Explaining the Temperature Dependence of Spirilloxanthin’s S*\(^*\) Signal by an Inhomogeneous Ground State Model

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ABSTRACT: We investigate the nature of the S*\(^*\) excited state in carotenoids by performing a series of pump–probe experiments with sub-20 fs time resolution on spirilloxanthin in a polymethylmethacrylate matrix varying the sample temperature. Following photocexitation, we observe sub-200 fs internal conversion of the bright S2 state into the lower-lying S1 and S*\(^*\) states, which in turn relax to the ground state on a picosecond time scale. Upon cooling down the sample to 77 K, we observe a systematic decrease of the S*/S1 ratio. This result can be explained by assuming two thermally populated ground state isomers. The higher lying one generates the S*\(^*\) state, which can then be effectively frozen out by cooling. These findings are supported by quantum chemical modeling and provide strong evidence for the existence and importance of ground state isomers in the photophysics of carotenoids.

INTRODUCTION

Carotenoids and chlorophylls are the two fundamental building blocks of natural light harvesting complexes. The role of carotenoids is 2-fold: on one hand, they absorb and utilize sunlight in the blue–green spectral region; on the other hand, they photoprotect the light harvesting complex by quenching excited state triplet- and oxygen singlet-states. Most of the remarkable properties of carotenoids can be explained by their delocalized π-electron system along their polyenic backbone. The lowest excited state in carotenoids, S1, is dark with respect to the ground state due to symmetry reasons. The first optically allowed transition to S2 is broad and shows strong vibronic modulation in the 18 200–25 000 cm\(^{-1}\) region. After the initial excitation event, population transfer between S1 and S2 occurs on a sub-200 fs time scale. The excited state absorption (ESA) signal from S1 is red-shifted compared to the ground state transition and exhibits the strongest transition dipole moment found in naturally abundant molecules. The electronic structure and energy deactivation pathways of carotenoids are a matter of ongoing debate.\(^1\) Employed experimental methods range from sub-10 fs pump–probe,\(^3\) fs-Raman,\(^4\) four-wave-mixing methods,\(^5–10\) electronic 2D-spectroscopy,\(^11–13\) and several other techniques.\(^1,2\) Despite such efforts, no consensus has yet been reached, as summarized in a review article by Polivka and Sundström.\(^2\) The models proposed by different research teams deviate substantially in the interpretation of an electronic state named S*. S* manifests itself as a high-energy shoulder of the S1-ESA band. Depending on the chain length of the investigated carotenoid, the S* lifetime can be substantially longer or on the same time scale as S1. As first described for long-chain \(\beta\)-carotene homologues,\(^14\) S* has been interpreted as a separate electronic excited state,\(^15–20\) an excited state isomer,\(^21\) a vibrationally hot ground state, populated by either Impulsive Stimulated Raman Scattering (ISRS)\(^22,23\) or relaxation form S1,\(^24,25\) the product of different ground state isomers,\(^11,26,27\) or as the result of chemical impurities.\(^28\) We note that none of the proposed energy level models is able to explain all experimental findings in literature. For example: the fact that depletion of S2 via an near-infrared (NIR)-pulse affects only S1, but not S*, has led to the hypothesis that S* stems from a vibrationally hot ground state (hot S0), populated via an ISRS mechanism. However, experiments with varying excitation pulse bandwidth leave the S* signal unchanged,\(^29\) which is not expected for ISRS-processes. Additionally, the hot ground state cannot be populated by the pump pulse in the linear regime of excitation. ISRS will rather produce coherence between S0 and S*,\(^30\) or relaxation form S1,\(^24,25\) and hot S0 will not populate hot S*, as required for an ESA signal.\(^11\) These problems are averted in a model where S* is populated after relaxation from S1;\(^24\) however, such an...
assumption fails in explaining the ultrafast rise time of $S^*$. If $S^*$ is assumed to be the product of an isomerization process on $S_1$, the $S^*$ signal has again the correct ESA character and should be independent of temperature: in such a scenario, the activation energy for isomerization is not thermal, but provided by relaxation from $S_2$. This is in contrast to the observation of temperature effects on $S^*$. In a recent theoretical work, Lukes et al. tried to combine all these findings in a model based on an inhomogeneous ground state. Briefly, the authors interpreted $S^*$ and $S_1$ as the lowest lying excited states of two stable ground state conformers. Such a model correctly predicts the properties of all pump–probe signals and their temperature dependence. Additionally, pump–deplete–probe results can be explained assuming that the $S_2$ spectra of these two isomers differ; an NIR depletion pulse may then selectively deplete only one of them. Lukes et al. showed that rotation of one of the end-groups of $\beta$-carotene is a promising candidate for the origin of such inhomogeneities. It was demonstrated that $\beta$-carotene’s electronic ground state surface shows two thermally populated minima, where the global minimum is slightly asymmetric across the inversion center of the molecule, and the higher lying isomer shows $C_{2v}$ symmetry. It is interesting to note here that the ground state bleach (GSB) signal of carotenoids after the relaxation of $S_1$ but before depopulation of $S^*$ was reported to be more symmetric and more structured than the absorption spectrum. It was therefore speculated that $S^*$ might stem from a subset of molecules showing higher symmetry than the $S_1$-forming ensemble. The higher lying isomer found upon end-group rotation with its $C_{2v}$ symmetry is a promising candidate for such a planar $S^*$-forming subset.

In this work, we put the inhomogeneous ground state hypothesis to a crucial test: if there are indeed two thermally populated ground state isomers (the energetically lower one forming $S_1$ and the other forming $S^*$), then a decrease in temperature should enhance the ESA-signal from $S_1$ with respect to the signal associated with $S^*$. Accordingly, we performed a series of pump–probe experiments at variable temperature on spirilloxanthin, which is the longest naturally occurring carotenoid, with 13 conjugated double bonds. To provide similar environments at any temperature ranging from room temperature (293 K, RT) down to liquid nitrogen (77 K), we could not dissolve spirilloxanthin in a solvent: we instead dispersed it in a polymethyl-methacrylate (PMMA) matrix, which is solid also at room temperature and does not provide similar environments at any temperature ranging from 0.1 to 0.6 as measured by UV absorption spectroscopy. The actual sample studied in this work had OD $= 0.15$ at 540 nm, which is the excitation central wavelength. Carotenoid extractions and preparation of PMMA slides were performed in near darkness. Purity of the sample was confirmed by high performance liquid chromatography (HPLC).

**Ultrafast Spectroscopy.** The high time resolution experimental apparatus is based on two synchronized nonlinear optical parametric amplifiers (NOPAs), pumped by a regeneratively amplified mode-locked Ti:Sapphire laser system delivering pulses with 150 fs duration, 500 μJ energy, 1 kHz repetition rate, and 780 nm central wavelength. The first NOPA generates 15 fs pulses peaked at 540 nm (Pump in Figure 1), in resonance with the $S_0 \rightarrow S_2$ transition of spirilloxanthin. The second NOPA provides ultrabroadband pulses that were then resuspended in acetone, stirred, and centrifuged again at 429 × g for 3 min at 4 °C. This step was repeated until all carotenoid had been extracted from the pellet. The acetone/carotenoid mixture was then added to a separating funnel and mixed with a half volume of PET ether (40:60 b.p.) followed by an excess volume of warm, salty water. Carotenoids preferentially partitioned into the PET ether layer, which was collected and evaporated to dryness using a rotary evaporator.

**Materials and Methods**

**Synthesis.** Spirilloxanthin was extracted from *Rhodospirillum rubrum* cells grown anaerobically in the light in C-succinate media. Cells were harvested and chromatophores prepared as described previously. Membranes were suspended in acetone, stirred, and centrifuged at 429 × g for 3 min at 4 °C. The supernatant was discarded, and the pellet suspended in methanol, stirred, and centrifuged as before. This step was repeated until the majority bacteriochlorophyll a had been extracted (evident by the absence of blue in the pellet). Pellets were then resuspended in acetone, stirred, and centrifuged again at 429 × g for 3 min at 4 °C. This step was repeated until all carotenoid had been extracted from the pellet. The acetone/carotenoid mixture was then added to a separating funnel and mixed with a half volume of PET ether (40:60 b.p.) followed by an excess volume of warm, salty water. Carotenoids preferentially partitioned into the PET ether layer, which was collected and evaporated to dryness using a rotary evaporator.

**Electrochemical Experiments.** Electrochemical experiments were performed using a conventional three-electrode cell consisting of a Pt working electrode, Ag/AgCl reference electrode, and a Pt counter electrode. The working electrode was polished with 1 μm alumina slurry and ultrasonically cleaned in acetone before each experiment. The electrolyte used for polarization was 0.1 M sodium hydroxide. The electrochemical analysis was performed at a scan rate of 100 mV/s. The CVs were recorded from -1 to +1 V vs. Ag/AgCl.

**Absorption Spectroscopy.** Absorption spectroscopy was performed using a Shimadzu UV-1800 spectrophotometer. The samples were diluted in ethanol to an absorbance of 0.1 at the maximum absorption wavelength. The absorbance spectra were recorded from 300 to 900 nm.

**Figure 1.** (a) Molecular structure of spirilloxanthin. (b) Normalized absorption spectrum of spirilloxanthin in toluene (gray circles) and PMMA matrix (blue circles) and pulse intensity spectra used in the experiment: the pump pulse (green shaded) and the probe pulse covering the visible (Probe, yellow area).
probe pulses with ∼7 fs duration spanning the 500–700 nm wavelength range (yellow filled spectrum in Figure 1). Both NOPAs are compressed to their transform-limited duration by multiple bounces on custom-designed chirped mirrors. The beams are focused onto the sample contained in a liquid nitrogen cryostat with 200 μm thick fused-silica entrance window. After the sample, the probe beam is selected by an iris and focused onto the entrance slit of a spectrometer with single-shot detection capability at 1 kHz.37 Recording of the probe spectrum with and without the pump pulse, one can obtain the differential transmission (∆T/T) spectrum, defined as ∆T/T(λ,τ) = (T_{off}(λ,τ) − T_{on}(λ))/T_{off}(λ), as a function of probe wavelength λ and pump–probe delay τ.

**Data Analysis.** For each sample temperature, we analyzed the measured two-dimensional ∆T/T maps using a homemade target analysis software, based on the approach described by van Stokkum et al.38 We employed a kinetic scheme with 4 components (S2, hot S1, S1*, and S0) and 5 rate constants (named k1−k5), resulting in the estimated species associated difference spectra (SADS). In order to take into account the sharp features around time zero due to coherent artifacts such as cross-phase modulation, we also introduced two additional components with a delta-like (less than 5 fs duration) temporal evolution. The concentration profiles (and the above-mentioned components associated with the coherent artifacts) were convoluted with the instrument response function, which was fitted to a Gaussian of 18 fs duration (full width at half-maximum).

**Quantum Chemical Methods.** The B3LYP and/or BHLYP (Becke’s half and half exchange functional with the LYP correlation functional) density functionals were applied in the Density Functional Theory (DFT) calculations of optimal electronic ground state geometries. The B3LYP functional includes Becke’s three parameter mixing of the nonlocal exchange potential and the nonlocal correlation functional LYP proposed by Lee, Yang, and Parr.39,40 On the basis of the optimized geometries, the electronic transitions were calculated using the time-dependent (TD)-DFT,41 ab initio Complete Active Space Self-Consistent Field (CAS-SCF),42 and Multi-reference Configuration Interaction (MRCI)43 methods. In the ab initio multireference approaches, all valence electrons were correlated, and the reference space generation started by allowing all single and double excitations from the three highest occupied molecular orbitals to the three lowest unoccupied molecular orbitals. All quantum chemical calculations were performed with the ORCA 2.9.1 package.44 We employed the split valence basis sets SV(P)45,46 with a polarization d-function on carbon and oxygen atoms. The combination of the B3LYP functional with SV(P) or SVP basis sets offers a reliable description of torsional barriers, energy minima, and optimal geometries as demonstrated for various organic molecules.47−49

■ RESULTS

Figure 2 shows experimental two-dimensional ∆T/T maps for spirilloxanthin in PMMA at RT (a) and at 77 K (b). In both cases, the pump wavelength (540 nm) was tuned to the red of the S0→S1 absorption, in order to minimize vibronic relaxation effects. We observe at early times a positive signal (in the high-energy part of the spectrum, red in Figure 2), which is assigned to the superposition of GSB (for wavelengths shorter than ~560 nm) and of stimulated emission (SE, for wavelengths longer than ~560 nm) from the S1 state. The ∆T/T signal rapidly changes in sign to form, within ~500 fs, a broad ESA band (ESA1, blue area in Figure 2 peaking at ~606 nm) that is assigned to the S1→S2 transition. This band narrows and blue-shifts on a longer time scale. The ESA1 band then decays on a picosecond time scale (outside the measurement window shown in Figure 2).

The observed photoinduced dynamics were fitted using the target analysis procedure detailed in the Materials and Methods section. Both at RT and at 77 K, it was possible to satisfactorily reproduce the data using four excited states: S2, hot S1, S1*, and S0*, according to the energy level scheme shown in Figure 3a.

**Figure 2.** ∆T/T maps for spirilloxanthin as a function of probe wavelength and delay at room temperature (a) and at 77 K (b). Time traces at selected probe wavelengths (c,d). Excitation was at 540 nm.

**Figure 3.** (a) Energy level scheme for spirilloxanthin used in the target analysis model to fit the temperature-dependent transient absorption spectra. (b) Inhomogeneous ground state model introduced to explain the temperature dependence of the signals.

Figure 3b shows the physical energy-level scheme, according to the inhomogeneous ground state model: two separate ground state isomers (S0 and S0*), energetically separated by ∆ES, are excited to their respective optically allowed states (S1 and S1*). Consequently, S2 and S2* decay to hot S1 and S0*, respectively. In our target analysis, we found that the model in Figure 3a suffices to explain our measurements, i.e., a common S2 state branches to give hot S1 and S0*. If S2 and S2* are similar in both their SADS and decay time constants (k1 + k2 in Figure 3b), the two models in Figure 3 become mathematically equivalent. This is why we chose the simpler model in Figure 3a for the target analysis described below.

The extracted SADS are shown in Figure 4. Let us first discuss the RT results (Figure 4a). The first SADS can be identified as the S2 state of spirilloxanthin and shows clear features of SE, i.e., the spectral positions of the vibronic replicas with mirror symmetry with the respective absorption bands. At the blue tail of the spectrum (wavelengths shorter than...
performed the same target analysis on all the retrieved maps, RT and acquiring, for each temperature, the measurements varying the sample temperature from 77 K to ∼180 fs time constant into the relaxed S1 state, which subsequently decays to the ground state with a 1.2 ps time constant. The S* state, however, decays to the ground state with a significantly slower time constant of 4.4 ps. The overall rates extracted from global analysis are shown in Table 1 and are in agreement with those we derived from a reference measurement of spirilloxanthin solvated in toluene under the same experimental conditions (not reported here) and with previous ultrafast spectroscopy studies of spirilloxanthin.30,32,50

Figure 4. SADS extracted for spirilloxanthin in PMMA at room temperature (a) and at 77 K (b).

When moving to 77 K, the SADS look qualitatively the same (see Figure 4b), with the spectral signatures of S0, hot S1, S1, and S* clearly recognizable and very similar to those found at RT. However, by a closer inspection of the results, two significant differences become apparent: (i) The overall dynamics becomes slower at 77 K (see Table 1), with the rate constants for the internal conversion processes decreasing by 10 to 20%; this is in agreement with previous studies of carotenoids at cryogenic temperatures19,30 and can be explained by the temperature dependence of system–bath interactions.53 (ii) The relative weight of the S* and S1 SADS changes upon cooling the sample; in particular, the parameter \( r(T) = \left( \frac{\text{ESA}_{S*}(\lambda, T) \, d\lambda}{\text{ESA}_{S1}(\lambda, T) \, d\lambda} \right) \), defined as the ratio of the integrals of the S* and S1 SADS (limited to the negative portions of the spectra), decreases from \( r(\text{RT}) = 0.5 \) to \( r(77\text{K}) = 0.25 \). Assuming that the absorption cross-sections do not change significantly with temperature, this suggests that the relative weight (spectral amplitude) of the S* state decreases at 77 K, i.e., that the state is frozen out.

To confirm this last finding, we performed a systematic series of measurements varying the sample temperature from 77 K to RT and acquiring, for each temperature, the \( \Delta T/T \) map. By performing the same target analysis on all the retrieved maps, we could extract the temperature dependence of the parameter \( r(T) \), which is shown in Figure 5 as diamonds. Despite the uncertainties introduced by the fitting procedure, the figure shows a clear trend of decrease of \( r \) with lowering temperature.

This temperature dependence can be rationalized in the framework of the inhomogeneous ground state model. Let us consider two ground state isomers, named \( S_0 \) (the low-energy global minimum) and \( S'_0 \) (the thermally activated local minimum at an energy higher by \( \Delta E \)) as in Figure 3b. Following the Boltzmann statistics, at a given temperature, the total population \( N_{\text{tot}} \) will be found partially in \( S_0 \) with population \( N_{S_0}(T) = N_{\text{tot}}/(1 + \exp(-\Delta E/k_B T)) \), and partially in \( S'_0 \), with population \( N_{S'_0}(T) = (N_{\text{tot}}\exp(-\Delta E/k_B T))/(1 + \exp(-\Delta E/k_B T)) \). Assuming that transitions starting from the global minimum \( S_0 \) populate the S1 state, while those starting from the higher energy isomer \( S'_0 \) populate S* (see Figure 4b), we can write

where \( \sigma_s(\lambda) \) and \( \sigma_{S1}(\lambda) \) are the (temperature-independent) ESA cross-sections for S* and S1, respectively. Given that these values are unknown, we fit only the shape of \( r(T) \) and scale the amplitude to match the experimentally retrieved values. The result, shown in Figure 5 as a solid line, is in very good agreement with the experimental data and allows us to retrieve a value of \( \Delta E = 0.68 \text{ kJ/mol} \) (which is the only free parameter in the fitting procedure).

In order to determine the nature of the two isomers, we conducted a quantum chemical analysis of spirilloxanthin, similar to a previous work on \( \beta \)-carotene.27 The structural study of the investigated molecule started with the structure where the mutual orientation of the lateral parts is \( \Theta_1 = \Theta_2 = 244^\circ \). This structure belongs to the \( C_2 \) symmetry point group, and the dihedral angle(s) \( \Theta_{(12)} \) is the dihedral angle defined between bonds 1, 2, 3 or 1’, 2’, 3’, respectively, as indicated in Figure 1. In Figure 6, we show the one-dimensional B3LYP/SV(P) potential energy cut for a fixed angle \( \Theta_2 = 244^\circ \) and variation of \( \Theta_1 \). We retrieve two minima at 119° and 244° and two maxima at \( \Theta_1 = 178^\circ \) and planar arrangements (\( \Theta_2 = 0/360^\circ \)). As can be seen in Figure 6, the lowest energy barriers between two minimum-energy conformations are 9.8 kJ·mol\(^{-1}\) (from the \( S_0 \)-
oscillator strengths of 6.99 (B3LYP) and 6.67 (BHLYP). The dominating electronic transition is connected with the excitations from the highest occupied molecular orbital (HOMO) to the lowest unoccupied molecular orbital (LUMO). The HOMO orbital is regularly delocalized over the carbon–carbon double bonds between the outer methyl groups of the polyenic chain (see Figure 7). Its irreducible representation is A. However, the LUMO orbital is delocalized over the single C–C bonds. Its irreducible representation is B. The HOMO to LUMO transition with 96% contribution dominates in this optically allowed vertical transition. The second transition of 2^A symmetry comes from the HOMO to LUMO+1 and HOMO−1 to LUMO orbitals. In this case, the LUMO+1 and HOMO−1 orbitals are symmetrically split over bonds connected with the methyl groups on the polyenic chain. The third optical transition of 2^B symmetry is connected mainly with the HOMO−2 to LUMO orbitals. The correct order of optical transitions with respect to the oscillator strength can be obtained using the multireference ab initio calculations. As indicated in Table 2, both conformations exhibit one forbidden transition at 3.4 eV for CAS-SCF method and two forbidden transitions for MRCI method at 1.8 and 2.0 eV.

The value of the energy difference ΔE of 1.68 kJ mol^-1 between the local and the global minimum is considerably higher than the value of 0.68 kJ/mol obtained from the fit to the experimental data in Figure 5. One obvious reason for this difference is that, in the fit in Figure 5, the ratio between the transition strengths of S_1 and S_1^* is unknown and was scaled arbitrarily. Knowledge of this factor might change the value obtained for ΔE. Another factor explaining the discrepancy to the B3LYP/SV(P) calculations is the neglect of solvent effects. Consideration of solute–solvent interactions might alter the potential curve in Figure 6 drastically.

**DISCUSSION**

Ground state conformers of carotenoids have been employed by several groups to explain ultrafast spectroscopic measurements. In an investigation of light harvesting complex 1, Papagiannakis et al. discussed the intensity dependence of S_1^* in terms of two molecular ensembles with different transition dipole moments from the electronic ground state. Such a possibility was disregarded by the same authors in favor of a so-called two photon model, involving an excited state transition in the visible from S_2 to S_2^*. Recently, Kosumi et al. conducted an intensity dependent study of spirilloxanthin in solution and as part of a light harvesting complex. In agreement with Papagiannakis et al., Kosumi et al. found that the intensity dependences of S_1 and S_1^* are similar and therefore ruled out the involvement of different ground conformers as they would need similar ground state transition dipole moments in order to exhibit the same intensity dependent behavior. Our quantum chemical calculations, however, show that the transition dipole moments μ for the global and the local minimum in Figure 6 are equal down to a level of 0.2% (see Table 2, CAS-SCF results). This makes it unfeasible to distinguish them in an intensity dependent study.

The inhomogeneous ground state model does not only explain the temperature dependence of the S1^*/S1 ratio discussed in Figure 5. The model also rationalizes the shape of the bleaching signal after decay of S_1. The GSB signal after the depopulation of S_1 but before the decay of S_1^* is more structured than the absorption spectrum or the GSB signal within the S_1-lifetime. Chabera et al. invoked a planar ground-state subpopulation as the source of S_1^* to explain their findings. The higher lying symmetric minimum in Figure 6 is an ideal candidate for such a planar local minimum, as it is closer to C_{2h} symmetry than the more twisted lower lying minimum at Θ_2 = 119°. The same conclusions were drawn from a quantum chemical study on β-carotene.27
We note that excited state isomers as discussed by Niedzwiedzki et al.\(^{21,30}\) are less suited for explaining temperature-dependent effects on the amplitude of \(S^*\): if the isomers are assumed to be formed on \(S_1\) rather than on \(S_0\) they would be populated with an excess energy defined by the energy difference between \(S_2\) and \(S_1\), not by thermal excess energy as for ground state isomers. A decrease in temperature should therefore only affect ground and not excited state isomers.

## CONCLUSIONS AND OUTLOOK

In this work, we systematically investigated the temperature dependence of \(S^*\) in relation to \(S_1\) in spirilloxanthin. The PMMA matrix provides a similar environment at both RT and 77 K. We see a clear trend of decreasing \(S^*/S_1\) ratio as the temperature is lowered. This trend follows roughly a Boltzmann distribution, adding further proof to the concept of an inhomogeneous ground state in spirilloxanthin: \(S_1\) is formed by an energetically lower-lying isomer, while \(S^*\) is the lowest-lying singlet state of a local minimum on the electronic ground state. This hypothesis is experimentally corroborated by the fact that the \(S^*\) signal can be frozen out. We consider this model to be general, not limited to spirilloxanthin but applicable to all carotenoids with an \(S^*\) signal. As determined by the low energy barrier between the minima in Figure 6, the rate of exchange between the two isomers is in the nanosecond time range,\(^{27,27}\) which makes attempts to chemically purify the sample unfeasible. In order to explain pump–deplete–probe results\(^{27}\) within the framework of the inhomogeneous ground state model, the two isomers are expected to exhibit different \(S_1\) spectra in the NIR region. Specifically, a 1000 nm depletion pulse was shown to selectively deplete \(S_1\) but not \(S^*\). Therefore, we expect the \(S_1\)-forming \(S_1\) state to absorb more strongly in this region with respect to the \(S_1^*\) state (see Figure 3b). Such a pump–NIR–probe experiment at different temperatures is the subject of ongoing investigations.

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**Notes**
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