compartments to interact directly through membrane proteins or osmotic flows of water and thereby enable the engineering of collective properties such as long-range electrical communication or macroscopic deformation. We used the well-characterized eHL pore and simple salt solutions to achieve cooperative action between droplets in a printed droplet network. Additional membrane proteins and their engineered forms (5, 6) should allow printed networks to transduce a wider range of signals, and stimulus-responsive osmolytes might offer greater control of folding. An interesting challenge is the integration of droplet networks with living organisms. The outer surface of a printed network might be engineered to interact in a designed way with a physiological environment; for example, to deliver drugs upon a specific signal (7–9). More sophisticated networks might be interfaced with failing tissues to support their functions. Alternatively, cells could be included inside the droplets during printing for various applications, such as to immobilize cells within a defined 3D dimensional scaffold for tissue engineering (22).

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Acknowledgments: The authors thank Q. Li for the eHL protein and A. Wainman for assistance with confocal microscopy. This work was supported by grants from the National Institutes of Health and the European Commission’s Seventh Framework Programme Revolutionary Approaches and Devices for Nucleic Acid Analysis Consortium. G.V. was supported by an Engineering and Physical Sciences Research Council Life Sciences Interface Doctoral Training Centre studentship. A.D.G. was supported by a Biotechnology and Biological Sciences Research Council Doctoral Training Programme in Molecular Biochemistry and Chemical Biology studentship. The authors have submitted a patent application related to this work. The authors declare no competing financial interests. G.V., A.D.G., and H.B. planned the research. G.V. and A.D.G. designed the experimental system and performed the experiments. G.V. wrote the software, performed the modeling, and analyzed the data. G.V. and H.B. wrote the paper.

Supplementary Materials
www.sciencemag.org/cgi/content/full/340/6128/48/DC1
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29 August 2012; accepted 24 January 2013
10.1126/science.1229495

Broadband 2D Electronic Spectroscopy Reveals a Carotenoid Dark State in Purple Bacteria

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Although the energy transfer processes in natural light-harvesting systems have been intensively studied for the past 60 years, certain details of the underlying mechanisms remain controversial. We performed broadband two-dimensional (2D) electronic spectroscopy measurements on light-harvesting proteins from purple bacteria and isolated carotenoids in order to characterize in more detail the excited-state manifold of carotenoids, which channel energy to bacteriochlorophyll molecules. The data revealed a well-resolved signal consistent with a previously postulated carotenoid dark state, the presence of which was confirmed by global kinetic analysis. The results point to this state’s role in mediating energy flow from carotenoid to bacteriochlorophyll.

Carotenoids (Cars) play an important role in the photoreactions of photosynthesis (1). Being accessory pigments, they absorb light in the spectral region not accessible to chlorophylls (Chls) and, by subsequent energy transfer to Chls, they increase the spectral cross-section for photosynthetic activity. Moreover, in some organisms, most of the light that drives photosynthesis is absorbed by Cars (2). However, in spite of many years of studies, the exact mechanism of energy transfer from Cars to Chls remains controversial (3, 4), owing to the complex electronic structure of Cars.

Traditionally, Car photophysics is described by a three-level model, in which the ground state–to–S1 (2A1g) state transition is symmetry-forbidden and the light is absorbed by the S2 (1B2u) state. After excitation, the S2 state rapidly decays in a few hundred femtoseconds to the “dark” S1 state, which then decays back to the ground state (S0) or 1A1g) on a picosecond time scale. However, the picture becomes more complicated for Cars with nine or more conjugated double bonds, for which Tavan and Schulten predicted the appearance of other dark states between the S2 and S1 states (5), in particular, the 1B2u state. In 2002 Cerullo et al. (6), by using sub-10-fs pulses to excite isolated Cars, reported the presence of a kinetic intermediate phase (called S0) that was populated by decay of the S2 state and then decayed into the S1 state. However, the interpretation of this phase as the dark electronic state has been controversially discussed in later studies (7, 8). Moreover, it remained unclear whether such an intermediate electronic state is actually involved in Car-to-Chl energy transfer.

In this work we investigated how the complex manifold of Car electronic states in the peripheral light-harvesting complex LH2 from the purple bacteria Rhodopseudomonas acidiphila strain 10050 and Rhodobacter sphaeroides strain 2.4.1 couple to the Q0 state of bacteriochlorophyll (BChl). By comparing the 2D spectra of LH2 complexes with spectra of isolated Cars, we deduce the presence of a Car dark state in the vicinity of the Q0 state. A global kinetic analysis suggests several energy transfer pathways associated with this dark state. The results demand reexamination of the intermolecular energy transfer mechanisms in light-harvesting proteins.

The molecular structure of the LH2 complex from Rps. acidiphila and corresponding ladder of the
to the lowest-lying Car excited state, the dark S1 excited state of the Car. In order to study the cited states of BChl-a (BChl) and the high-lying overall Car-to-BChl energy transfer efficiency are achieved in Rba. sphaeroides (~90%), where a substantial fraction of the energy is transferred from the S1 state (14, 15). Theoretical studies, however, predicted only 20% efficiency for the overall Car-to-BChl energy transfer (16). The disagreement between theoretical predictions and experimental observations is ascribed to the small spectral overlap of S2 emission and Qx absorption bands, which results from the large energy gap between these transitions.

It is a challenge to elucidate questions concerning the nature and significance of dark electronic states using normal femtosecond pump-probe experiments, because their signals are overwhelmed by contributions from the strongly allowed transitions: ground-state bleach (GSB) and stimulated emission (SE) from the Car S2 and BChl Qx states, as well as excited-state absorption (ESA) from the Car S1 state. We took advantage of the combined time and frequency resolution attainable in 2D electronic spectroscopy (17, 18) that enables sensitive detection of coupling among states via cross-peaks. Therefore, we could detect not only the spectral signatures and kinetics of various electronic states, but also their interaction pathways.

In our experiment, excitation pulses of broad bandwidth (full width at half maximum 50 to 60 THz, 13 to 15 fs) were applied in the 500- to 650-nm spectral range. The LH2 complexes of Rps. acidophila and Rba. sphaeroides were selected because of the relatively small energy gap between the S2 state and Qx state (65 to 80 nm), which allowed our excitation pulses to overlap the transitions to these two states simultaneously. The spectral width and central wavelength of the pulses were optimized carefully to cover both the Car S2 and BChl Qx transitions in the LH2 absorption spectrum. The 2D spectra were obtained by measuring the spectral response of the system at different delays between the first two pulses (t1, the so-called coherence time). The resulting 2D data were Fourier-transformed with respect to t1, yielding the excitation frequency scale. This procedure was repeated for different delays between second and third pulses (t2, the so-called population time) in order to obtain the temporal evolution of the spectral response. Figure 2, A and B, shows 2D spectra of Rba. sphaeroides and Rps. acidophila taken at t2 = 215 and 158 fs, respectively.

The excitation pulse spectra are shown in Fig. 2, C and D, together with the absorption spectra of the corresponding LH2 complexes. Several well-resolved features are evident in the 2D spectra. Three positive peaks on the diagonal are due to the GSB signal. In accordance with the stationary absorption spectra, the two peaks at 530 and 580 nm are assigned to excitation to the Car S2 and BChl Qx states, respectively. Owing to stronger overlap of the excitation pulse with the S2 absorption band in Rps. acidophila, the intensity of the corresponding diagonal peak is much more pronounced than in Rba. sphaeroides. The off-diagonal signal with negative amplitude located below the diagonal is due to the ESA signal from the Car S1 state. This feature of Cars is well known from transient absorption studies (12) and reflects the S2→S1 energy transfer pathway. The negative off-diagonal signal located
above the diagonal in the *Rba. sphaeroides* spectrum might be due either to ESA from the Car S₂ state, populated from the BChl Qₓ state as was proposed by Kosum et al. (19), or due to ESA from BChl Qᵧ, populated by internal conversion from the Qₓ state (20). The observed signal could also be a product of both of these two signals. In order to distinguish between these two contributions, a kinetic analysis has to be applied. In 2D spectra recorded for *Rps. acidophila*, only one ESA signal is observable. It is located below the diagonal and is associated with excitation of the Car S₂ state. The ESA signal above the diagonal is generally a weaker feature, and it is obscured in raw data owing to overlap with positive Qₓ/S₂ and Qₓ/X cross-peaks, which have higher amplitude in *Rps. acidophila* than in *Rba. sphaeroides*. However, as will be shown below, the extraction of the oscillatory component and application of the kinetic analysis uncover a weak S₁ ESA signal due to Qₓ→S₁ energy transfer [Fig. 4D and (21)]. The double-peak shape of the ESA in Fig. 2B has the same origin, because it overlaps with the positive S₂/X cross-peak.

Positive cross-peaks in 2D spectroscopy are caused either by coupling of the corresponding states (i.e., via a common ground state) or by energy transfer between the states. Cross-peaks between S₂, Qₓ, and X states can be observed at different t₂ delay times and are shown in Figs. S5 to S8 as well as in movies S1 and S2 (these auxiliary measurements were performed over a 0- to 400-fs time range, with 5-fs time steps). These data illustrate two points. One is that the 2D spectra are complicated because of the oscillations and the number of transitions that contribute to the spectroscopic map. The second point is that, despite the complexity, there are a sufficient number of clearly identified transitions to allow fitting of these data using a global target analysis. First we point out some qualitative clues to the origin of the X state.

The diagonal signal marked X as well as the cross-peaks at ~560 nm excitation and detection have not been observed previously (e.g., in transient absorption studies). In order to elucidate the origin of the corresponding state, namely to determine whether it is due to Car or BChl and whether it is a vibrational sublevel or an electronic state, we performed Fourier analysis of the data along the t₂ time variable. The time traces taken at points along the diagonal of the 2D spectrum of *Rps. acidophila* at the peak positions (at 530-, 560-, and 585-nm excitation/detection wavelengths, see Fig. S9A) reveal pronounced oscillations. The oscillations at the 530- and 560-nm peaks show clear beating, indicating the presence of more than one frequency. The Fourier spectra of these two traces are almost identical and reveal three high-frequency modes (Fig. S9B). These three modes of 990, 1195, and 1590 cm⁻¹ (CH₃ rocking mode, C-C stretching mode, and C=C stretching mode, respectively) are known to be signature vibrational frequencies of the Car ground state (10, 22).

In order to ensure that the X state is indeed a Car feature, we performed 2D spectroscopy measurements on the isolated Cars rhodopin glucoside (from *Rps. acidophila*) and spheroidene (from *Rba. sphaeroides*), both in acetone solution. These Cars have different conjugation length, which causes differences in their spectroscopic features. Figure 3 shows 2D spectra of the two Cars taken at t₂ = 100 fs and 150 to 160 fs. The Cars were excited at the very red edge of the absorption spectrum in order to minimize the contribution of the S₂ state GSB in the 2D spectrum (Fig. 3E). For such excitation conditions, the S₂ GSB signal is observed at 520 nm for spheroidene and at 540 nm for rhodopin glucoside. This shift is mostly due to the longer conjugation length of rhodopin glucoside (N = 11) as compared to spheroidene (N = 10). However, the exact position of the S₂ peak in the 2D spectrum depends on the spectrum of the excitation pulse, because the Cars are not excited at the absorption maximum but rather at the red shoulder of the S₂ band (Fig. 3E).

The second notable contribution to these 2D spectra is the off-diagonal negative signal of the
S₁ ESA. A significant feature of that ESA signal is its elongation at longer excitation wavelengths: A substantial shoulder of the ESA is present at 560-nm excitation wavelength in Fig. 3A, and a separate negative peak can be observed at 570- to 580-nm excitation wavelengths in Fig. 3, C and D. The ESA signals at these longer wavelengths indicate an additional state (the X state) from which the excitation energy is transferred to the S₁ state, giving rise to the S₁ ESA.

Even firmer evidence for the X state is the diagonal peak and the cross-peak in Fig. 3B. We found this signal to be very weak and observed only at several t₂ delay times, whereas at all other t₂ delays, it is obscured by S₁ ESA (see movies S3 and S4 for 2D spectra at all t₂ delay times). The absence of the X bleach signal in rhodopin glucoside is due to the different electronic properties of the two molecules. As shown in Fig. 3E, the S₂ absorption band of rhodopin glucoside is not only shifted more to the red but is also broader. As a result, the overlap of the laser pulse spectrum and Car absorption spectrum is larger in rhodopin glucoside. In our experiments, the Cars were specifically excited at the very edge of the S₂ absorption in order to minimize signals arising after S₂ excitation (Fig. 3E). No diagonal X signal, or corresponding ESA signals, were observed when the S₂ was excited more to the blue (λₑₒₓ − λₜ₂ < 50 nm for spheroidene and <60 nm for rhodopin glucoside).

Global target analysis is a well-established approach for kinetics-based analysis of time-resolved spectra, usually fluorescence or pump-probe data (24–26). It involves deconvolution of spectral signatures of interconverting components (e.g., populations of excited electronic states) connected to a kinetics model. We report here a global target analysis of the 2D data recorded for Rps. acidophila, shown in Fig. 2B. Details of the analysis procedures are described in (21). In the target analysis for 2D spectra, we find how spectral features evolve globally (i.e., across the 2D spectrum as a function of t₂ time) according to the kinetic scheme. Cross-peaks provide strong constraints on the fitting, because cross-peaks contain information on the history of populations (i.e., how they interconvert).

We found that a sequential kinetic scheme for the population dynamics of each kinetic component yielded the most reasonable results [see (21) for discussion]. The features observed in the raw 2D spectra are a product of the overlap of coherent oscillatory amplitude and incoherent relaxation signals. The coherent signals arise from the broadband excitation and can obscure spectral features by modulating the amplitude of bleach and excited-state absorption. Applying a global target analysis to the 2D data required separation of the coherent and incoherent signals. The oscillatory evolution was calculated separately by simulating the experimental Fourier spectra (fig. S9B). We then self-consistently fitted the damped oscillations and the population kinetics to the experimental 2D data. Selected experimental data as a function of population time, together with the corresponding traces obtained after global analysis, are shown in fig. S12.

The results of the global target analysis are presented in terms of population dynamics (Fig. 4E) and their amplitudes in 2D space: The 2D evolutionary-associated spectra (2DEAS) shown in Fig. 4, A to D. The term “evolutionary-associated spectra” means we assumed a sequential kinetic scheme. Owing to the complex kinetics and close values of some of the relaxation rates, each component (species) in this evolutionary kinetic model can be a compartment of several physical states, combined according to the similarity of their temporal properties. Nevertheless, this model allows elucidation of the main features in the 2D spectra and a quantitative estimate of the underlying dynamics. Here we highlight our principal results, whereas the detailed assignment of the 2DEAS spectra can be found in (21), where we also discuss our rationale behind our choice of the scheme and its limitations.

Four species were found to yield a good fit with 2DEAS that correspond most closely to spectral indicators of the Car and BCHl states that are interacting by energy transfer and radiationless transitions. The main signals are due to three Car states S₂, X, and S₁ and one BCHl Qₓ state. The S₂ and S₁ features have the strongest amplitude, owing to strong transition dipole moments of the corresponding transitions. The features of the X and Qₓ states have much lower intensity, but they can be observed in all four 2DEAS. The most evident signals of the intermediate X state are the bleach features on the diagonal of the 2DEAS-1 and -3 spectra (marked as X in Fig. 4, A and C). These bleach signals indicate depopulation of the ground state and are present in all 2DEAS. However, in 2DEAS-2 and -4 they are obscured by the negative S₁ ESA signal (21). Rather more informative are the cross-peaks at 560-nm excitation or detection wavelengths. In 2DEAS-1, a strong S₂/X cross-peak is observed (Fig. 4A). This cross-peak is due to SE from the X state after S₂→X energy transfer. This process has been previously observed in the stationary fluorescence spectra and transient absorption spectra of isolated Cars (27) and agrees
well with our results. This ultrafast energy transfer (~48 ps 1 rate) leads to efficient population of the X state. That process is followed by further energy transfer from the X state to lower excited states as indicated in the 2DEAS-2 and -4 spectra (Fig. 4, B and D). Here the negative X/S signal corresponds to the X=S1 energy transfer, whereas the positive X/Q feature is due to Car-to-BChl energy transfer. These energy transfer pathways point to the important role of the intermediate X state in the photoinduced dynamics of the LH2 protein.

Another interesting result is the presence of the negative S1 ESA signal in 2DEAS-2 and -3. Conventional transient absorption spectroscopy reveals a ~350-fs lifetime of the hot S1 state (11, 28, 29), which is well in agreement with the 2DEAS-4 (Fig. 4D). The observation of the S1 ESA on earlier time scales might be due to higher vibrational levels of the S1 state. Whether these S1, vibrational levels are different, or the same level, which mixes into both 2DEAS-2 and -3, is not clear, and further studies are necessary to answer that question. Consideration of the X state and higher-lying vibrational levels of the S1 state below the S2 state might explain the deviation of the S2 relaxation rate from the energy gap law, observed in several studies of Cars (30, 31).

The data we have reported here clearly demonstrate the presence of a weakly allowed transition (the X state) in both isolated Cars and in LH2 proteins. The global analysis confirmed that the X feature is not a product of the overlap of coherent oscillations with signals from S2 or S1 states, but is a separate state that decays on an ultrafast time scale. One of the possible origins of the X state is a higher-energy vibrational level (second or fourth level in the vibrational progression) of the S1 state. However, in that case, no bleach signal should be observed after excitation of the X state, because the ESA from the X state is weaker than the S1 ESA signal, as follows from 2DEAS-2 and -4. In contrast, a clear diagonal bleach peak is observed in 2DEAS-1 and -3. Therefore, a much more reasonable assignment of the X state is to the B1 state, predicted by theoretical calculations to be located below the S2 state for Cars with conjugation length ≥9 (3, 32). As a consequence of symmetry properties, the ground state to B1 state transition is optically forbidden. In some molecules, transitions to the lower optically forbidden electronic state can borrow intensity from higher optically allowed electronic states via nontotally symmetric vibrations according to the Herzberg-Teller mechanism (33–35). This process of borrowing dipole strength can lead to a weak B1 signal in Cars.

The intermediate Car dark state (B1 or S1 state) was proposed in several experimental studies (6, 27, 36, 37). These reports were based on excited-state absorption signals in pump-probe spectra. However, the assignment of the ESA signals was questioned in later studies. It was shown in several specially designed experiments that the ESA signals can be confused with nonlinear excitation effects, such as two-photon absorption or impulsive Raman scattering (7, 8, 38). Thus, until now, the existence of the B1 state remained controversial. In contrast to previous reports, in this work we observed the B1 state via its GSB signal. Because a GSB signal gives the energetic position of the excited state relative to the ground state, this observation is compelling evidence of the presence of the B1 state.

In 2002, Cerullo et al. suggested that the intermediate Car state, located between the Car S2 and BChl Q states, plays an important role in light-harvesting proteins (6). The clearest indication of the contribution of the B1 state is the disagreement between theoretically calculated and experimentally measured efficiencies of Car-to-BChl energy transfer in LH2 complexes. The calculations performed on the LH2 complex of Rps. acidophila took into account the main features of the BChl and Car molecules by precise description of the size and shape of their transition densities (16). This method allowed the calculation of electronic couplings and energy transfer rates with high precision; nevertheless, the obtained values were less than half the experimentally measured values (9–12). This deviation was explained by a large energy gap and small spectral overlap between the S1 and Q states. The cross-peaks observed in 2D spectra in the current work (Fig. 2 and figs. S2 to S5) clearly show evidence for electronic coupling between the B1 state and both Car S2 and BChl Q states. This coupling increases the spectral overlap and therefore the Car-BChl energy transfer efficiency. The present study suggests that the disagreement in energy transfer efficiencies is due to the absence in the theoretical model of the B1 state, which closes the energy gap and promotes Car-to-BChl energy transfer.

The energy transfer pathways within light-harvesting proteins critically depend on the molecular structure of the interacting chromophores as well as their orientation within the protein. In this work, the presence of the Car intermediate state has been demonstrated for two LH2 proteins, containing Cars with 10 and 11 conjugated C=C double bonds. Therefore, thorough studies (both theoretical and experimental) are necessary to revise the understanding of interaction between Cars and Chls. In this respect, 2D electronic spectroscopy in combination with global analysis has proven to be a preeminent tool for studying complex multichromophore systems, providing insights into the energy conversion processes and revealing spectral properties invisible to other techniques.