Biochimica et Biophysica Acta, 974 (1989) 44–53
Elsevier

BBA 42955

Isolation and characterization of the 47 kDa protein and the D1-D2-cytochrome *b*-559 complex

D.F. Ghanotakis [1], J.C. de Paula [2], D.M. Demetriou [3], N.R. Bowlby [3], J. Petersen [3],
G.T. Babcock [2] and C.F. Yocum [3]

[1] *Department of Chemistry, University of Crete, Iraklion, Crete (Greece),* [2] *Department of Chemistry, Michigan State University, East Lansing, MI (U.S.A.) and* [3] *Departments of Biology and Chemistry, University of Michigan, Ann Arbor, MI (U.S.A.)*

(Received 4 July 1988)
(Revised manuscript received 27 October 1988)

Key words: Photosystem II; Reaction Center; Chlorophyll binding protein; ESR; Raman spectroscopy

The 47 kDa polypeptide and a protein complex consisting of the D1 (32 kDa), D2 (34 kDa) and cytochrome *b*-559 (9 kDa) species were isolated from a Tris-washed Photosystem II core complex solubilized with dodecylmaltoside in the presence of $LiClO_4$. Although the 43 kDa chlorophyll-binding protein is readily dissociated from the Photosystem II complex under our conditions, two cycles of exposure to high concentrations of detergent and $LiClO_4$ were required for complete removal of the 47 kDa chlorophyll-binding protein from the D1-D2-cytochrome *b*-559 complex. Spectroscopic characterization of these two species revealed that the 47 kDa protein binds chlorophyll *a*, whereas the D1-D2-cytochrome *b*-559 complex shows an enrichment in Pheo *a* and heme on a chlorophyll basis. A spin-polarized EPR triplet can be observed at liquid helium temperatures in the D1-D2-cytochrome *b*-559 complex, but no such triplet is observed in the purified 47 kDa species. The zero-field splitting parameters of the P-680$^+$ triplet indicate that the triplet spin is localized onto one chlorophyll molecule. Resonance Raman spectroscopy showed that: (i) β-carotene is bound to the reaction center in its all-*trans* conformation; (ii) all chlorophyll *a* molecules are five-coordinate; and (iii) the C-9 keto group of one of the chlorin pigments is hydrogen-bonded. Our results support the proposal that the D1-D2 complex binds the P-680$^+$ and Pheo *a* species that are involved in the primary charge separation.

Introduction

Work by various groups has produced highly refined preparations of the PS II 'core' complex, which has made it possible to study the polypeptides whose presence constitutes the hydrophobic domain of PS II and the components of photochemical electron transfer associated with this domain [1–4]. An analysis of the polypeptide composition of 'core' complexes by polyacrylamide gel electrophoresis in the presence of SDS and urea resolves five major polypeptides. More specifically, these species have been identified as the Chl-binding polypeptides of apparent molecular masses of 43 and 47 kDa, 32 and 34 kDa proteins, denoted as 'D1' and 'D2', and the 9 kDa polypeptide identified as the species which binds the heme of cytochrome *b*-559.

Both the 43 and 47 kDa proteins have been purified under conditions whereby bound Chl is retained. Work by Nakatani et al. [5] with the isolated 47 kDa protein has revealed the presence of fluorescence and pheophytin absorbance changes, findings that led to the suggestion that the 47 kDa polypeptide might contain the binding sites for the reaction center photochemistry of PS II. Tang and Satoh [6] showed the presence of 695 nm fluorescence band associated with their preparation of 47 kDa protein. The polypeptide, as isolated by the procedure reported by Tang and Satoh, contains 3 Chl *a*, 1 Pheo *a* per 3 47 kDa; no PQ-9 was detected and the isolated protein is inactive in the DPC → DCPIP assay.

More recently, the discovery of a set of very close functional and structural similarities between the electron-acceptor side of the purple-bacteria reaction center

---

Abbreviations: Chl, chlorophyll; EPR, electron paramagnetic resonance; PS, Photosystem; Pheo *a*, pheophytin *a*; Mes, 4-morpholineethanesulfonic acid; Bistris, 2-[Bis(2-hydroxyethyl)amino]-2-hydroxymethyl)-propane-1,3-diol.

Correspondence: D.F. Ghanotakis, Department of Chemistry, University of Crete, P.O. Box 1470, Iraklion, Crete, Greece.

and that of PS II [7], along with the amino-acid sequence similarity between the D1 and D2 subunits and the L and M subunits of the purple bacteria has led to the proposal that the so-called D1 and D2 polypeptides might constitute a complex that is capable of binding the primary donor and acceptor of PS II [8,9]. Recently Nanba and Satoh [10] isolated a Photosystem II complex consisting of the D1, D2 and Cyt *b*-559 species. The complex was isolated by exposure of oxygen-evolving PS II membranes from spinach thylakoids to 4% Triton X-100 followed by ion-exchange chromatography. The complex prepared by this procedure was found to contain five chlorophyll *a*, two Pheo *a*, one β-carotene and one or two Cyt *b*-559. A spectroscopic characterization of the complex revealed the accumulation of reduced pheophytin when the system was illuminated at 5 ° C, and low-temperature EPR spectroscopy demonstrated the presence of a spin-polarized triplet [10,11] species characteristic of the interaction between the oxidized primary donor chlorophyll and a pheophytin anion species.

A subsequent report by Akabori et al. [12] has reported a chromatographic method for isolation of various fractions from PS II core material which avoids the exposure to large amounts of Triton X-100 utilized in the earlier preparation; among these fractions, is a preparation of the D1/D2 heterodimer. Data presented by Seibert et al. [13] indicate that the preparation of Nanba and Satoh [10] is unstable.

In the present paper we describe a new method for the isolation of the 47 kDa protein and the D1-D2-Cyt *b*-559 complex that uses the non-ionic detergent dodecylmaltoside in combination with high concentrations of lithium perchlorate to dissociate polypeptides of the PS II complex, followed by FPLC ion-exchange chromatography to separate these polypeptides. By carrying out a low-temperature EPR study of the two systems, we support the proposal that the D1-D2 complex, and not the 47 kDa polypeptide, is the species which bind the reaction center of PS II. The EPR signal from the spin-polarized triplet was also used as a probe of the stability of the D1-D2-Cyt *b*-559 complex. It was found that more than 80% of the EPR signal intensity from the spin-polarized triplet could still be observed after incubation of our D1-D2-Cyt *b*-559 complex in the dark at room temperature for 5 h. Resonance Raman spectroscopy has been used to investigate the structure of the D1-D2-Cyt *b*-559 complex in more detail and reveals that β-carotene is bound to the reaction center in the all-trans conformation, that the chlorophylls in the reaction center are five-coordinate, and that the C-9 keto group of one or more of the chlorin pigments is hydrogen-bonded, possibly to an amino-acid group. Fujiwara et al. [36] arrived at similar conclusions from their resonance Raman studies of Nanba-Satoh preparations.

Materials and Methods

Subchloroplast PS II membranes and the PS II oxygen-evolving core complex were prepared as described in Ref. 4. Tris treatment of the complex was carried out by a 20 min exposure to a 0.8 M Tris buffer (pH 8.0) followed by centrifugation and subsequent resuspension in a medium containing 0.4 M sucrose/50 mM Mes (pH 6.0)/10 mM NaCl.

Antibodies against the 47 kDa and the D1 subunits were kind gifts of Dr. Nathan Nelson (47 kDa) and R. Debus (D1). Immunoblotting was carried out with a Bio-Rad transblotting apparatus according to the manufacturer's instructions. Proteins transferred to nitrocellulose were detected by using goat anti-rabbit secondary antibodies conjugated with horseradish peroxidase; antigens reacting with the secondary antibody were detected by a color-generating reaction in which H_2O_2 and 4-chloro-1-naphthol were the chromogenic substrates for the peroxidase.

Gel electrophoresis was carried out as described in Ref. 14. EPR spectra were recorded at liquid-helium temperature on a Bruker ER200D spectrometer operating at X-band and equipped with an Oxford ESR-900 liquid helium cryostat. A Hewlett Packard 5245L electronic counter with a 5255A frequency converter plug-in and a Bruker ER035M gaussmeter were used to measure microwave frequency and magnetic field, respectively. Optical spectroscopy was carried out on a Perkin-Elmer Lambda 5 UV/Vis spectrophotometer.

Resonance Raman measurements were performed in a backscattering geometry with a SPEX 1401 scanning double-monochromator instrument equipped with photon-counting detection and interfaced to a DEC LSI11-2 computer for signal averaging. The exciting radiation from either a Coherent INNOVA-90K Kr^+ (406.7 nm, 10 mW) or a Spectra-Physics Model 164 Ar^+ (488.0 nm, 20 mW) laser was used. Samples ([Chl] = 0.4 mg/ml) in a spinning EPR tube were frozen to about -140 ° C in a N_2-gas flow apparatus with liquid N_2 as the coolant (see Ref. 15 for a description). Potential damage to the sample by the laser beam was assessed by monitoring the electron-transfer activity before and after the Raman experiment. It was found that 20 min of exposure to 10 mW of 406.7 nm radiation did not impair the ability to generate the EPR signal emanating from the spin-polarized triplet of the reaction-center molecule. This EPR signal can only be obtained if the reaction center can support electron transfer from P-680 to Pheo *a*. We chose 20 min of exposure time for our laser damage study, even though a typical experiment necessitated more than 1 h of signal-averaging time. We can justify our choice on the strength of the observation that the Raman spectral features of the first and last scans (10 min acquisition time per scan) were identical in all respects.

Results

Isolation of the 47 kDa protein and the D1-D2-Cyt b-559 complex

A Tris-treated preparation of the PS II core complex (approx. 1.5 mg Chl/ml), resuspended in a medium containing 0.4 M sucrose/50 mM Mes (pH 6.0)/10 mM NaCl, was mixed with an equal volume of a solution containing 20 mM Bistris (pH 6.0)/15% dodecylmaltoside/1.5% taurine/4 M $LiClO_4$. After 10 min incubation in the dark at 4 ° C, the solubilized complex was desalted by a 2 h dialysis step against a solution containing 20 mM Bistris (pH 6.0) and subsequently loaded onto a Mono-S Pharmacia column (attached to a Pharmacia FPLC system) that had been previously equilibrated with a solution containing 20 mM Bistris/1.5% taurine/0.05% dodecylmaltoside/3 mM $LiClO_4$. The fraction that did not bind on the Mono-S column was loaded onto a Mono-Q column; the column was subjected to a gradient of $LiClO_4$ in the presence of 20 mM Bistris (pH 6.0)/0.05% dodecylmaltoside/1.5% taurine. As shown in Fig. 1, the elution profile from the Mono-Q column consists of five main fractions. Table I lists the polypeptides found in each fraction, as determined from SDS polyacrylamide gel electrophoresis of individual fractions. As shown in the table, fraction p1 contains the Chl-binding polypeptide of apparent molecular mass 43 kDa, while fraction p2 contains the 28 kDa species, another Chl-binding protein that was isolated and characterized in Ref. 4. The fractions designated as p3 and p4 are of primary interest because they contain the 47 kDa (p3) and the D1-D2-Cyt *b*-559 (p4) species. The fraction denoted p5 was concentrated in a dialysis bag placed in solid polyethylene glycol 6000 to about 1.5 mg Chl *a* ml. This fraction was then purified by repeating the solubilization and isolation procedure as described above starting with exposure to 1.5 M $LiClO_4$ and 7% dodecylmaltoside to yield a preparation devoid of the 47 kDa protein.

TABLE I

Polypeptide content of various fractions

See the elution profile shown in Fig. 1.

Fraction	Polypeptides
p1	43 kDa
p2	28 kDa
p3	47 kDa
p4	$D_1 + D_2$ + Cyt *b*-559
p5	$D_1 + D_2$ + 47 kDa + Cyt *b*-559

The polypeptide content of some of the fractions, as revealed by SDS-polyacrylamide gel electrophoresis and subsequent staining with Coomassie blue, is shown in Fig. 2. The 47 kDa and the D1 species were also identified by use of antibodies against these proteins.

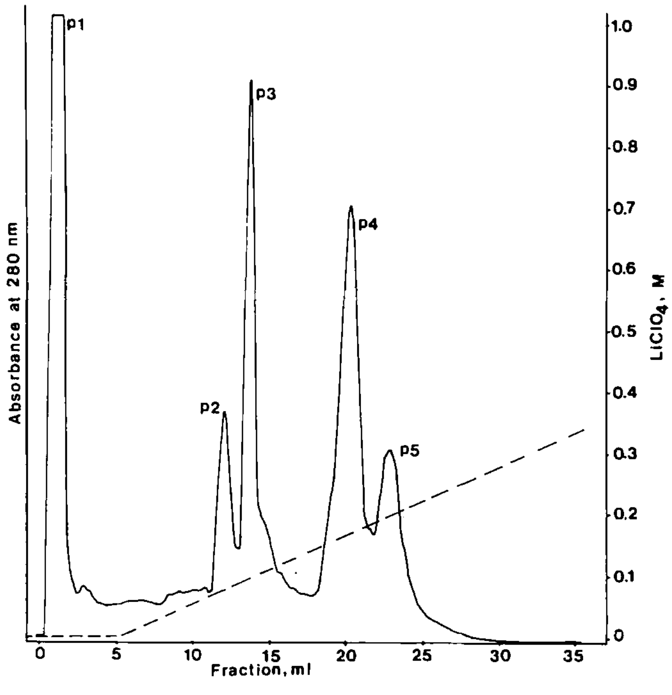

Fig. 1. Elution profile of the solubilized PS II core complex monitored by absorption at 280 nm. The conditions of the experiment are described in the text.

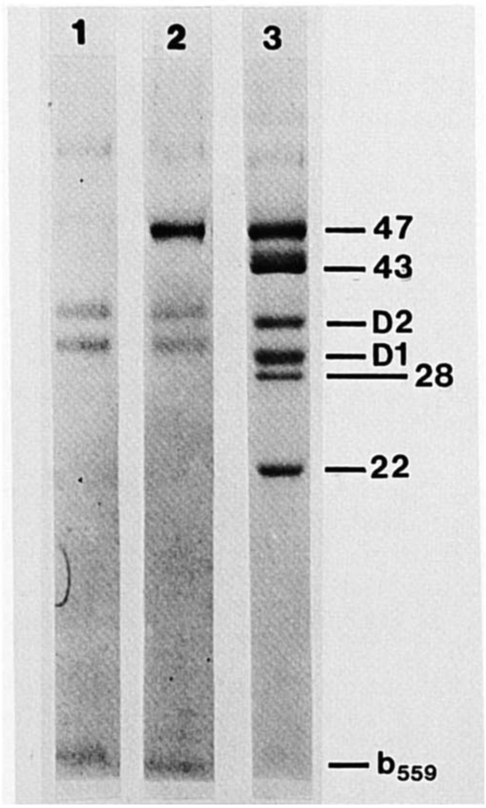

Fig. 2. Polypeptides of selected PS II fractions after SDS-gel electrophoresis. The gel contained 6.5 M urea; proteins were stained with Coomassie blue. The fractions shown are: (1) p4, (2) p5 and (3) PS II core complex.

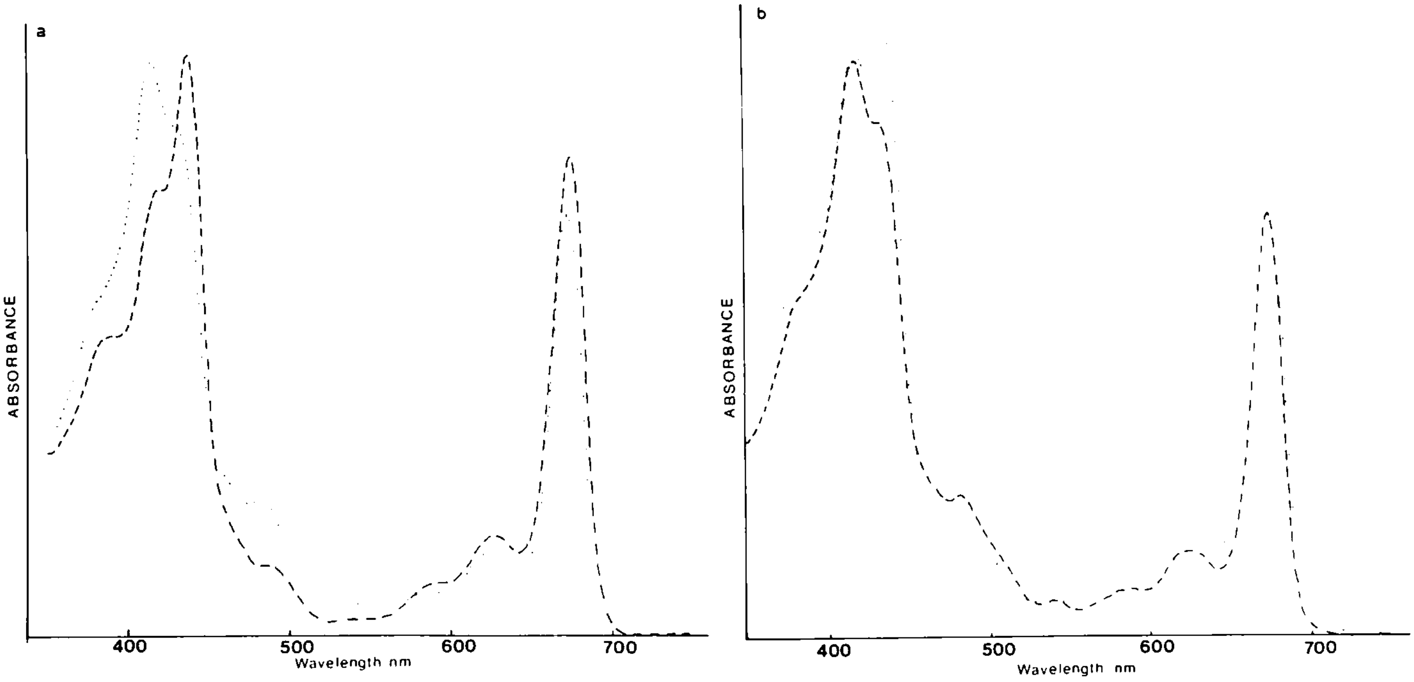

Fig. 3. (a) Room-temperature absorption spectra of the 47 kDa (– – –) and the D1-D2-Cyt *b*-559 complex (······). (b) Room-temperature absorption spectra of the D1-D2-Cyt *b*-559 complex (– – –) and the D1-D2-47 kDa-Cyt *b*-559 complex (······).

The reactivity of blotted protein bands with antibodies against the 47 kDa protein revealed that the D1-D2-Cyt *b*-559 complex contains only small traces of the 47 kDa species (data not shown).

Absorption spectroscopy

The absorption spectra of the isolated fractions p3, p4 and p5 are shown in Figs. 3a and b; it is apparent that none of these fractions contains optically detectable amounts of Chl *b*. An enrichment in the D1-D2 complex is accompanied by the corresponding appearance of a discrete peak at 417 nm in the absorption spectrum of this fraction. The 437 nm Soret band is due to the absorption by Chl *a*, whereas the 417 nm peak arises from absorption contributions from Pheo *a* [16] as well as from the Soret band of oxidized Cyt *b*-559 [17]. As shown in Fig. 4, reduction of the D1-D2-Cyt *b*-559 complex with dithionite produced a distinctive red-shift of the Soret band at 417 nm; as reported in Ref. 17, reduction of Cyt *b*-559 by dithionite is accompanied by a 14 nm red-shift in the Soret region and a weak absorption increase at 559 nm. The presence of absorption bands due to Chl *a* makes it difficult to discriminate between contributions to the 417 nm band which arise from Cyt *b*-559 as well as from Pheo *a*. Similarly, the presence of pheo *a* complicates attempts to assess the pigment content of the D1-D2-Cyt *b*-559 complex; our preliminary estimates indicate that the complex contains six pigments (Chl *a* plus Pheo *a*) per Cyt *b*-559.

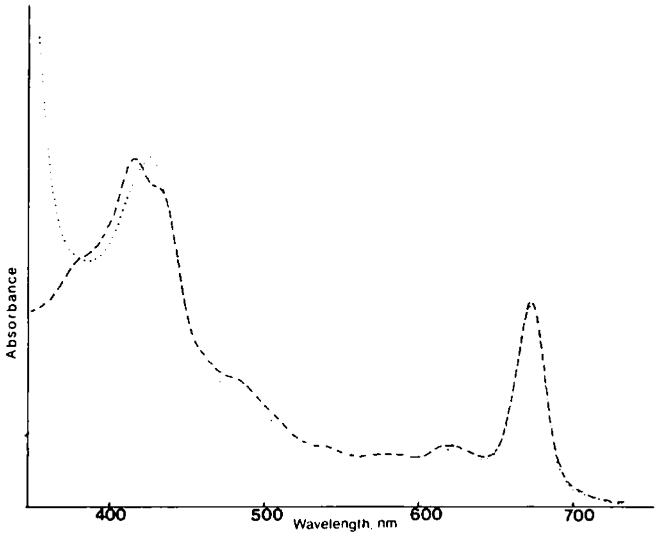

Fig. 4. Room-temperature absorption spectra of the D1-D2-Cyt *b*-559 complex as isolated (– – –) and reduced by 4 mM dithionite (······).

EPR spectroscopy

To investigate further the question of which protein(s) bind(s) the primary donor and acceptor of PS II, we carried out a low-temperature EPR study of the purified 47 kDa species and of the D1-D2-Cyt *b*-559 complex. As shown in Fig. 5, a spin-polarized triplet was observed in the D1-D2-Cyt *b*-559 complex, but no such triplet was present in the 47 kDa polypeptide. Reduction of both preparations with dithionite had no significant effect on the observed signals (data not shown).

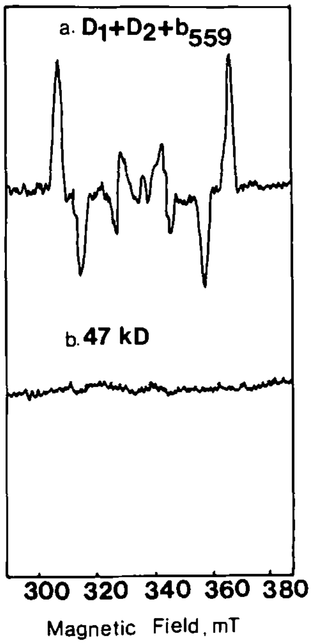

Fig. 5. (a) EPR spectrum of the light-induced triplet in the D1-D2-Cyt *b*-559 complex. The experiment was carried out at 4.2 K. Instrument conditions: $\nu = 9.4674$ GHz, modulation amplitude = 25 Gpp, microwave power = 36 μW, Chl concentration = 0.75 mg/ml. (b) The same as in (a) but with the 47 kDa polypeptide at a Chl concentration of 2.25 mg/ml.

Recently, Okamura et al. [11] observed the same triplet in their preparation of D1-D2-Cyt *b*-559 complex. Because the spin-polarized triplet EPR signal is a sensitive probe of both structure and function of the reaction-center molecule, P-680, we analyzed its properties in detail. From the point of view of structure, the zero-field splitting parameters $|D|$ and $|E|$, which can be determined from the EPR spectrum, are very informative. The splitting between the two outermost lines in the EPR spectrum is equal to $2|D|$, whereas the splitting between the next two lines is equal to $|D| + 3|E|$. We thus obtain values of $|D| = 0.0273 \pm 0.0004$ cm^{-1} and $|E| = 0.00392 \pm 0.00002$ cm^{-1}, which are lower than those reported either by Rutherford et al. [18] for the P-680 triplet in non-oxygen-evolving PS II particles ($|D| = 0.0290$ cm^{-1}, $|E| = 0.0040$ cm^{-1}) or by Okamura et al. [11] for D1-D2-Cyt *b*-559 complexes prepared with Triton X-100 ($|D| = 0.0287$ cm^{-1}, $|E| = 0.0043$ cm^{-1}). In spite of these differences, all reported values for the zero-field splitting parameters of the P-680 triplet are much higher than those reported for the triplet of the special pair of *Rb. sphaeroides*, where $|D| = 0.0187$ cm^{-1} and $|E| = 0.0034$ cm^{-1} [19]. A value of $|D| = 0.0273$ cm^{-1} agrees well with the zero-field splitting of monomeric Chl *a* in solution obtained by Thurnauer et al. [19] ($|D| = 0.0275$ cm^{-1}). We can state, however, that the spin-polarized triplet observed in our D1-D2-Cyt *b*-559 complexes does not originate from loosely bound, non-functional chlorophyll because the observed polarization pattern in our preparations is that expected from a spin-polarized triplet created by radical-pair recombination, that is AEE AAE, where A and E denote absorptive and emissive transitions, respectively. The chlorophyll *a* triplet in solution is expected to show an EEE AAA pattern [19].

The P-680 triplet EPR signal was further used as an assay of the stability of our D1-D2-Cyt *b*-559 preparations. We determined the intensity of the EPR signal as the sum of the intensities of the two highest-field and the two lowest-field peaks, as they are the farthest removed from the spectrally dense $g = 2.0$ region. We found that even after a dark-incubation period of 5 h at room temperature the intensity of the P-680 triplet EPR spectrum decreased by only 15.5%. Akabori et al. [12] have measured optically the formation of the P-680 triplet in D1-D2-Cyt *b*-559 complexes. They found that complexes prepared according to Nanba and Satoh [10] with Triton X-100 as detergent were relatively unstable, as they lost about 85% of the triplet signal after 5 h of incubation at 25 °C. Akabori et al. [12] also found that the use of milder detergents, such as *n*-octyl glucopyranoside and *n*-octyl-D-thioglucopyranoside afforded more stable complexes, which lost only about 15% of the triplet signal after a 5 h incubation period at 25 °C. Therefore, our protocol, which uses dodecylmaltoside as detergent, yields reaction-center complexes whose stability is comparable to that found by Akabori et al. [12] in their preparations.

Resonance Raman spectroscopy

Fig. 6 shows the resonance Raman spectrum of D1-D2-Cyt *b*-559 complexes at −140 °C in the region between 1300–1800 cm^{-1} with excitation at 406.7 nm (Fig. 6a) and 488.0 nm (Fig. 6b). Excitation at 406.7 nm (Fig. 6a) is expected to enhance vibrations from all pigments in the reaction center, because the Soret bands of Chl *a*, Pheo *a*, and oxidized low-potential Cyt *b*-559 all lie in this region of the absorption spectrum. In addition, the strongly scattering β-carotene molecule should also contribute significantly to the Raman spectrum under 406.7 nm excitation.

In order to distinguish between chlorin and carotenoid modes, a resonance Raman spectrum was obtained with excitation at 488.0 nm, a wavelength that is coincident with the strong $^1B_u \rightarrow {}^1A_g$ electronic transition of β-carotene (Fig. 6b). Thus, by comparison with published data on the vibrational spectra of carotenoids, it is seen that the weak band at 1442 cm^{-2} and the strong band at 1529 cm^{-1} correspond to mostly β-carotene modes, even though Chl *a* modes are also expected to appear at those frequencies.

The contributions from Cyt *b*-559 to the resonance Raman spectrum in Fig. 6a can be determined by monitoring shifts upon reduction of the heme. These changes are shown in Fig. 7. Fig. 7a shows the reso-

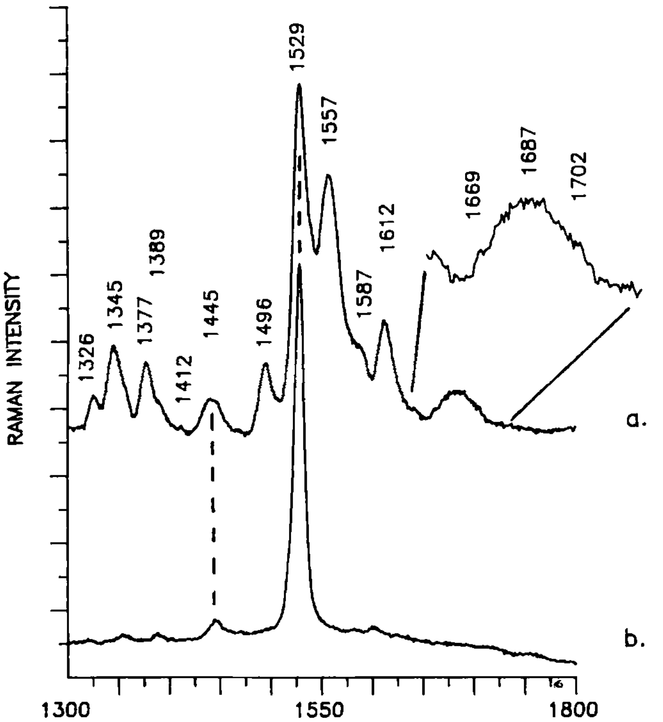

Fig. 6. Resonance Raman spectra of the D1-D2-Cyt *b*-559 complex with excitation at (a) 406.7 nm (10 mW) and (b) 488 nm (20 mW). Spectral resolution: 6 cm^{-1}; sample temperature: −140 ° C; sample concentration: 0.4 mg Chl *a* ml.

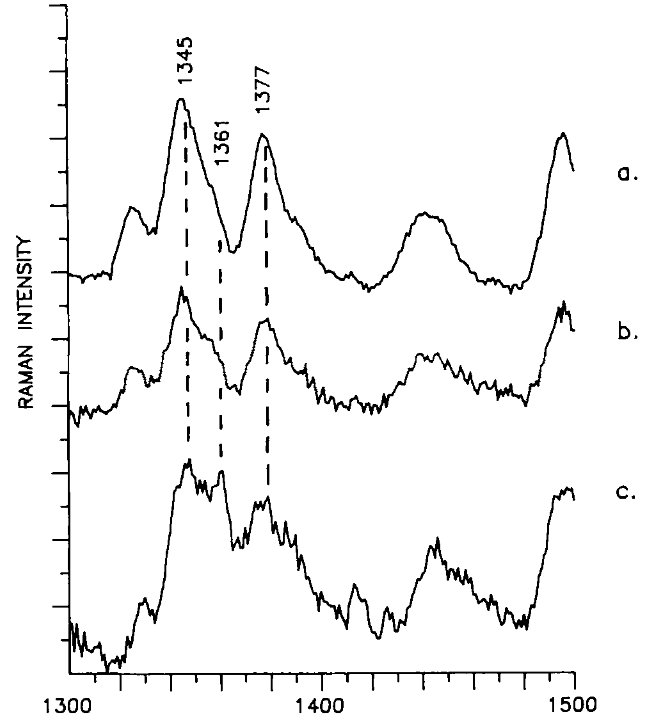

Fig. 7. Resonance Raman spectra of the D1-D2-Cyt *b*-559 complex with excitation at 406.7 nm: (a) sample treated with 5 mM Fe(CN)$_6^{3-}$; (b) no treatment; (c) sample treated with excess sodium dithionite. Instrument conditions as in Fig. 6.

nance Raman spectrum (406.7 nm excitation) of ferricyanide-treated D1-D2-Cyt *b*-559 complexes, where Cyt *b*-559 is oxidized. If no ferricyanide is present, a shoulder at 1360 cm^{-1} appears (Fig. 7b); this shoulder is cleanly resolved into a peak if the sample is reduced with excess sodium dithionite (Fig. 7c). These data can be interpreted in light of the results obtained by Babcock et al. [17] on the resonance Raman spectra of isolated low-potential Cyt *b*-559. They found that the oxidation state marker band, ν_4, of cyt *b*-559 shifted from 1372 to 1355 cm^{-1} upon reduction of the heme with dithionite. Furthermore, they observed that oxidized low-potential Cyt *b*-559 can be partially reduced in the presence of a laser beam. This photoreduction is apparent in Fig. 7b and further strengthens our assignment of the 1361 cm^{-1} band to ν_4 of reduced low-potential Cyt *b*-559. No other contributions to the resonance Raman spectrum of D1-D2-Cyt *b*-559 complexes from Cyt *b*-559 can be discerned by dithionite treatment. This is entirely reasonable, since the strongest band expected from Cyt *b*-559, ν_4 (see Ref. 17) is already weak in the resonance Raman spectrum of D1-D2-Cyt *b*-559 complexes.

Therefore, by singling out those peaks in the resonance Raman spectrum originating from β-carotene and Cyt *b*-559, we can make assignments for the chlorin pigments, Chl *a* and Pheo *a*. Table II summarizes our assignments, which are based on extensive studies performed on Chl *a* and Pheo *a* in solution and in the solid state (for reviews, see Lutz [20] and Tasumi and Fujiwara [21]). Several conclusions can be drawn from our data. First, it is well-established that several bands

TABLE II

Principal vibrational frequencies (cm^{-1}) for D1-D2-Cyt b-559 complexes

All stretching vibrations (denoted as ν) have mostly C = C character, except where noted. Assignments from Tasumi and Fujiwara [21] and Babcock et al. [17].

Raman shift (cm^{-1})	Assignment ^{a}
1326	Chl *a* and Pheo *a*
1345	Mostly Chl *a*
1361	Cyt *b*-559 (reduced, low-potential)
1377	Chl *a* and Cyt *b*-559 (oxidized, low-potential)
1389	Chl *a* - ν(C-H)
1412	Pheo *a*
1442	β-carotene and Chl *a*
1455	Pheo *a*
1496	Chl *a* and Pheo *a*
1529	β-carotene
1557	Chl *a*
1587	Mostly Pheo *a*
1612	Chl *a*
1669	Chl *a* and/or Pheo *a* - ν(C = O)
1687	Chl *a* and/or Pheo *a* - ν(C = O)
1702	Chl *a* and/or Pheo *a* - ν(C = O)

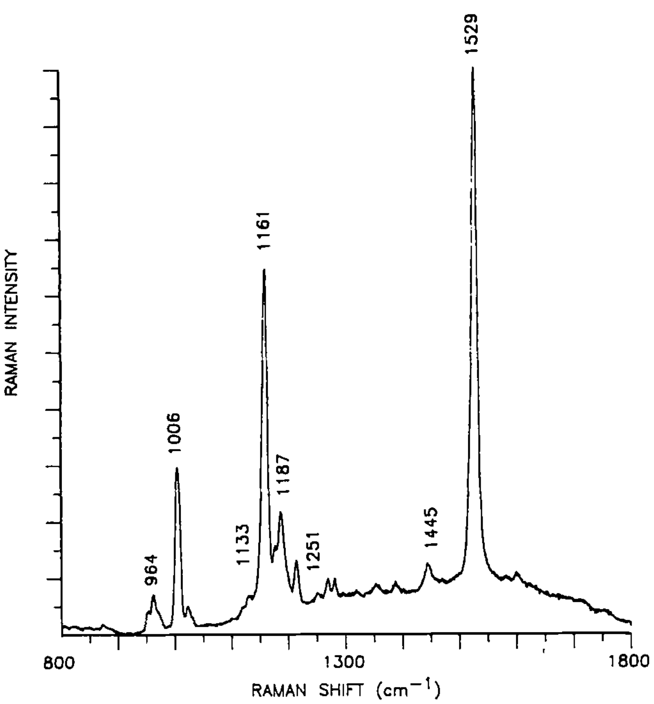

Fig. 8. Resonance Raman spectrum of the D1-D2-Cyt *b*-559 complex with excitation at 488 nm (20 mW). Instrument conditions as in Fig. 6.

in the vibrational spectra of Chl *a* can yield information on its coordination state [21]. Thus, the bands at 1557 and 1612 cm^{-1} are strongly indicative of five-coordination state for the central Mg^{2+} of Chl *a* in D1-D2-Cyt *b*-559 complexes; these models should downshift by about 6 and 12 cm^{-1}, respectively, if the central Mg^{2+} is six-coordinate [21]. Second, the carbonyl region of the resonance Raman spectrum of D1-D2-Cyt *b*-559 complexes is broad and appears to be composed of at least three peaks at 1669, 1687 and 1702 cm^{-1} The latter two peaks are near the free-vibrator frequencies for the C-9 keto carbonyl of Chl *a* and Pheo *a*, respectively. The lower frequency peak at 1669 cm^{-1}, however, cannot be assigned to a free keto carbonyl and is in the frequency range expected for a hydrogen-bonded C-9 keto carbonyl [22].

The resonance Raman spectrum of β-carotene can also provide valuable insight into its conformation in the reaction center. Fig. 8 shows a more extensive view of the resonance Raman spectrum of D1-D2-Cyt *b*-559 complexes upon excitation at 488 nm. The regions of the spectrum richest in structural information are around 1160–1190 cm^{-1} (the so-called ν_2 region) and around 1530 cm^{-1} (the so-called ν_1 region). Koyama et al. [23] have studied the vibrational properties of different conformers of β-carotene in hexane solutions, and have concluded that the ν_1 and ν_2 regions are very sensitive to the number and location of *cis* double bonds in the molecule. The resonance Raman spectrum of β-carotene in D1-D2-Cyt *b*-559 complexes is characterized by a ν_1 band at 1529 cm^{-1}, a weak band at 1177 cm^{-1}, and the absence of strong bands at 1060 and 1240 cm^{-1}. According to Koyama et al. [23], these features suggest an all-*trans* conformation for β-carotene. Hence, we conclude that β-carotene is in the all-*trans* form in D1-D2-Cyt *b*-559 complexes. This is surprising because it is believed that carotenoids are bound to bacterial reaction centers in a twisted 15-*cis* conformation [23,24]. It is only in light-harvesting proteins that carotenoids seem to assume the unfolded all-*trans* conformation [25].

Discussion

The procedure we have reported in this communication allows the isolation of both the 47 kDa polypeptide and the D1-D2-Cyt *b*-559 complex. Our method uses the non-ionic detergent dodecylmaltoside instead of Triton X-100, and the recovery of the 47 kDa and the D1-D2-Cyt *b*-559 complex under these conditions of separation and purification is accomplished with yields approaching 80%, based on the chlorophyll content of the starting material.

In developing a stepwise procedure for the isolation of the hydrophobic polypeptide components of the PS II core we have noted repeatedly that under our conditions of separation and purification, the 43 kDa Chl-binding protein behaves as if it were more loosely bound to the 'core' than the 47 kDa species. This is seen in the initial dissociation step, where the combination of dodecylmaltoside and $LiClO_4$ produces the 43 kDa species in a free, pure state after anion-exchange chromatography. In this first dissociation step, on the other hand, substantial amounts of the 47 kDa protein remain bound with the D1-D2-Cyt *b*-559 complex, and a second cycle of detergent/$LiClO_4$ exposure is necessary to effect a separation of the two species. These observations indicate that the 47 kDa protein is more intimately associated with the photochemical reaction center than is the 43 kDa; it is possible that in higher plants this association is essential for some aspect of electron transfer within the reaction center.

From this work and that of Akabori et al. [12], it is clear that the use of mild detergents, such as dodecylmaltoside and *n*-octyl glucopyranoside, results in preparations that are more stable than those prepared with Triton X-100 [10], at least in cases where the criterion for stability is the ability to generate the P-680 triplet. This increased stability makes the preparations more amenable to spectroscopic studies.

The observation that the D1-D2-Cyt *b*-559 complex shows the spin-polarized triplet strongly suggests, in agreement with Nanba and Satoh [10], that this complex contains the binding sites for P-680 and Pheo *a*. Since a parallel study of the 47 kDa species, under the same conditions, failed to demonstrate the P-680^{+} triplet even in the presence of a strong reductant such as dithionite, we believe that previous proposals linking the 47 kDa protein to the site of the reaction center function in PS II are incorrect.

The similarities in the primary reactions of PS II and purple bacteria, accompanied by the demonstrated similarity between the 32 and 34 kDa polypeptides of higher plants and the L and M subunits of purple bacteria, have led to the idea that the recently solved crystal structures of *Rps. viridis* [26] and *Rb. sphaeroides* [27] can be used as viable structural models for the reaction center of PS II. Although the similarities are undeniable and have been used effectively [28], there are also significant spectroscopic differences between the reaction centers of PS II and photosynthetic bacteria which pose some limits as to how far the analogy can be extended.

An analysis of the spin-polarized triplet of PS II reveals some of these differences. Our estimate of the zero-field splitting parameter for the P-680 triplet, $|D| = 0.0273$ cm^{-1}, qualitatively agrees with previous measurements in PS II preparations [11,18], and is consistent with a monomeric Chl *a* structure. It is also suggested [29] that the P-680 triplet is oriented parallel to the membrane. In contrast, the triplet from the reaction-center molecule of *Rb. sphaeroides*, P-860, which is oriented perpendicular to the membrane [30], showed a $|D|$ value consistent with a dimeric BChl *a* structure [19]. Therefore, the properties of the excited state of P-860 agree well with the properties of the ground state, which were determined by X-ray crystallography [27].

The properties of the triplet excited state of P-680$^+$ might reflect the properties of the ground state, as in *Rb. sphaeroides*. One would then infer from the data described above that P-680 is a Chl *a* monomer oriented parallel to the membrane. As pointed out by Rutherford et al. [18] and Levanon and Norris [31], however, a $|D|$ value similar to that of a monomer can be obtained if the axes of the two molecules in a dimer are parallel. Furthermore, the reaction-center molecule of *Rps. viridis*, which is a BChl *b* dimer in the ground state [26], has a triplet excited state with characteristics of a monomer, the spin localized to the BChl *b* closest to the BPheo *b* electron acceptor [32]. Thus, we cannot dismiss the possibility that P-680 is a Chl *a* dimer whose triplet is localized either on one of the Chl *a* molecules forming the putative special pair or on one of the so-called accessory chlorophylls that supposedly bridge the special pair and the Pheos (Rutherford, A.W., personal communication). The latter hypothesis is attractive in that it can explain the parallel orientation of the P-680 triplet in the membrane.

In an attempt to obtain more information on the ground state structure of Chl and Pheo in the PS II reaction center, we performed resonance Raman experiments. Fujiwara et al. (1987) have already studied the resonance Raman spectra of D1-D2-Cyt *b*-559 complexes prepared according to the Nanba and Satoh [10] protocol, with excitation at 441.6 nm. Experimentally, our studies differ from those of Fujiwara et al. [36] in that (i) our D1-D2-Cyt *b*-559 complexes are stable, as revealed by EPR (see above); and (ii) our choice of excitation wavelength (406.7 nm) allows for easy observation of chlorin and heme modes relative to β-carotene modes.

Extensive studies of the vibrational spectra of Chl *a* in different coordination and aggregation states allow us to pinpoint specific structural characteristics of the chlorin pigments in the PS II reaction center. The modes appearing at 1557 and 1612 cm^{-1}, which are indicative of a coordination number of 5 for Chl *a* in the reaction center, were mentioned in the previous section. In this respect, the bacterial and plant systems are similar because Zhou et al. [33] have shown that, at least in *Rb. sphaeroides* and *Rsp. rubrum*, the BChls are five-coordinate.

Koyama et al. [22] have studied the effect of solvents on the resonance Raman spectra Chl *a*. Their results are applicable to the analysis of the resonance Raman spectrum of the D1-D2-Cyt *b*-559 complexes with excitation in the Soret band. Particularly striking is the similarity of the spectrum presented in Fig. 6a with that which Koyama et al. [22] reported for a Chl *a* form seen in a dimethyl sulfoxide/water mixture where the dimethyl sulfoxide content was 10%. This Chl *a* form is characterized by: (i) a Q_y absorption maximum at 672 nm; (ii) five-coordination of the central Mg^{2+}, as evidenced by the bands at 1555 and 1611 cm^{-1}; (iii) a $C_9 = 0$ stretch at 1672 cm^{-1}, which reflects a hydrogen bond to a water molecule; and (iv) an overall vibrational pattern that is indicative of a monomer structure. Because Koyama et al. [22] used an excitation wavelength of 457.9 nm in their studies and we excited our PS II reaction centers at 406.7 nm, a direct comparison of relative intensities between spectra is not warranted. For this reason, we compare the peak positions of only those Raman bands of Chl *a* that are found both in solution and bound to PS II. Hence, inspection of the absorption and resonance Raman spectra of D1-D2-Cyt *b*-559 complexes (Figs. 4a and 6a, respectively) shows that the properties of the Chl *a* monomer in DMSO/H_2O closely match those of the Chl *a* molecules bound to the reaction center. The carbonyl region of the resonance Raman spectrum (Fig. 6a) shows some heterogeneity, however, as two free $C_9 = 0$ stretches are observed in addition to the H-bonded $C_9 = 0$ stretch. At this point of our investigation, it is impossible for us to discern clearly which chromophore is participating in this H-bond interaction. However, Zhou et al. [33] and Robert and Lutz [34] have observed similar H-bonding interactions in bacterial reaction centers and, by acquiring the resonance Raman spectra under varying illumination conditions, were able to assign the features to specific interactions between carbonyl groups of the special pair and the protein. On the other hand, Tavitian et al. [35], working with spinach PS II-enriched particles, have detected a positive 1665 cm^{-1} band in the difference FT-IR spectrum of P-680^+ – $Pheo^-$ minus P-680^+ – Pheo, possibly related to a negative band at 1681 cm^{-1}, which can arise from a hydrogen-bonded $C_9 = 0$ group of Pheo *a*. Clearly, more work is necessary in order to distinguish between these two possibilities.

With respect to β-carotene in the D1-D2-Cyt *b*-559 complexes, our results are consistent with an all-*trans* conformation for β-carotene in the PS II reaction center. This contrasts with the suggestion that carotenoids assume a *cis* conformation when bound to bacterial reaction centers [23,24]. It has been observed that, when dissociated from the reaction center, carotenoids undergo BChl-sensitized photoisomerization to the all-*trans* conformation (see, for example, Ref. 24). This raises two possible problems with respect to our conclusion that β-carotene is in the all-*trans* form in the PS II reaction center. First, through an artifact of the preparation, β-carotene may become partially uncoupled from the reaction center, thus undergoing photoisomerization from a *cis* to the all-*trans* conformation when exposed to 20 mW of 488 nm light from the Ar^+ laser. Second, the same photoisomerization effect could occur if one of the intrinsic properties of the PS II reaction center is that it binds β-carotene more loosely, and thus cannot withstand the actinic effect of the laser light. The above two points notwithstanding, we note that laser irradiation caused no deleterious effects and that, consequently, β-carotene was performing its function as a photoprotector of the chlorin pigments even in the all-*trans* conformation.

Comparing the resonance Raman studies presented here with those published by Fujiwara et al. [36], we conclude that the coordination state of Chl and the conformation of β-carotene are the same in D1-D2-Cyt *b*-559 complexes solubilized with either Triton X-100 (10,36) or dodecylmaltoside (this study). This indicates that the instability of Triton-solubilized preparations does not originate from changes in the structures of the pigments imparted by the isolation procedure.

Acknowledgements

This work was supported by grants from the National Science Foundation (DMB-8515932) to CFY and from the United States Department of Agriculture Competitive Grants Office and NIH (GM25480) to G.T.B. J.C.P. is supported by an NIH postdoctoral fellowship (GM12383).

References

1 Satoh, K. and Butler, W.L. (1978) Plant Physiol. 61, 373–379.
2 Satoh, K., Nakatani, H.Y., Steinback, K.E., Watson, J. and Arntzen, C.J. (1983) Biochim. Biohys. Acta 724, 142–150.
3 Diner, B.A. and Wollman, F.A. (1980) Eur. J. Biochem. 110, 521–526.
4 Ghanotakis, D.F., Demetriou, D.M. and Yocum, C.F. (1987) Biochim. Biophys. Acta 891, 15–21.
5 Nakatani, H.Y., Ke, B., Dolan, E. and Arntzen, C.J. (1984) Biochim. Biophys. Acta 765, 347–352.
6 Tang, X-S. and Satoh, K. (1984) Plant Cell Physiol. 25, 935–945.
7 Rutherford, A.W. (1986) Biochem. Soc. Trans. 14, 15–17.
8 Michel, H. and Deisenhofer, P. (1986) in Encyclopedia of Plant Physiology: Photosynthesis III (Staehelin, A.C. and Arntzen, C.J., eds.), pp. 372–381, Springer, Berlin.
9 Trebst, A. (1986) Z. Naturforsch. C. 41, 240–245.
10 Nanba, O. and Satoh, K. (1987) Proc. Natl. Acad. Sci. USA 84, 109–112.
11 Okamura, M.Y., Satoh, K., Isaacson, R.A. and Feher, G. (1987) in Progress in Photosynthesis Research (Biggins, J., ed.), Vol. I, pp. 379–381, Martinus Nijhoff, Dordrecht.
12 Akabori, K., Tsukamoto, H., Tsukihara, J., Nagatsuka, T., Motokawa, O. and Toyoshima, Y. (1988) Biochim. Biophys. Acta 932, 345–357.
13 Seibert, M., Picorel, R., Rubin, A.B. and Connolly, J.S. (1989) Plant Physiol. in press.
14 Ghanotakis, D.F. and Yocum, C.F. (1986) FEBS Lett. 197, 244–248.
15 Oertling, W.A. (1987) Ph.D. Thesis, Michigan State University.
16 Fujita, I., Davis, M.S. and Fajer, J. (1978) J. Am. Chem. Soc. 100, 6280–6282.
17 Babcock, G.T., Widger, W.R., Cramer, W.A., Oertling, W.A. and Metz, J.G. (1985) Biochemistry 24, 3638–3645.
18 Rutherford, A.W., Paterson, D.R. and Mullet, J.E. (1981) Biochim. Biophys. Acta 635, 205–214.
19 Thurnauer, M.C., Katz, J.J. and Norris, J.R. (1975) Proc. Natl. Acad. Sci. USA 72, 3270–3274.
20 Lutz, M. (1984) in Advances in Infrared and Raman Spectroscopy (Clark, R.J.H. and Hester, R.H., eds.), pp. 211–300, Vol. 11, J. Wiley & Sons, Heyden.
21 Tasumi, M. and Fujiwara, M. (1987) in Spectroscopy of Inorganic-Based Materials (Clark, R.J.H. and Hester, R.E., eds.), pp. 407–428, J. Wiley & Sons.
22 Koyama, Y., Umemoto, Y.and Akamatsu, A. (1986) J. Mol. Struc. 146, 273–387.
23 Koyama, Y., Takii, T., Kito, M., Saiki, K., Tsukida, K. and Yamashita, J. (1982) in Raman Spectroscopy - Linear and Nonlinear (Lascombe, J. and Huong, P.V., eds.), pp. 789–786, J. Wiley & Sons.
24 Lutz, M., Szponarski, W., Berger, G., Robert, B. and Neumann, J.-M. (1987) Biochim. Biophys. Acta 894, 423–433.
25 Lutz, M., Agalidis, I., Hervo, G., Cogdell, R.J.and Reiss-Husson, F. (1978) Biochim. Biophys. Acta 503, 287.
26 Deisenhoffer, J., Epp, O., Milk, K., Huber, R. and Michel, H. (1984) J. Mol. Biol. 180, 385–398.
27 Allen, J.P., Feher, G., Yeates, T.O., Komyia, H. and Rees, D.C. (1987) Proc. Natl. Acad. Sci USA 84, 5730–5734.
28 Debus, R.J., Barry, B., Babcock, G.T. and McIntosh, L. (1988) Proc. Natl. Acad. Sci. USA 85, 427–430.
29 Rutherford, A.W. (1986) Biochim. Biophys. Acta 807, 189–201.
30 Breton, J and Vermeglio, A. (1982) in Photosynthetic Energy Conversion in Plants and Bacteria (Govindjee, ed.) Vol. 1, pp. 153–194.
31 Levanon, H. and Norris, J.R. (1978) Chem. Rev. 78, 185–198.

32 Norris, J.R., Budil, D.E., Crespi, H.L., Bowman, M.K., Gast, P., Lin, C.P., Chang, C.H.a nd Schiffer, M. (1985) in Antennas and Reaction Centers of Photosynthetic Bacteria Structure, Interactions and Dynamics (Michel-Beyerle, M.E., ed.), pp. 147–149, Springer-Verlag, Berlin.

33 Zhou, Q., Robert, B. and Lutz, M. (1987) in Progress in Photosynthesis Research (Biggins, J., ed.) Vol. 1, pp. 395–398, Martinus Nijhoff, Dordrecht.

34 Robert, B. and Lutz, M. (1986) Biochemistry 25, 2303–2309.

35 Tavitian, B.A., Nabedryk, E., Mantele, W. and Breton, J. (1987) in Progress in Photosynthesis Research (Biggins, J., ed.), Vol. 1, pp. 261–264, Martinus Nijhoff, Dordrecht.

36 Fujiwara, M., Hayashi, H., Tasumi, M., Kanaji, M., Koyama, Y. and Satoh, K. (1987) Chem. Lett. 2005–2008.