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Meech, S.R.; Hoff, A.J.

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EVIDENCE FOR A VERY EARLY INTERMEDIATE IN BACTERIAL PHOTOSYNTHESIS. A PHOTON-ECHO AND HOLE-BURNING STUDY OF THE PRIMARY DONOR BAND IN *RHODOPSEUDOMONAS SPHAEROIDES**

S.R. MEECH^a, A.J. HOFF^b and D.A. WIERSMA^a

^a Picosecond Laser and Spectroscopy Laboratory, Department of Chemistry, State University of Groningen, Nijenborgh 16, 9747 AG Groningen, The Netherlands

^b Department of Biophysics, Huygens Laboratory of the State University, P.O. Box 9504, 2300 RA Leyden, The Netherlands

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Two coherent spectroscopic methods, accumulated photon echo and population bottleneck hole-burning, have been employed in a study of the decay rate of the primary donor (P) of *Rhodospseudomonas sphaeroides* at 1.5 K. The decay rate is instrument-limited in the photon-echo experiment, implying a population relaxation time < 100 fs. The hole-burning study revealed the P absorption at 900 nm to be largely *homogeneously* broadened, from which a decay time of ≈ 25 fs was inferred. Comparison of these data with a photon-echo study of the bacteriochlorophyll a monomer suggests that this ultrafast process is not due to vibrational relaxation within P*, but to an excited state electronic decay mechanism. It is suggested that the initial event after excitation in P is a very rapid charge separation within the dimer pair, prior to the electron-transfer process, which occurs on a much longer timescale.

1. Introduction

Light energy is converted into chemical energy by the process of photosynthesis. The first step is thought to be a charge-separation reaction: upon excitation a specialized chlorophyll (Chl) or bacteriochlorophyll (BChl) pigment complex, the so-called primary electron donor, reduces a nearby acceptor:



In subsequent dark reactions the charges migrate along a chain of donors and acceptors, so that after a certain time, they are stabilized and their remaining potential energy may be used in metabolic reactions. The pigments involved in (1) and subsequent dark reactions are encased in the so-called reaction center (RC) protein, which for several photosynthetic bacteria can be

isolated in pure form. The RC of purple bacteria contain 4 BChl, 2 bacteriopheophytins (Bph), 2 quinone molecules, and one iron atom [1]. Two of the BChl form a dimer making up the primary donor, P.

Recently, an X-ray crystallographic study of the RC of the purple bacterium *Rhodospseudomonas (Rps.) viridis* revealed that the pigment cores are organized with a two-fold symmetry, the C_2 axis running from P to the iron atom [2]. Despite this apparent two-fold symmetry it is known [3] that only one of the Bph, H_A , is involved in electron transfer from P to the first quinone acceptor, Q_A . One of the two BChl not involved in P, B_A , lies close to P and H_A .

It is now generally accepted that in bacterial RC the primary charge separation (1), that is to say photo-oxidation of P and reduction of H_A , occurs in a few picoseconds [4]. The possibility, however, of an earlier acceptor than H_A , e.g. B_A , is still much debated [5]. To resolve the earliest events of the charge separation process it would clearly be of great interest to assess the lifetime of the excited state of P, $^1P^*$, as this would be determined by the formation of intermediary states prior to reduction of H_A .

* A preliminary account of this work was presented at the International Conference on Dynamical Processes in Excited States of Solids, DPC-85, July 2-4, Villeurbanne, France.

A recently developed coherent optical technique that is particularly suitable for the determination of lifetime of rapidly decaying excited states is that of the accumulated photon echo (APE) [6]. This method is similar to the conventional photon echo [7], in which chromophores are coherently excited and the loss of optical coherence is measured by the ability to generate a photon echo. The lifetime of the photon echo is determined by the optical dephasing time T_2 , which, in the absence of pure dephasing processes, equals $2T_1$, where T_1 is the lifetime of the excited state. A necessary prerequisite to the measurement of an accumulated photon-echo signal is the presence of a bottleneck in the optical pumping cycle. In the case of the RC of purple bacteria the long-lived (several milliseconds at 1.5 K) charge separated state functions as such.

An alternative method to measure the lifetime of an excited state is the technique of population or photochemical hole-burning [8]. Narrow-band laser excitation at very low temperature (1.5 K) of an inhomogeneously broadened absorption band leads to the bleaching of those pigments whose transition frequencies lie within the laser bandwidth. If this bandwidth is sufficiently narrow, then the hole thus formed has a width corresponding to the coherence lifetime (T_2) of the excited state [8,9].

In this communication we report on the application of APE and hole-burning to bacterial photosynthetic reaction centers. We show that the loss of coherence in the excited state of P occurs in a time of less than our instrumental time resolution, currently about 200 fs. Hole-burning within the absorption band of P at 880 nm leads to bleaching of almost the whole absorption band, i.e. the width of the hole is approximately the width of the absorption band. This means that the band is predominantly homogeneously broadened, and that the coherence lifetime of the excited state of P is of the order of tens of femtoseconds.

We conclude that almost instantaneously after excitation of the photosynthetic reaction center in the absorption band of the primary donor a new excited state is formed. The great speed of formation of this state seems to exclude a vibrationally relaxed state of $^1P^*$; this idea is strongly supported by photon-echo experiments on BChl embedded in a glass. We suggest that the new state represents a charge-transfer state, P^\pm , within the dimeric BChl complex making up the

primary donor, and that this state is the precursor of the much slower process of electron donation to H_A , either directly or via B_A .

2. Experimental

Reaction centers of *Rhodospseudomonas sphaeroides* R-26 were isolated according to the procedure of Feher and Okamura [1] and the samples prepared as described by then. The accumulated photon-echo experiments were performed as previously using both coherent [6] and stochastic [10] excitation fields. The synchronously pumped dye laser used to pump the reaction centre contained Styryl 9-M in a 4/1 mixture of ethylene glycol and propylene carbonate at lasing medium. IR-140 was added to this mixture as a hybrid mode-locking dye until no further pulse shortening was observed. As a pump laser a CR-6 mode-locked Ar-ion laser was used. With this combination the shortest pulses obtained had an autocorrelation width of 0.65 ps, which for a sech^2 pulse shape, deconvolutes to a 420 fs pulse. These pulses were obtained with a 5 μm pellicle as tuning element in the dye laser. The spectrally widest pulses were obtained using a single plate birefringent filter as tuning element and slightly detuning the laser cavity length. Under these conditions a bandwidth of $\approx 100 \text{ cm}^{-1}$ was obtained at around 870 nm, which implies a field correlation time of 150 fs.

For the hole-burning experiments the dye-laser output was narrowed to 0.2 nm by inserting a two-plate birefringent filter into the dye-laser cavity. In these experiments the power incident on the sample was 5–20 mW/cm^2 .

For excitation of BChl itself pyridine-2 with HITC as the mode-locking dye, in the same solvent mixture, was used as the lasing medium. The shortest pulses were obtained at 775 nm and had an autocorrelation width of 0.6 ps. The dye laser with this dye mixture could be tuned in the near IR over the wavelength region 770–800 nm.

The RC samples were slowly cooled through the glass transition of the water–glycerol (1/3–2/3) medium and were finally immersed in superfluid helium at a temperature of $\approx 1.5 \text{ K}$.

For the photon-echo experiments the pump and probe beams were focused to a 100 μm diameter spot. As a result of the very large signals observed in these

experiments very low laser powers could be used (40–100 μW in the pump beam).

The absorption spectra were obtained by using a 1 m Spex monochromator with 10 cm^{-1} bandpass to predisperse the light falling onto the cooled RC. The transmitted light was detected by using a photodiode whose output was amplified with a home-built dc amplifier.

3. Results and discussion

Part A of fig. 1 shows the well known absorption spectrum of reaction centers of *Rps. sphaeroides* embedded in a water–glycerol glass at 1.5 K. Accumulated photon-echo experiments were performed over the whole tuning range of the dye laser, which extended from 890 to 810 nm. At not a single point in the tuning range did we observe any asymmetry of the echo correlation trace, either using coherent or stochastic excitation of the RC. Typical echo traces are shown

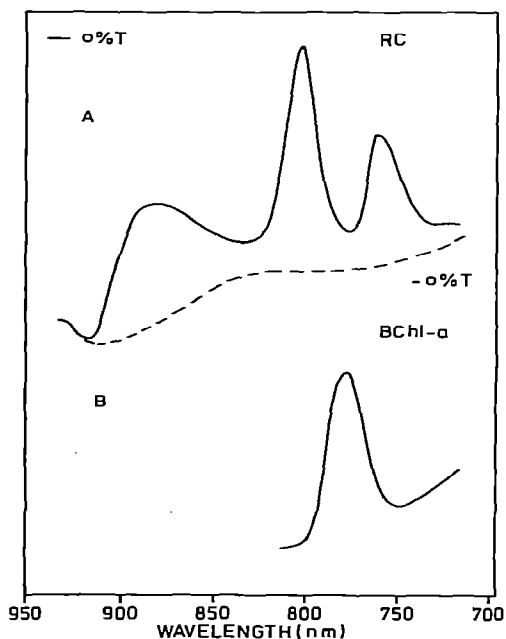


Fig. 1. Transmission spectra of: (A) RC in 3:1 glycerol:buffer and (B) BChl-a in MTHF at 1.5 K. Dashed line represents 100% transmission.

in fig. 2. The upper one (A) was obtained at 865 nm, using coherent excitation, and the lower one (B) for stochastic excitation at 875 nm. The accumulated nature of these traces was ascertained by noticing a distinct effect of the modulation frequency of the pump beam on the intensity of the observed signal. The conclusion from these experiments must be that the echo decay is faster than can be observed for the present excitation conditions, implying an optical T_2 for the P transition shorter than 200 fs.

Next to the shape of the echo trace the intensity of the echo was also monitored over the wavelength region 880 to 810 nm. In the wavelength region of 880 to 825 nm the echo intensity followed the absorption strength; at shorter wavelengths the echo intensity fell below the absorption curve indicating a lower efficiency of accumulation. This observation is in agreement with previous suggestions of a second dimer component underlying the BChl-monomer absorption band [11]. This is a point of further investigation.

In order to obtain more information on the homogeneous lineshape of the P_{890} transition we decided to perform transient (population bottleneck) hole-burning experiments. Fig. 3 shows what happens to the absorption spectrum of the RC when a narrow-band (0.2 nm bandwidth) laser is tuned to 880 nm. It seems that under these conditions virtually the whole P-absorption band bleaches. This is brought out more clearly in a modulation type experiment, a typical result of which is shown in the lower part of fig. 3, where the difference spectrum between laser on and laser off is given. It is clear that the hole burnt in the absorption line encompasses, to a large extent, the whole band. The narrowest hole obtained had a width of 400 cm^{-1} which is only slightly less than the 450 cm^{-1} width of the P band. Further, the position and shape of the hole that was burnt was independent of the excitation wavelength which was varied between 865 and 890 nm. Qualitatively the same behaviour as in fig. 3A was observed at 77 K. At room temperature the bleaching was much more effective, which reflects the increase in the bottleneck lifetime observed when the sample goes from the glass to the fluid phase. Finally we note that the hole shape had essentially the same shape as the P band which is described by neither a Lorentzian nor a Gaussian.

In order to analyze our results we have to ascertain

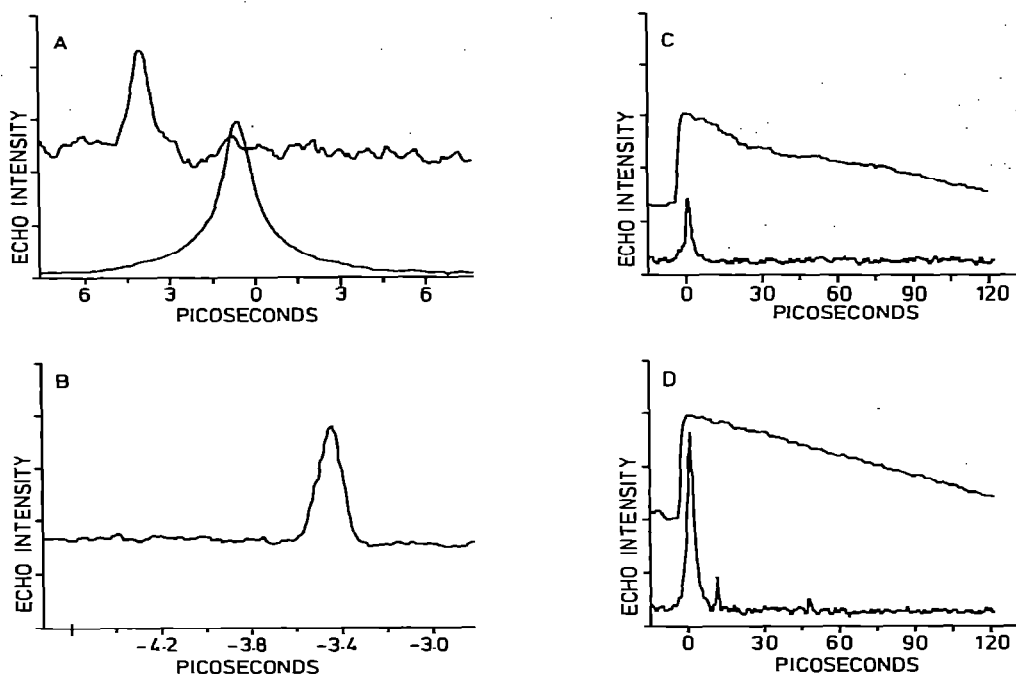
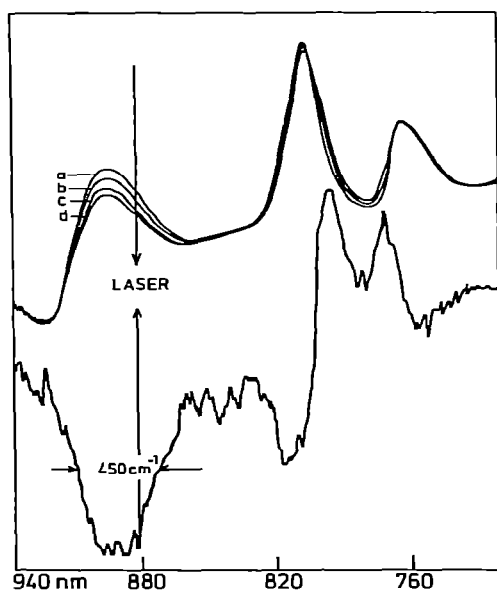


Fig. 2. APE signals of RC (A, B) and BChl-a (C,D) at 1.5 K. (A) Time domain APE of RC. The laser wavelength was 865 nm. The autocorrelation (lower trace) width was 1.3 ps. The symmetrical echo (upper trace) signal has a width of about 1 ps. This is less than the width of the autocorrelation, and closer to the correlation time of the excitation light; the laser pulses were not transform limited. The ≈ 3 ps offset between the two traces results from a neutral density filter placed in the probe beam to provide the proper pump/probe intensity ratio. (B) APE signal following stochastic excitation at 875 nm. The correlation time of the excitation light was 130 fs. (C,D) APE traces of BChl-a in MTHF at 768 and 782 nm respectively. The ≈ 100 ps decay time decreases slightly with increasing excitation energy. The observed decays are not strictly exponential; whether or not this is an experimental artefact is a subject of further investigation.



that the hole burnt is not artificially broadened by the presence of the bottleneck. Recently this type of hole-burning was analyzed in great detail [9]. Using the theoretical expressions from that paper for the hole width with the conditions of our experiments, and using the fact that $T_2 \lesssim 200$ fs we can safely conclude that the hole we observe is not power-broadened and that its width of $425 \pm 25 \text{ cm}^{-1}$ reflects a maximum of two times the homogeneous width [9] of the P_{890} transition. Such a homogeneous width $\Delta\nu_h$ corresponds, for a Lorentzian lineshape, to an optical T_2 of 50 fs, via the relation $\Delta\nu_h = (\pi T_2)^{-1}$, where

◀ Fig. 3. Hole-burning studies of RC. The upper trace is the transmission spectrum taken with (a) 0.0, (b) 0.2, (c) 0.8, (d) 1.6 mW of laser energy directed through a 3 mm^2 aperture placed before the sample. The lower trace is a light minus dark spectrum taken with 0.4 mW of power.

$$\frac{1}{T_2} = \frac{1}{T_2^*} + \frac{1}{2T_1} \quad (2)$$

In this expression T_2^* is the time constant for pure dephasing processes and T_1 is the population relaxation time constant. At low temperature pure dephasing processes are frozen out (at least on a picosecond time scale) which implies via (2) that the optical T_2 under these conditions should equal $2T_1$. We therefore conclude that in the excited state of P a relaxation process occurs in about 25 fs. A more precise determination of this number awaits a more detailed study of the aforementioned complex lineshape of the P transition, which could either be due to different sources of spectral inhomogeneity or to intramolecular coupling effects.

The important question that now arises is whether this ≈ 25 fs relaxation process reflects the electronic decay of the excited dimer or whether it presents a measure of vibrational relaxation in this excited state. In order to explore this latter point we have performed accumulated photon-echo experiments on BChl itself dissolved in a 2-methyltetrahydrofuran (MTHF) glass. In this case the triplet state of the molecule acts as the bottleneck. The absorption spectrum of this system is shown in the lower part of fig. 1 where-by we note that the BChl monomer in the RC is some 400 cm^{-1} red-shifted compared to isolated BChl in a glass. Typical photon-echo traces observed for this system are shown in traces C and D of fig. 2. Throughout the whole absorption band echo decays are observed in the range of 70–120 ps which is four orders of magnitude slower than is observed for the BChl dimer (P) in the RC. This further shows that most of the transition strength in BChl is of a pure electronic nature and therefore the vibronic part is negligible.

Previous hole-burning experiments on dimers of free base porphyrin in MTHF have shown that dephasing of the dimers is only slightly faster than observed for the monomer [12]. We therefore conclude that vibrational relaxation is not a viable option for the explanation of the calculated 25 fs relaxation process in the excited state of P. The conclusion must then be that this ultra-fast relaxation process is related to the electronic decay of the excited dimer state. We suggest that the state formed on this time scale is an intramolecular charge transfer state of the BChl dimer. From the fact that the fluorescence spectrum

of the (reduced) reaction center is close to being the mirror image of the P band [13] one has further to conclude that this charge transfer state must be underlying the P band. An alternative and equivalent interpretation of the 25 fs decay time then is to assign this time scale to the process of intramolecular dephasing of the congested level structure underlying the P band which is due to strong mixing of a Frenkel-type dimer state and an intramolecular charge transfer state. The presence of a close lying charge transfer state has also recently been suggested for *Rps. viridis* on the basis of a shoulder on the red side of the absorption of the P band in that system [14]. The experiments reported in this paper show that it is unlikely that a charge transfer state exists as a separate entity next to the Frenkel-type dimer state. Irrespective of this point the experiments reported in this paper present the first *direct* evidence for the importance of charge separation in P. It therefore seems that the presence of a dimer in these photosynthetic reaction centers arises partly from its function in the initial step in the process of charge separation. The role of B_A and the surrounding proteins in the whole electron transfer process remains a point of great interest. We end by noting that recently reported Stark effect measurements on the near infrared absorption bands of the reaction centre *Rps. sphaeroides* [15] show that a large change in dipole moment occurs when the dimer (P-band) is excited. This finding strongly supports our suggestion that the initial charge separation reaction occurs at the dimer.

4. Summary and conclusions

Low-temperature photon-echo and hole-burning experiments show that the excited state of the special pair (P) in the photosynthetic bacterium *Rhodospseudomonas sphaeroides* R26 decays in ≈ 25 fs.

Photon-echo experiments on isolated bacteriochlorophyll show that it is highly unlikely that this ultra-fast process can be associated with vibrational relaxation in the excited state of P. The conclusion is that the 25 fs relaxation time is associated with relaxation from the Frenkel-type dimer state into an intramolecular charge transfer state of P. It is proposed that the initial charge separation occurs in the dimer, where the local C_2 symmetry is broken by the non-symmetric protein environment.

Note added

After this manuscript was written we were informed that Dr. S. Boxer and co-workers of Stanford University have also performed hole-burning experiments on the P transition of the RC of *Rps. sphaeroides* [16].

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