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A picosecond circular dichroism study of photosynthetic reaction centers from *Rhodobacter sphaeroides*

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Picosecond transient circular dichroism spectra are reported for the primary intermediates in the photocycle of reaction centers isolated from *Rhodobacter sphaeroides*. The time-resolved circular dichroism spectra of the two electron transfer intermediates $(BChl_2)^+BPh_L^-Q_A$ and $(BChl_2)^+BPh_LQ_A^-$ reveal a large, nonconservative, and fairly stationary CD band at 800 nm. These results suggests that mechanisms other than exciton interactions need to be included in order to explain the optical activity of this biological system.

Introduction

The conversion of light energy to chemical energy during photosynthesis involves the transfer of electrons between pigments embedded in a membrane protein. Light energy gathered in antenna pigments is transferred and trapped into a photosynthetic reaction center (RC) where these fast electron transfers occur with high quantum efficiency [1–3]. Recently the three dimensional structures of the photosynthetic reaction centers of the bacteria *Rhodopseudomonas viridis* [4] and *Rhodobacter sphaeroides* [5,6] were reported, enabling a molecular description of the primary events of the photosynthetic process.

The RCs of photosynthetic bacteria consist of polypeptide chains of molecular weight 20000–30000, designated H, L and M, of which the H subunit is less essential to the photochemistry. The L and M subunits enclose four bacteriochlorophylls (BChl) two bacteriopheophytins (BPh), two quinones and a nonheme iron atom. The spatial arrangement of these cofactors are shown in Fig. 1.Two quasi-symmetrically related branches extend from the special pair of bacteriochlorophylls ($BChl_{LP}$ and $BChl_{MP}$) to a quinone (Q_A in the L branch or Q_B in the M branch) via a BChl ($BChl_{LA}$ or $BChl_{MB}$) and a BPh (BPh_L or BPh_M).

Picosecond time-resolved spectroscopies have advanced our knowledge of the primary electron-transfer process in RCs [3,7–11]. The generally accepted route of electron transfer and the associated time-scales at room temperature are also illustrated in Fig. 1. Intriguingly, the electron transfer occurs only along the L branch [3]. Upon photoexcitation of the $BChl_2$ dimer, the electron is transferred to the BPh in the L branch with a time constant of 3 ps [8–11]. The role of the BChl monomer on the electron-transfer step is currently a topic of great debate [10–14]. The subsequent electron

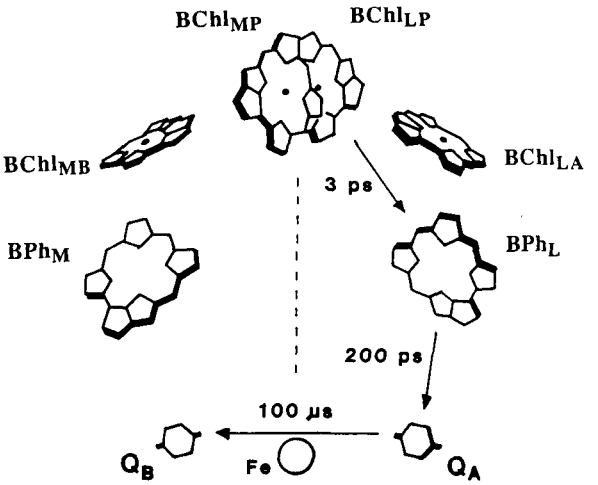

Fig. 1. Arrangement of the BChls, BPhs and quinones in the RC of *Rb. sphaeroides* determined in the X-ray crystal structure[3]. The dynamics of the electron-transfer reactions are also shown. The transfer times given are for room temperature.

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transfer from the BPh^- to Q_A has a time constant of 200 ps [7]. Many features of these electron transfer processes are still poorly understood. In particular, questions concerning the role of protein conformational motion in the RC remain to be answered.

Protein conformational motion could influence the electron transfer process in two major ways. First, fluctuations in structure could modulate the redox potentials, thereby assisting the forward electron transfer processes [10,15,16]. Secondly, structural relaxation that might occur following or accompanying the electron transfer processes could stabilize the charge transfer intermediates. These structural changes might act in turn to inhibit the system from the back charge recombination processes. In principle, CD in the visible and near-IR spectral regions is sensitive to both the relative orientations of the pigments and the location of the pigments with respect to various aromatic residues of the surrounding protein.

The evidence of a light-induced structural changes in the RC of *Rb. sphaeroides* was discussed by Kleinfeld et al. [17]. They compared the kinetics of charge recombination, $(BChl)_2^+BPh_LQ_A^- \rightarrow (BChl)_2BPh_LQ_A$ in RCs cooled in the dark with those cooled under illumination (i.e., in the charge-separation state) and found that the rate of back electron transfer is significantly lower in the later case. This was attributed to protein structural changes which stabilize the charge separation. Evidence for volume changes also comes from calorimetric studies of Arata et al. [18]. Their data suggest that the light-induced charge separation is accompanied by a decrease in the volume of the RC-solvent complex. To examine dynamical changes in structure, transient techniques with sensitivity to molecular conformation are needed. Among attempts along this line, Kirmaier et al. have used picosecond linear dichroism experiments along with transient absorption experiments [1,19,20,21]. These workers have suggested evidence for the structural relaxation accompanying the formation of $(BChl)_2^+BPh_L^-$ and $(BChl)_2^+BPh_LQ_A^-$ [20].

In order to study conformational changes of RCs using time-resolved CD spectroscopy, the origin of the CD of this system needs to be fully understood. Many features of CD spectra, as well as many other electronic properties of this complex system remain to be understood. In this paper, a technique for recording CD spectra with picosecond time resolution is presented. The CD spectra of the RCs of *Rb. sphaeroides* at various times during the electron transfer process are examined. Our transient CD data provide new clues to the origin of the optical activity and the electronic structure of bacterial photosynthetic reaction centers.

The organization of this paper is as follows. In the next section, previous experimental and theoretical studies of the steady-state CD of reaction centers are discussed. This is followed by a description of the picosecond CD spectrometer. The final section examines transient absorption and CD spectra and compares the results with the theoretical models previously discussed.

Circular dichroism properties of reaction centers

As mentioned above and shown in Fig. 1, the RC consists of six interacting pigments in close contact and in near electronic resonance. The visible and near-IR absorption spectrum of the RC of *Rb. sphaeroides* at room temperature and cryogenic temperature have been extensively studied [1–3,22,23]. The spectrum cannot be described as a simple sum of the absorption bands of the individual chromophores. The current view is that the electronic structure of the special pair ($BChl_{MP}$ and $BChl_{LP}$) can be described by an excitonic dimer [22], whereas the remaining BChls and BPhs are only subject to minor perturbations from the surrounding pigments [23].

From a theoretical point of view [24,25], excitonic splitting of a molecular dimer arises from the interaction of two identical or near-resonant excited states. The energy separation between the pair of exciton bands depends on the orientation of the two chromophores; this energy gap decreases as the cube of the distance between the two chromophores and increases as the product of the transition dipole moments of the two chromophores. With respect to the CD spectrum, the rotational strengths of the two exciton bands should be equal in magnitude but different in sign. The resulting CD spectrum is conservative, i.e., the integrated area of the two exciton bands is zero. The rotational strength is also sensitive to the relative orientations of the monomers. The fact that exciton coupling between a pair of near-resonant electronic states results in two CD bands of opposite sign makes this form of spectroscopy unique in addressing the importance of excitonic interactions in determining optical properties of complex systems.

There have been many reports on the steady state CD spectra of RCs [26–30]. In addition, there have been several theoretical calculations of the CD spectrum [31–35]. The ground state CD spectrum (see Fig. 4, vide infra) reveals a new band at 810 nm, which is hidden in the absorption spectrum. This band has been the subject of extensive discussion [26,34,35]. Based on a variety of spectroscopic data, this band has been assigned to the high-energy exciton band of the special pair. This is consistent with the observation that this feature vanishes in the CD spectrum of chemically oxidized RCs, $(BChl)_2^+BPh_LQ_A$ [27]. Similar CD spectra of *Rps. viridis* have also been reported [26,28,30]. To date, neither CD spectra of RCs under actinic excitation (i.e., CD of $(BChl)_2^+BPh_LQ_A^-$), nor time-resolved CD spectra have been reported. This paper represents, to our knowledge, the first such report.

With the positions and orientations of the porphyrin chromophores obtained from the X-ray structure, one can use various levels of theory to calculate optical spectra, e.g., absorption, CD, and linear dichroism. The simplest approach is to use electronic exciton theory and model all other interactions via adjustable line width parameters. For such calculations, one must evaluate only the electronic site energies and exchange matrix elements explicitly; the latter can be obtained from a point dipole or point monopole approximation. This approach was first adopted by Fischer and coworkers [31,32] and was successful at reproducing a variety of spectroscopic results with reasonable agreement. However, there are a large number of phenomenological adjustable parameters in their calculations, some of which are not easily interpreted physically. Warshel and Parson have also attempted to calculate the spectra of *Rps. viridis* [33,34]. Their approach starts

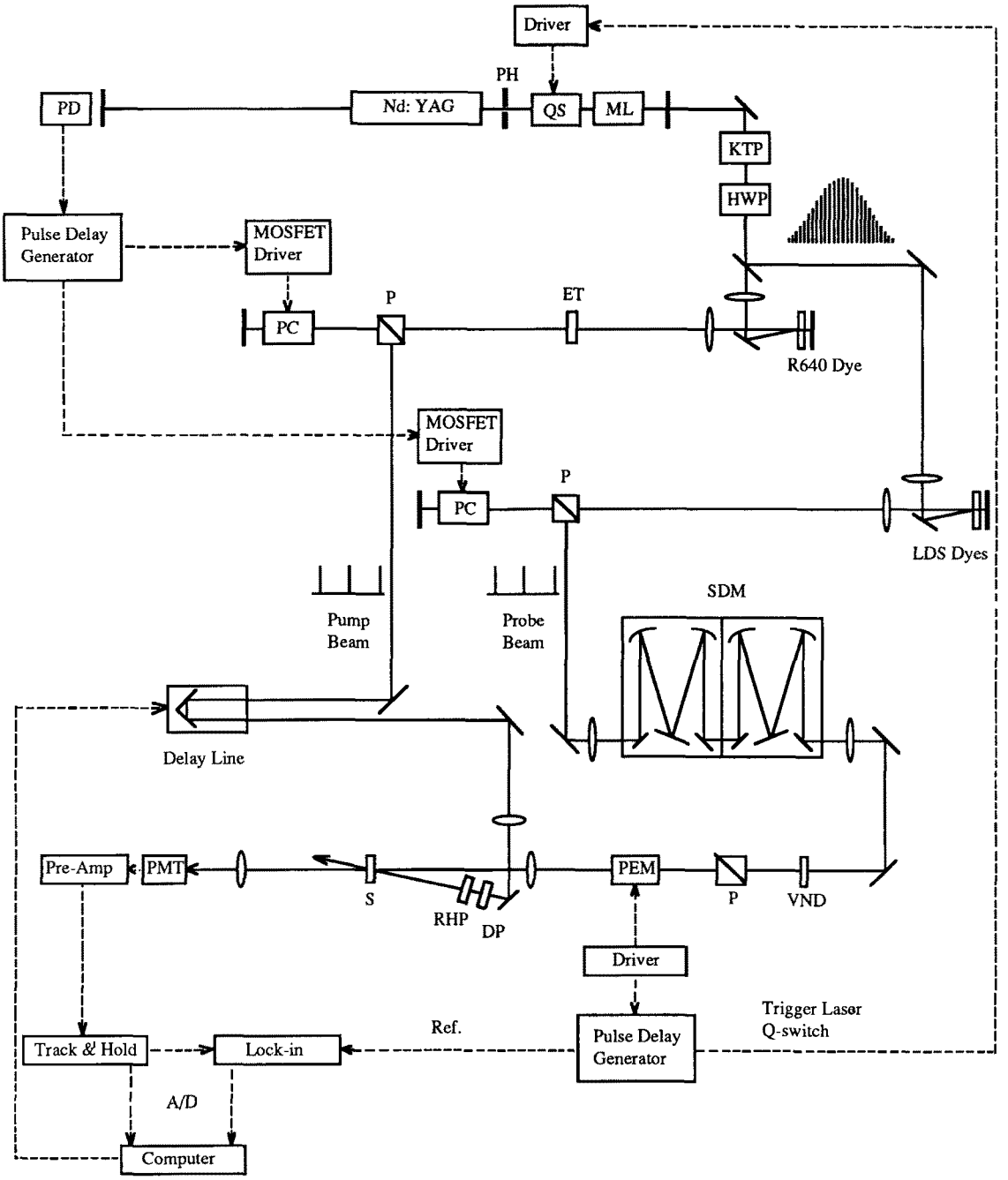

Fig. 2. Transient picosecond circular dichroism spectrometer. Abbreviations used: PD, photodiode; ML, acousto-optic mode-locker; QS, acoust-optic Q-switch; PH, pinhole; KTP, $KTiOPO_4$ nonlinear crystal; PC, electro-optic Pockels cell; ET, etalon; P, polarizer; PEM, piezo-electric modulator; RHP, rotating half wave plate; DP, depolarizer; SDM, subtractive double monochrometer; VND, variable neutral density filter; S, sample; PMT, photomultiplier tube.

with the basis set of states of the individual BChls and BPhs from which the combined electronic states for the oligomeric system are constructed at the self-consistent-field/ configuration-interaction (SCF/CI) level. Unlike the previous calculations, charge-transfer interactions are included in this model. A point monopole approximation is used to treat the interactions between pigments. The most recent calculations are those by Won and Friesner [35]. In this model, a set of strongly coupled molecular vibrational modes are explicitly included in the Hamiltonian and utilized in calculating optical spectra.

All reported CD calculations have focused on the RC from *Rps. viridis* [31–35]. The various approaches have been used to generate absorption, CD and linear dichroism spectra, all of which are similar to the experimental data. These calculations have impacted on our understanding of the electronic properties of the system. In particular, all the bands in the absorption spectrum are linear combinations of the eigenstates of the individual pigments (and some have contributions from the charge transfer states of the dimer; for example, the low-energy exciton band of the dimer has considerable contributions from charge transfer transitions [34]).

The calculations also yield rotational strengths of all the major bands in the CD spectrum [31–35]. The CD spectra derived from calculations have the qualitative features of experimental spectra. In accord with the standard excitonic theory, all these calculations predict a conservative CD spectrum. In addition, all the calculations show a underestimated rotational strength for the CD band of the monomeric BChls at 830 nm for *Rps. viridis* (corresponding to the 800 nm band for *Rb. sphaeroides*). These issues will be discussed in detail in the following sections along with the implications of the time resolved CD data on the origin of the CD signal for RCs.

Experimental details

The data discussed in this paper were recorded in the near-IR spectral region where the RC complex has a large CD signal. To detect a CD signal of $\Delta\epsilon/\epsilon=10^{-3}$, a sample absorbance of about 4 is needed at the desired probe wavelength in order to obtain a reasonable signal to noise ratio. In the experiments described in this paper a RC concentration of 0.1 mM was used ($A \approx 4$ at 800 nm in a 2 mm sample cell).

The experimental apparatus used to collect the transient CD spectra is shown in Fig. 2. A mode-locked and Q-switched Nd : YAG laser was used to pump synchronously two cavity-dumped tunable dye lasers with a pulse repetition rate of up to 1 kHz [36,37]. The outputs of the two dye lasers served as the pump and probe light pulses, respectively. The probe light pulses are sequentially switched between left and right circular polarization using a piezoelectric modulator. These polarization modulated pulses are then passed through the sample, and detected using phase-sensitive detection.

The pump laser pulses travel down a variable optical delay line and are focused onto a spinning (or flowing) sample. Prior to excitation, the pump light is passed through a depolarizer and a spinning half wave plate. These optics serve to remove all unwanted pump induced linear dichroism contributions to the detected signal. A complete optical theory which relates the observed signal to the transient CD measurement and discusses the importance of removing pump induced linear dichroism effects has recently appeared [38].

In the present experiments, the probe dye laser is operated without a tuning element. An empirically determined mixture of LDS750, LDS756, LDS821 and LDS865 dyes (Exciton) is used to generate a broad band light pulse which extends from 750 to 840 nm. An automated subtractive double monochrometer (SPEX Model 1672) is used to scan the output of the broad band dye laser. The slits of the monochrometer were set to give a spectral resolution of 1 nm. The monochrometer does not temporally broaden the pulses. Transient absorption experiments at various wavelengths within the tuning range of the probe pulse also indicate essentially no temporal dispersion in the arrival time at the sample with respect to the excitation pulse.

In selecting the excitation wavelength to trigger the electron transfer process, one can either excite the low energy exciton band or the Q_x band of the dimer. We chose to excite the latter at 610 nm as the absorbance of the sample at this wavelength (≈ 1) enables the excitation pulse to penetrate through the entire sample. With a pulse energy of 22 μJ at 610 nm (R640 dye laser) most of the sample (88–90%) can be excited. A problem associated with excitation of the Q_x band of the special pair is that this band (610 nm) overlaps the Q_x bands of the two monomeric BChls. Fortunately, excitation of these pigments results in fast (< 100 fs) energy transfer [39] to the special pair.

Another problem associated with the 610 nm excitation is the resulting fluorescence of the RC samples. Although this fluorescence is weak, the lack of any dispersive optics after the sample (see Fig. 2) means that the total fluorescence is detected by the PMT along with the signal of the monochromic probe pulses. About 60% of the fluorescence signal is in the region from 700 to 770 nm [27]. Since this is outside the spectral region of interest, this light was eliminated using a colored glass filter. Under these conditions, the ratio of the intensity of the probe pulses to the background emission was greater than 10 to 1.

The RC system undergoes slow charge recombination [1]. One needs to take this into serious consideration when studying these systems with a high repetition

rate laser. The back electron transfer from Q_B^- to $(BChl)_2^+$ has a time constant of 1 s. In order to accelerate the recovery of the sample one can, in principle, block electron transfer from Q_A^- to Q_B using various inhibitors. In the present studies, a 2.0 mM solution of 1,10-phenathrorin, was used. The back electron transfer from Q_A^- to $(BChl)_2^+$ has a time constant of 0.1 s, and thus at a laser repetition rate of 250 Hz (used for all data reported) and using a spinning sample cell, the photoexcited RCs should recover between successive passes through the excitation beam. Unfortunately, a small portion of the photoexcited RCs ($\approx 1\%$/shot) still form Q_B^- and thus recover on a much longer time-scale. At a repetition rate of 250 Hz, this causes a substantial steady state concentration of Q_B^- to build up, despite the presence of the inhibitor. When using a rotating sample cell (2 inch diameter, spun at 7 Hz), we observe that only about 70% of the sample recovered at negative pump-probe delay time. We also attempted to flow the sample through a thin optical cell (2.0 mm). The flow cell was mounted on an automated translational stage which moved back and forth horizontally during data collection. In this case, about 90% fresh sample is observed for each laser shot. In the case of the RC with reduced Q_A (back transfer time of tens of nanoseconds), there is complete recovery.

Experimental results and discussion

(A) Transient absorption studies

Before conducting the time-resolved CD experiments, transient absorption experiments were performed and compared with previous reports. This was done to assure that our experimental conditions were consistent with past reports. In particular, the effect of the transient spectrum on photolysis energy was checked. Nonlinear effects associated with intense excitation pulses plagued early picosecond work on this system [40]. As discussed in detail elsewhere [41], a depolarized pump beam is used in our transient absorption experiments in order to reduce the nonlinear effects observed around zero delay time. Fig. 3 shows the transient difference spectrum taken at zero time delay for RCs with Q_A chemically reduced. As previously reported [20], the low energy exciton band of the bacteriochlorophyll dimer at 860 nm bleaches and the absorption decrease at 810 nm results from both the bleaching of this exciton band and a blue shift of the absorption spectrum of the monomer bacteriochlorophyll. The absorption increase around 785 nm has been attributed to the blue shift of the Q_y bands of the monomeric BChls resulting from either changes in the interactions between the various pigments or an electrochromic effect [3]. In collecting this transient spectrum, a pump energy of 20 μJ/pulse was used. Reducing the pump power to 2 μJ/pulse does not change the shape

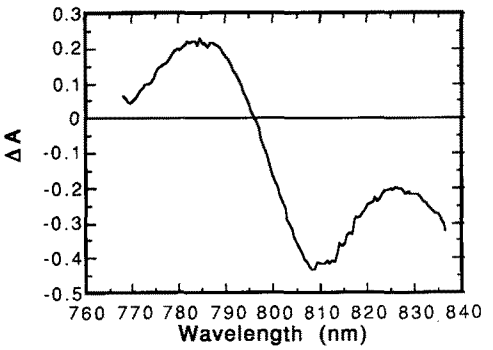

Fig. 3. The transient difference spectrum for RCs with Q_A reduced taken at zero time delay. ΔA is to the absorbance of the sample with excitation minus the absorbance without excitation. The data are independent of photolysis energy (2–20 μJ), indicating there are no multiphoton effects contributing to the transient spectra.

of the transient difference spectrum, indicating there are no multiphoton effects contributing to the transient spectra.

(B) Transient CD spectra

To demonstrate that our apparatus accurately measures CD signals, Fig. 4 shows the steady-state CD spectra of the RC of *Rb. sphaeroides* with both Q_A and Q_B, with Q_B removed, and with Q_A reduced by sodium dithionite in BOG buffer recorded by our apparatus. The three spectra are identical, indicating that Q_A and Q_B have little effect on the CD in this spectral region. There is also an excellent agreement between these spectra and those recorded for the RC with Q_B using conventional techniques [27,29].

Fig. 5 shows the transient CD spectra taken at a zero (35 ps experimental resolution) and 2 ns time delay. The 800 nm band probed by these measurements has been assigned to the Q_y transition of both BChls [2,3]. With respect to the rates of reactions shown in Fig. 1, these spectra represent the CD of the electron transfer inter-

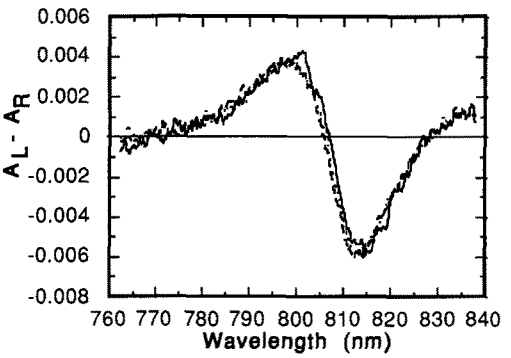

Fig. 4. Steady state near-IR CD spectra of *Rb. sphaeroides* RCs with both Q_A and Q_B (———), with Q_B removed (· · · · · ·), and with Q_A reduced by sodium dithionite in BOG buffer (- - - - - -) recorded by the picosecond CD spectrometer with the excitation beam blocked. The three spectra are the same, indicating that the Q_A and Q_B have a negligible effect on the CD in this spectral region.

mediates, $(BChl_2)^+BPh_L^-Q_A$ and $(BChl_2)^+BPh_LQ_A^-$, respectively. The incomplete vanishing of the negative CD signal at 816 nm is due to unexcited RCs (15%). The data indicate that the CD bands at 850 nm and 810 nm disappear within our instrumental response time of 35 ps. In comparison with Fig. 4, the red edge of the transient CD band shown in Fig. 5 reaches a value of zero at around 810, approximately 5 nm to the red of the zero-crossing in the ground state spectrum. This results does not indicate that the transient CD band is shifted from that observed in the ground state. This change is solely due to the disappearance of the negative band at 816 nm, which effects the location of the zero-crossing in the ground state spectrum.

As revealed by the high-energy edge of the transient CD bands, there may be a small red shift of the CD band of the BChls associated with the electron transfer from BPh_L to Q_A. Although improved signal to noise is needed to confirm this observation, it is consistent with other previous time resolved spectroscopic observations in which the 795 nm absorption band red shifts following the electron transfer from BPh_L^- to Q_A [3]. These spectral shifts have been explained by both an electrochromic effect associated with changing charges around the monomer and the changing interactions between the electronic states of the pigments [3]. Moreover, given that the kinetics measured at 795 nm (150 ps time constant) are faster than both the recovery of the bleached Q_y band of the BPh_L and the disappearance of the BPh_L^- anion absorption between 620 and 720 nm (both 250 ps), Kirmaier et al. [3,20] suggested that spectral evolution of the monomeric BChl band might result from movements of nearby charged groups on the protein and/or the movements of the pigments in response to the charge separation. Fig. 6 shows a transient CD spectrum obtained with a slowly rotating sample cell. This data are recorded in such a way that about 60% of the RCs in the focal volume do not recover prior to the arrival of the next excitation pulse. Therefore, the

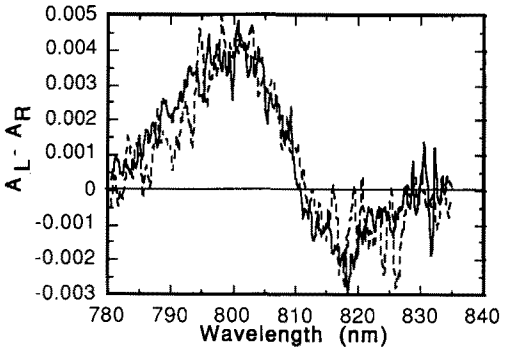

Fig. 5. Transient CD spectra taken at zero (———) and 2 ns (- - - - - -) time delay. These spectra represent the CD of the electron transfer intermediates, $(BChl_2)^+BPh_L^-$ and $(BChl_2)^+BPh_LQ_A^-$, respectively. The incomplete vanishing of the negative CD signal at 816 nm is due to unexcited RCs (15%).

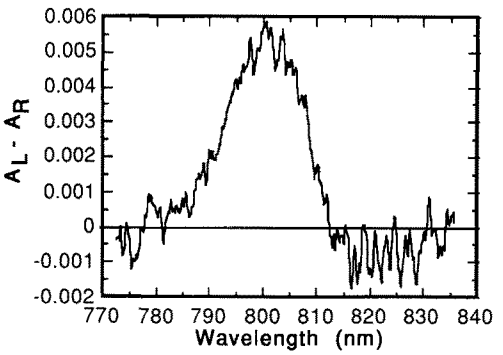

Fig. 6. The transient CD spectrum obtained with a slowly rotating sample cell. This spectrum is recorded in such a way that about 60% of the RCs in the focal volume do not recover prior to the arrival of the next excitation pulse. Therefore the spectrum reflects the transient species that exists milliseconds after photoexcitation. The 800 nm band such obtained has about 15% more intensity than those shown in Fig. 4 because of the complete excitation of the RCs in the focal volume. These data indicate that there is no evolution of the transient CD spectrum between 2 ns and the millisecond time-scale.

spectrum shown mimics continuous actinic conditions and reflects mainly the transient that exists milliseconds after photoexcitation, $(BChl_2)^+BPh_LQ_A^-$. The 800 nm band such obtained is about 15% more intense than that observed at earlier times because of the complete excitation of the RCs in the focal volume. These data indicate that there is no evolution of the CD spectrum between 2 ns and the millisecond time-scale.

The data also shows that the two intermediates, $(BChl_2)^+BPh_L^-Q_A$ and $(BChl_2)^+BPh_LQ_A^-$, have positive CD bands with similar rotational strength in the 800 nm region. These transient spectra are also similar in intensity and shape to that reported for the chemically oxidized RC, $(BChl_2)^+BPh_LQ_A$ [27].

(C) The high-energy exciton band of the special pair

The spectral region for which we have collected the time-resolved CD spectra has been a subject of intensive discussions [26,30,35,42,43]. The low-temperature absorption spectrum of *Rb. sphaeroides* reveals a shoulder at 812 nm on the 800 nm band [44]. Similarly, *Rps. viridis* has a 850 nm shoulder on the 830 nm band. Shuvalov and Asadov's CD study on the *Rps. viridis* assigns the 830 nm band to $BChl_{LA}$ and the 850 nm spectral features to $BChl_{MA}$ [30]. Kiramaier et al. have adopted this assignment in their picosecond-photodichroism studies of related bands in *Rb. sphaeroides* [3]. However, these later workers pointed out that one cannot account for the absorption changes between 780 and 820 nm entirely on the basis of a blue-shift or loss of intensity in the 800 and 812 nm bands of the BChls [20]. In light of the crystal structure data, a low temperature linear dichroism study of oriented *Rps. viridis* sample reported by Breton [26] assigned the 850 nm band to the high-energy exciton state of the special pair (P_+ state). This conclusion was based on the fact that a

distinctive linear dichroism band at 850 nm disappears upon oxidation of the special pair. This is in agreement with an earlier assignment by Vermeglio et al. based on low-temperature absorption data [42]. With respect to recent theoretical efforts, Fischer et al. [31,32] calculated electronic spectra of *Rps. viridis* using a simple excitonic model. They pointed out that the upper exciton state of the special pair, P_+, carries practically no intensity in an absorption spectrum, but is vital for an understanding of the CD spectrum. A calculation of the same RC by Warshel and Parson [33], indicates that it is not accurate to consider the 850 nm band as being primarily a pairing exciton band of the $BChl_{LP}$ and $BChl_{MP}$. Although the Q_y transitions of the $BChl_{LP}$ and $BChl_{MP}$ do make a major contribution to this band (coefficients of -0.4138 and -0.4455 respectively), the contribution from $BChl_{LP}$ is calculated to be smaller than that from BCh_{LA} (-0.6058), and about the same as that due to $BChl_{MA}$ (-0.4185) and BPh_L (-0.2452) [33]. Contrary to this, calculations by Won and Friesner [35], have confirmed the existence of the P_+ exciton band. Diagonalization of the resultant 6×6 exciton matrix shows that the P_+ component has 44.5% $BChl_{MP}$, 45% $BChl_{LP}$, 5.4% $BChl_{MA}$, 4.4% $BChl_{LA}$, and negligible BPh character. No theoretical calculations on *Rb. sphaeroides* have been reported.

Our time-resolved CD data show that upon photoexcitation, the negative CD band at 814 nm and the positive CD band at 860 nm disappear. The instantaneous (< 100 fs) bleaching of the 860 nm absorption band has been confirmed by recent femtosecond work [8–11]. The vanishing of the two biphasic CD bands upon photoexcitation indicates that the 814 nm CD band must have a major contribution from the special pair. This provides more experimental evidence consistent with and supportive of the existence of the high energy exciton band at about 810 nm for *Rb. sphaeroides*.

(D) A reexamination of the origin of optical activity of reactioncenters

The time-resolved CD data in Fig. 5 show that both $(BChl_2)^+BPh_L^-Q_A$ and $(BChl_2) + BPh_LQ_A^-$ are characterized by a large CD band at 800 nm with similar rotational strength (within 10%). The spectra are also nearly identical to the CD spectrum of the chemically oxidized RC [27]. These spectra also have a distinct nonconservative feature, contrary to an excitonic description. In fact, the band position and rotational strength of this transient CD band is nearly identical to the corresponding band of the unexcited RCs (shown in Fig. 4). The slight intensity reduction of this band in the ground state spectrum compared with the time-resolved measurements is due to contributions from the negative CD band of the high energy exciton state of the dimer. It is very interesting that this CD band remains unchanged regardless of whether the bacteriochlorophyll dimer is oxidized or the BPh_L is reduced. The radical cation of the special pair has a weak absorption band at 1250 nm [44] and the BPh_L^- anion has an absorption between 620 and 720 nm [3]. Both ionic intermediate should have less excitonic interactions with the two monomeric BChls than the corresponding uncharged species. In addition, if the CD band arose from an excitonic interaction of the two monomers, the band must be conservative, in contrast to the data. Given the two-fold symmetry of RCs, it is hard to imagine that only excitonic interaction with BPh_M results in the 800 nm CD band. These observations cast serious doubts on an excitonic origin of this CD band.

CD calculations for the various electron transfer intermediates have not been reported. Standard excitonic theory predicts strictly conservative CD bands [25], and all the calculations on *Rps. viridis* RCs have yielded rigorously conservative CD spectra [31–35]. When compared to the experimental data, however, these calculation all underestimate the rotational strength of the monomeric BChls band. One of the possible reasons for such a discrepancy (especially in the calculations only including the Q_y states of the pigments [35]) involves the absence of contributions from either hyperchromic or hypochromic effects (gain or loss of oscillator strengths). Originally developed by Tinoco, the theory of hyperchromism [45,46] has been extensively applied to nucleic acids and proteins [24]. Hypochroism and hyperchroism arise from the interaction between one particular electronic excited state of a given chromophore and different electronic states of the neighboring chromophores. In a study of oligomers of BPh and BChl, Scherz and Parson [47] showed that the mixing of the BChl's (or BPh's) four main excited states (Q_y, Q_x, B_x and B_y) with the ground and excited states of the dimer can account for the intensity borrowing between bands and the nonconservative CD spectra experimentally observed for those dimers. In the recent calculation of Parson et al. on RCs [33], all these higher states and their interactions are included in the excitonic matrix. However, as pointed out by these authors, a possible source of the deviation from the experiment data is the use of simple first-order perturbation theory to find the ground-state wave function, thereby neglecting the contributions of doubly excited states (two chromophores excited) to the ground state [33]. This interaction has little effect on the energy of the ground state but could markedly alter the calculated CD spectra [47]. it is important to remember that the prediction of a nonconservative CD associated with hyperchromic phenomenon is valid only when one is considering the lowest electronic transitions. In principle, if the CD is due only to the excitonic interactions of transitions in different chromophores, the sum of the rotational strength for all the electronic transitions of the entire system is zero. Therefore, the CD of the entire spectral

region is conservative. One possible rationalization of the positive nonconservative 800 nm CD band for the two intermediates, $(BChl_2)^+BPh_L^-Q_A$ and $(BChl_2)^+BPh_LQ_A^-$, is that there are negative CD bands in a different region of the spectrum. However, the special pair in the photoexcited RCs of *Rb. sphaeroides* has a weak absorption at 1250 nm and a small *positive* CD band at 1300 nm has been found for chemically oxidized RCs of *Rps. viridis* [49]. The CD of chemically oxidized RC of *Rb. sphaeroides* in the 500–750 nm region is also very weak compared to that observed for the 800 nm band [27]. In addition, the absorption band of the radical anion of BPh_L in the 620–720 nm region has only about one-quarter the oscillator strength of the 800 nm band [49] and therefore cannot exhibit a large CD. The CD in the 300–420 nm region (B_x and B_y transitions) has not been well documented; however, it is unlikely that these signals will cancel the large positive rotational strength of the 800 nm band.

The foregoing discussion suggests that mechanisms other than exciton interaction need to be considered in accounting for the CD of the bacteriochlorophyll monomer transition at 800 nm. One possible mechanism is that the monomer BCls are intrinsically chiral, perhaps arising from structural properties such as a doming of the porphyrin due to the five coordinated Mg or the nonplanarity of the porphyrin side-chains. Such information could be available from a more refined crystal structure. Another potential mechanism which gives rise to CD is an interaction between the chromophores and the surrounding protein. As discussed in previous works, the coupled oscillator interaction between $\pi \rightarrow \pi^*$ transitions on the prosthetic groups and the aromatic side-chains of the protein can also contribute significantly to CD spectra [50,51]. In the RC, there are numerous phenylalanine, tyrosine and tryptophan residues in close vicinity of the pigments. Interactions with these groups could result in substantial contributions to the CD signal. Site-directed mutagenesis could be used to test this hypothesis. If the CD signals do arise from such an origin, transient CD spectroscopy on the entire series of optical transitions may serve as a valuable approach for studying the protein response to the charge separation processes.

Conclusions

In this paper, we have demonstrated that one can collect transient CD spectra with picosecond time-resolution. This provides a new spectroscopic approach for characterizing transient intermediates in biological systems.

Application of this technique to the primary electron transfer intermediates in the reaction centers of *Rb. sphaeroides* show that, contrary to the predictions of the standard exciton theory, the time-resolved CD spectra reveal a large, nonconservative, and fairly stationary CD band at 800 nm for the primary electron transfer intermediates: $(BChl_2)^+BPh_L^-Q_A$ and $(BChl_2)^+BPh_LQ_A^-$. This finding suggests that mechanisms other than exciton interactions have to be included in order to explain the optical activity of this biological system. This provides a challenge for future experimental and theoretical work, and a possible avenue for studying the protein relaxation accompanying the electron transfer reaction.

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