by the  $S_2$  state.

The simple model outlined above does not invoke any (non-existing) symmetry-based restrictions to justify the inactivity of the  $S_0 \rightarrow S_1$  transition, and it consistently explains the 'elusive' features of the  $S_1$  state. First of all, the  $S_1$  state appears 'variable' in terms of its energy and the position of the potential energy surface minimum. Hence,  $S_1$  is never accessible from the ground state because its energy is too high as for a single photon excitation. In the two-photon processes, either one electron configurations will be populated *via* virtual states and therefore they only sense  $S_1$  at low energy (significantly below  $S_2$ ), or they involve double excitations *via* Rydberg states (see above), and they occur with a very low probability.

Secondly, when being populated from  $S_2$ , the following factors influence the size of  $S_2$ - $S_1$  energy gap: (i) the Crt structure and length of the conjugated system, (ii) the interactions of the conjugated system with the environment, and (iii) the

protein-induced skeleton conformation. The first two factors are well known and understood, whereas the last effect is less appreciated. The conformational heterogeneity of Crts in photosynthetic complexes is indeed impressive (see above, figure 5), especially when considering the fact that the deviations from planar conformations involve a significant increase in the total energy, in the range of 50 kcal mol<sup>-1</sup>, even due to relatively modest distortions [10]. It is tempting to postulate that the Crt conformational flexibility, as facilitated by the methyl side groups, provides a convenient handle to control the photophysical properties of Crts, in particular the S<sub>1</sub> level, and hence the Crt functioning in photosynthetic proteins. It may also be responsible for a variety of singlet states ('hot' states, S\*, etc.) often detected below the S<sub>2</sub> state. Such a control mechanism is feasible owing to the helicality and modularity of Crt molecules, well seen, for instance, in the NMR data (figure 3) and in the computations [10]. The diverse twisted conformations assumed by Crts in LHCs may help to prepare these molecules to populate the S<sub>1</sub> state, e.g. *via* a decreasing of the energetic barrier for the in-skeleton sp<sup>2</sup> → sp<sup>3</sup> conversion. The S<sub>1</sub> energy relative to the energy levels of chlorophylls (Chls) will then determine the particular role the Crts fulfil. Indeed, a recent report shows a conformation–function correlation in Crts bound to LHCII [93]. In addition, the molecular deformations greatly enhance the polarizability of Crt molecules, even in the case of Lyc and β-Car, as reflected in the increase in their permanent dipole moments [10]. Owing to this effect, the electronic levels of the chromophore can be tuned via electrostatic interactions within the binding pocket in protein, in analogy to the tuning mechanism of retinals [94].

Our model is also valid when the S<sub>1</sub> state is directly populated via intermolecular energy transfer from excited Chl, formally from the Crt ground state [18]. First of all, the symmetry selection rules could possibly be considered only in the initial step (a photon absorption) of this multistep intermolecular process (1. Chl + hν → Chl\*, 2. Chl\* + Crt → Crt\* + Chl), whereas the step of excitation energy transfer does not involve a photon. In addition, it is an intermolecular process and thus the internal selection rules for electronic transitions simply do not apply. Other internally forbidden states, for instance triplets (strongly forbidden from the ground state), can also be populated via intermolecular processes of energy transfer to molecules formally in their ground state. Secondly, not only the S<sub>1</sub> energies in the acceptor Crt molecules are tuned via distortions but also their ground states are energetically placed much higher than those of undistorted Crts. In other words, differently distorted molecules will have different internal energies, which will affect, for instance, the intermolecular energy transfer [10]. Because it is hard to accurately determine the ground state energies of protein-bound Crts, the shifts in these energies are not shown on the scheme in figure 6. The energy levels of the excited states will vertically follow the ground state and the energetic gaps to the ground state may remain only slightly affected. If, out on many Crts in a

pigment–protein complex, only a few molecules assume significantly distorted conformations, then their S<sub>1</sub> state may even not be spectroscopically resolved.

## 7. Summary

As reviewed in the above, the experimental data obtained from the techniques sensitive to electron density distribution in carotenoid molecules, evidence that they are not centrosymmetric because their π-electron system is asymmetric. The CD activity (= chirality) of Crts in solution and protein-bound *in vivo* is the ultimate evidence for this asymmetry. Hence, because Crts lack the molecular inversion centre, i.e. the very factor that would impose physical restrictions on their electronic states, all the electronic transitions in these pigments are symmetry-allowed. Obviously, no molecular symmetry effects can be expected to play a role in the natural systems and in the functioning of these pigments, especially in the chiral environment of proteins. Thus, also given the universal asymmetry of the ground state wave function of Crts, the symmetry-based description of their electronic levels is totally inadequate, and it is pointless to apply symmetry group rules. Instead, we should be seeking for the factual grounds for the electronic peculiarities in Crts.

Yet, a question remains open to the origin of the dark states in Crts and related molecules. The experimental facts summarized in the present review allow us to propose a consistent model that explains the inactivity of the S<sub>1</sub> state in Crts. The peculiarity of this state results from the following: (i) contributions of double excitations of low probability, (ii) a too high and variable energy of the state, and (iii) non-verticality of the S<sub>0</sub> → S<sub>1</sub> transition, due to the breaking of Born–Oppenheimer approximation. The negative synergy of these powerful factors causes that S<sub>1</sub> is never accessible in one-photon transition from the ground state. This simple model requires a thorough theoretical and experimental verification. The inactive but symmetry-allowed S<sub>1</sub> state, being strongly coupled to the other electronic states in the molecule, must affect the properties of these states. Such a sophistication influences all photophysical and photochemical processes in which Crts participate, including intermolecular energy transfer. Consequently, many experimental results on Crts may need a reinterpretation or revision in order to take these facts into account. Surely, a comprehensive understanding of Crt photophysics still poses a great theoretical and experimental challenge.

**Data accessibility.** This article has no additional data.

**Authors' contributions.** L.F. and M.P. conceived the study. All authors discussed the results and wrote the manuscript.

**Competing interests.** We declare we have no competing interests.

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