Photosystem II of Green Plants: Topology of Core Pigments and Redox Cofactors As Inferred from Electrochromic Difference Spectra†

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ABSTRACT: Three electrochromic difference spectra induced by the deposition of (1) a negative charge on the primary quinone acceptor, Q_A, (2) a positive charge on (or near) Tyr161 of the D1 subunit (Y_Z), and (3) a positive charge on the manganese cluster were determined at room temperature in photosystem II (PSII) core particles from pea. They were deconvoluted into Gaussian components by Powell's numerical optimization procedure. All three spectra were fitted by four components, which we assigned to the Q_y absorption bands of two chlorophyll *a* molecules of the primary donor P, the accessory chlorophyll *a*, and the pheophytin *a* molecules on the D1 subunit. On the basis of the electrochromic properties of chlorins and our data, we suggest an arrangement of pigments and redox cofactors in PSII that differs from current structural models, which have been shaped like the reaction centers (RC) of purple bacteria. Our model is compatible with sequence data, with the spectroscopic and electrochemical properties of chlorophyll *a* and pheophytin *a*, and with the extremely positive redox potential of water oxidation. We conclude the following: (1) P is formed from two orthogonally oriented chlorophyll *a* molecules that peak at 681 and 677 nm. (2) The accessory chlorophyll *a* on D1 is oriented perpendicular to the membrane, with ring V pointing to Q_A. It is presumably attached to His118 of D1. (3) The mutual arrangement of pheophytin *a* on the D1 subunit and Q_A differs from that of their counterparts in bacterial RC. (4) The manganese cluster is located out of the axis that is formed by Y_Z (Tyr161 of D1), P, and Y_D (Tyr161 of D2).

Photosystem II of higher plants and cyanobacteria is a multisubunit protein−pigment complex that oxidizes two molecules of water and produces oxygen at the expense of four quanta of light. The minimal structures carrying out oxygen evolution, called core particles, contain more than 50 molecules of chlorophyll *a* per RC[1] (Ghanotakis et al., 1987). Further reduction to yield so-called D1D2 preparations is always accompanied by loss of the oxygen-evolving capacity, although these preparations promote the primary charge separation between a chlorophyll *a* moiety P (P680) and a molecule of pheophytin *a* (Nanba & Satoh, 1987; Tang et al., 1990). Besides the two polypeptides, D1 and D2 (both ∼40 kDa), the preparations contain 4−6 molecules of chlorophyll *a*, 2 of pheophytin *a*, and usually several smaller polypeptides of ∼5−10 kDa (Nanba & Satoh, 1987; Shuvalov et al., 1989; Tetenkin et al., 1989; Satoh, 1993; Seibert, 1993; Yruela et al., 1994; Tomo et al., 1995; Vacha et al., 1995). The primary and predicted secondary structures of D1 and D2 resemble the primary and secondary structures of the membrane-embedded L and M subunits of the photosynthetic reaction centers (RC) of purple bacteria (Michel et al., 1986; Trebst, 1986). The atomic structure of the latter was determined by X-ray diffraction for both *Rhodopseudomonas viridis* (Deisenhofer et al., 1984, 1995) and *Rhodobacter sphaeroides* (Allen et al., 1987; Chang et al., 1991; Ermler et al., 1994). Hydrophobicity plots predict that the D1 and D2 subunits are also formed from five transmembrane α-helices (Trebst, 1986). They are named A−E, each with a counterpart in the bacterial structure. Accordingly, it was proposed that the core of PSII is organized and functions in a similar way as the bacterial RC, with a structural pseudo-C_2-symmetry but functional asymmetry of its two electron transfer branches A (on D1) and B (on D2) (Trebst, 1986; Michel & Deisenhofer, 1988).

Photoexcitation and oxidation of P that is located close to the lumenal side of the thylakoid membrane results in transmembrane electron transfer via pheophytin *a* on branch A ($Pheo_A$) to the primary quinone Q_A and farther on to the secondary quinone acceptor Q_B. The electron vacancy on

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[1] Abbreviations: $\Delta\boldsymbol{\mu}$ ($\Delta\vec{\boldsymbol{\mu}}$), difference vector of permanent dipole moments of the ground and excited states of a chromophore; ADMR, absorbance-detected magnetic resonance; $BChl_A$, accessory chlorophyll on the L subunit of the bacterial RC; BP, primary donor of the bacterial RC; $BPheo_A$, pheophytin on the L subunit of the bacterial RC; BQ_A and BQ_B, primary and secondary quinone acceptors of the bacterial RC, respectively; Chl_A, accessory chlorophyll on the D1 subunit of PSII; ENDOR, electron nuclear double resonance; FTIR spectroscopy, Fourier transform infrared spectroscopy; INDO/s, spectroscopic intermediate neglect of differential overlap, a semiempirical molecular orbital method parametrized for porphyrins; P, primary donor of PSII; $Pheo_A$, pheophytin on the D1 subunit of PSII; PSI, photosystem I; PSII, photosystem II; Q_A and Q_B, primary and secondary quinone acceptors of PSII, respectively; RC, photosynthetic reaction center; Y_Z and Y_D, redox-active Tyr161 on the D1 and D2 subunits of PSII, respectively.

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P is filled in nanoseconds (Schlodder et al., 1984) by an electron from a redox-active tyrosine Y_Z [Tyr161 on the D1 subunit (Barry & Babcock, 1987; Debus et al., 1988)]. The latter is in turn reduced in micro- to milliseconds by the manganese cluster that catalyzes water oxidation (Dekker et al., 1984b). Clocked by quanta of light, the Mn cluster cycles through states $S_0 \rightharpoonup S_1 \rightharpoonup S_2 \rightharpoonup S_3 \rightharpoonup S_4 \rightarrow S_0$, with oxygen evolution associated with the last transitions $S_3 \rightharpoonup S_4 \rightarrow S_0$ (Joliot & Kok, 1975).

There is consensus on the location of Y_Z and its counterpart Y_D (Tyr161 on D2) close to P, supposedly in positions that are occupied by L-Arg135 and M-Arg162 (or M-His162 in some bacteria) on the L and M subunits of the bacterial RC (Barry et al., 1990; Vermaas et al., 1993). This position is compatible with the similarity between the estimated $Y_Z \rightharpoonup P$ distance (10−15 Å; Hoganson & Babcock, 1989; Rutherford & Boussac, 1992) and the actual distance of 13 Å between L-Arg135 and the nearest bacteriochlorophyll of the special pair (Deisenhofer et al., 1984, 1995) and with the data on the electrogenicity of the $Y_Z \rightharpoonup P^+$ electron transfer (Pokorny et al., 1994). The location of the Mn cluster, however, has remained enigmatic. Estimates based on EPR (Hoganson & Babcock, 1989; Kodera et al., 1994) place it closer to Y_Z than the Y_D, but do not provide the exact position.

The current models of PSII topology (Svensson et al., 1992; Ruffle et al., 1992) were obtained by energy minimization algorithms that were applied to polypeptide chains of the D1 and D2 subunits under the assumption that the innermost four chlorophylls *a* and two pheophytins *a* occupy the same positions as their bacterial counterparts. There are some inconsistencies in this approach. In the bacterial RC, electron transfer from the primary donor to bacteriopheophytin is mediated by the accessory bacteriochlorophylls that are shown in Figure 3a (Shuvalov et al., 1978; Holzapfel et al., 1990; Shuvalov, 1993). As already noted by Michel and Deisenhofer (1988), the supposed domains containing the accessory chlorophylls on D1 and D2 lack the coordinating histidine residues that are present in bacteria. Moreover, the absence of strong excitonic coupling in PSII, in contrast to its presence in bacterial RCs and PSI (van der Vos et al., 1992), invokes the possibility of an orthogonal rather than a parallel orientation of the chlorophyll *a* rings that form P (Tetenkin et al., 1989; Noguchi et al., 1993).

In this work, we investigated the topology of pigments and redox cofactors of PSII by measuring and analyzing the electrochromic response of its innermost chlorins to a negative charge placed on Q_A and to positive charges placed on Y_Z or on the Mn cluster (during transition $S_1 \rightharpoonup S_2$). That Q_A^- is an anion radical is well established from the EPR data (Nugent et al., 1981; Rutherford & Mathis, 1983). The oxidation of Mn upon the $S_1 \rightharpoonup S_2$ transition is also demonstrated (Dismukes & Siderer, 1981; Sauer et al., 1992). The oxidized Y_Z (which we denote as Y_Z^+ for simplicity), on the one hand, is a neutral radical according to EPR (Babcock et al., 1989). On the other hand, the appearance of a transient electrochromic band shift concomitant with the oxidation of Y_Z in nanoseconds and its reduction in micro- to milliseconds is also established (Lavergne, 1991). The positive charge apparently resides in the vicinity of Y_Z, similar to the proton rocking that has been proposed to follow the oxidation of Y_D (Rodriguez et al., 1987; Mino et al., 1993).

Figure 1 illustrates the structure of chlorophyll *a* that is

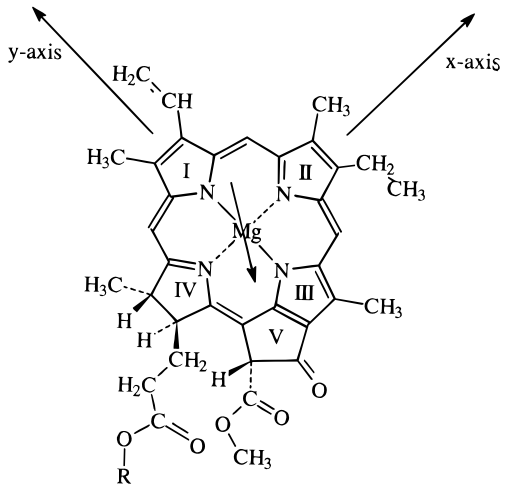

FIGURE 1: Structure of chlorophyll *a*. The orientation of the difference in the dipole moments between the first excited and ground states ($\Delta\boldsymbol{\mu}$) is indicated by an arrow (Lockhart & Boxer, 1987; Krawczyk, 1994).

asymmetric due to an additional ring V. Because of this asymmetry, the INDO/s calculations for bacteriochlorophylls *a*, *b*, and *g*, bacteriopheophytins *a* and *b*, and chlorophyll *a* (Hanson et al., 1987a,b; Fajer et al., 1992) predict a blue shift of the red spectral maximum of the chlorin if a positive charge is placed near ring III and a red shift for a positive charge at the opposite side near ring I. Inversion of the charge polarity reverses the direction of the shift. Placement of the probe charge above the ring or close to the line of the Q_x transition that connects rings II and IV causes much smaller electrochromic shifts of the Q_y band. The predicted directionality has been verified experimentally by comparison with spectra of derivatized chlorins [see Fajer et al. (1992) and references therein] and with electrochromic shifts in the RC of *Rps. viridis* (Hanson et al., 1987a,b). (See Appendix 1 for further discussion of the electrochromism of chlorins.)

In this work, we measured the difference spectra of the electrochromic band shifts in PSII. We concentrated on difference spectra in the red spectral region. Their counterparts in the blue are more complex and more difficult to interpret (Hanson, 1991). Electrochromic band shifts have been reported previously by various authors for different organisms and preparations. In this work, we present a coherent set of electrochromic difference spectra that was obtained in one and the same PSII preparation, namely, oxygen-evolving core particles from pea chloroplasts. We deconvoluted these spectra to yield the relative contributions of the innermost chromophores of PSII. The results of this deconvolution compared well with recently published spectra of charge-separated states in the oxygen-evolving core particles (Van Mieghem et al., 1995; Hillmann et al., 1995; Hillmann & Schlodder, 1995).

On the basis of the deconvolution of electrochromic difference spectra and their analyses, we modeled the arrangement of pigments and cofactors in PSII with the following main features: (1) The accessory chlorophylls are more remote from P than their counterparts in the bacterial RC. They are supposedly bound to the conserved His118 of helix B of the D1 and D2 subunits. At least the accessory chlorophyll on D1 is perpendicular to the membrane plane. (2) The mutual arrangement of $Pheo_A$ and Q_A differs from that of their bacterial counterparts. (3) The two chlorin rings

that form P are perpendicular, rather than coplanar as in bacteria. (4) The Mn cluster is located out of the axis formed by Y_Z, P, and Y_D.

MATERIALS AND METHODS

Oxygen-evolving PSII core particles were prepared from 12-day-old pea seedlings according to Ghanotakis et al. (1987), with the modifications outlined by Lübbers and Junge (1990). They were stored at −80 °C in a medium containing 10 mM $CaCl_2$, 5 mM MES (pH 6.0), and 400 mM sucrose until use. Prior to the measurements, the particles were suspended at 5 μM chlorophyll in a medium that contained 2 mM MES (pH 6.5) and 5 mM $CaCl_2$.

Flash spectrophotometric measurements were performed as described previously (Junge, 1976). The measuring light was passed through a monochromator, which was calibrated with the beam of a HeNe cw laser (633 nm). Saturating exciting flashes were provided by a xenon lamp (FWHM = 10 μs) filtered through Schott BG39+WB 560. For measurements with dark-adapted material, every train of flashes was recorded with a fresh sample that was automatically filled into the cuvette (optical path length = 1 cm) from a light-shielded reservoir. Samples were dark adapted for 15 min. The transients were digitized and averaged on a Nicolet Pro92 recorder and stored on a MicroVax computer. Typically 10 transients were averaged to improve the signal to noise ratio.

For some experiments the Mn cluster was deactivated by incubating the core particles with Tris according to the following protocol: Core particles were thawed and suspended in a medium containing 5 mM $CaCl_2$ and 800 mM Tris (pH 8) for 5 min at room temperature. They were collected by centrifugation (30000*g*, 30 min, 4 °C) and resuspended in 2 mM MES (pH 6.5) and 5 mM $CaCl_2$ prior to the measurements. After this treatment, the rate of oxygen evolution under continuous illumination was less than 10% that of the control (~1300 μmol of O_2 per mg of chlorophyll per h).

Flash-induced absorption transients were recorded over a wavelength interval ranging from 610 to 730 nm. Contributions of chlorophyll fluorescence were suppressed by spatial filtering and by the use of the strong fluorescence quencher DCBQ as the electron acceptor (Lavergne & Leci, 1994). As none of the long-lived redox cofactors (Q_A, Y_Z, and Mn cluster) had any appreciable absorption in this region, the transients were of purely electrochromic origin.

Several procedures, partly established in the literature (Dekker et al., 1984a,b; Lavergne, 1991), were used to obtain the particular difference spectra. (1) The difference spectrum attributable to the charging of the Mn cluster during the transition $S_1 \rightleftharpoons S_2$ was obtained from the extent of absorption transients taken 30 ms after a first flash, which was applied to dark-adapted oxygen-evolving samples in the presence of 100 μM DCBQ plus 1 mM hexacyanoferrate(III) as electron acceptors. Under these conditions, Q_A^- decays in less than 5 ms according to our observations (Bögershausen & Junge, 1995). The S_2 state, on the other hand, is stable for at least 2 s (Lübbers et al., 1993). By monitoring absorption transients at 320 nm induced by a train of nine flashes as described previously (Lübbers et al., 1993), we confirmed that about 90% of the centers were in S_1 and 10% in S_0 prior to the first flash. (2) The spectrum of the electrochromic response to Q_A^- was obtained as the difference between the spectrum of transient taken 5 ms after the first flash in the presence of 50 μM DCBQ plus 50 μM DCMU (under these conditions Q_A^- decays only slowly) and spectrum 1, as recorded with dark-adapted material. Alternatively, the $Q_A^- - Q_A$ difference spectrum was obtained from the extents of transients measured 5 ms after a flash under repetitive excitation (1 Hz) in the presence of only 35 μM DCBQ. Both methods yielded essentially the same spectrum. (3) The $Y_Z^+ - Y_Z$ spectrum was obtained with Tris-washed material in the presence of 100 μM DCBQ plus 1 mM hexacyanoferrate(III). The extent was determined 30 ms after a flash under repetitive excitation at low frequency (0.1 Hz). Under the given electron acceptor conditions, the half-time of the Y_Z^+ rereduction was about 300 ms. It is noteworthy that the similar spectrum (shown in Figure 1b) was obtained 2 ms after the third flash in *oxygen-evolving* core particles prepared according to van Leeuwen et al. (1991), in which the $S_3 \rightleftharpoons S_0$ transition proceeds more slowly than in the core particles prepared according to Ghanotakis et al. (1987), with a half-rise time of about 4.5 ms (Haumann et al., 1994). At 2 ms after the third flash, about 45% of the centers still contained Y_Z^+, starting with about 60% of S_3 prior to this flash (Bögershausen & Junge, 1995). The spectrum at 2 ms was about 2 times smaller than that obtained with Tris-washed material. From these results, we concluded that more than 90% of centers created Y_Z^+ in the latter material on the first flash. For the fittings of the spectra we took the raw extents of all spectra. The small scaling factors of about 1.2 and 1.1 for S_1 and Y_Z, respectively, were neglected (they were due to a miss factor of 0.1 and a 10% proportion of centers in the S_0 state prior to the flash).

The extents were calibrated in terms of extinction coefficients ($\Delta\epsilon$) by the following procedure. Absorption transients due to Q_A^- formation at 320 nm were measured under repetitive flash excitation (1 Hz). By taking a figure of $\Delta\epsilon_{320} = 13\ mM^{-1}\ cm^{-1}$ for $Q_A^- - Q_A$ from the literature (van Gorkom, 1974), we calculated a chlorophyll to reaction center ratio of 120:1 for our preparation. This ratio was used to calculate the extinction coefficients of electrochromism in the red spectral region.

The deconvolution of an experimental difference spectrum of electrochromic origin, which is composed of several different band shifts, involves four parameters for each spectral component, namely, the peak wavelength λ_0, the peak extinction coefficient A, the spectral band width w (the full widths of spectral bands at 1/e of their height are considered throughout this work), and the extent of the band shift $\Delta\lambda$. To simplify the calculation, we assumed that the Q_y band of each individual chromophore could be described by a single Gaussian both *in vitro* and *in vivo*. This is a fair approximation at room temperature. We also assumed equal oscillator strengths *in vitro* and *in vivo* for all chlorophyll *a* and pheophytin *a* molecules, independent of their exposure to an electric field. This reduced the number of variables for each component to three parameters, λ_0, w, and $\Delta\lambda$. The values of A_0 and w_0 in solution that were used in the calculations were 89 $mM^{-1}\ cm^{-1}$ and 25 nm for chlorophyll *a* and 57 $mM^{-1}\ cm^{-1}$ and 19 nm for phenophytin *a*, respectively (Goedheer, 1966). We took the Q_y absorption bands as symmetrical Gaussians:

$$A(\lambda) = (A_0 w_0/w)\ \exp(-4(\lambda - \lambda_0)^2/w^2) \qquad (1)$$

Here A_0 and w_0 are the *in vitro* values (see above), whereas

w is the band width *in vivo*. We neglected the asymmetry that may arise from a possible heterogeneity of the environment and from the internal properties of complex aromatic molecules.

The experimental electrochromic difference spectra, $B_k(\lambda_i)$, that have been measured with the electric charges located in three different positions in PSII ($k = 1-3$) at discrete wavelengths λ_i ($i = 1, 2, ..., n$) can be described as a superposition of electrochromic band shifts of independent pigment components ($j = 1, 2, ..., m$). If the absorption band of a given component j is Gaussian with center wavelength λ_j^0 and band width w_j, and if the band shift $\Delta\lambda_{kj}$ is small, then its contribution to the difference spectrum k is given by the first derivative of $A_j(\lambda)$ from eq 1:

$$\Delta A_{kj}(\lambda_i) = \frac{8A_j(\lambda_i)}{w_j^2}(\lambda_i - \lambda_j^0)\Delta\lambda_{kj} \equiv C_j(\lambda_i)\Delta\lambda_{kj} \quad (2)$$

The task is to find those values of λ_j^0, w_j, and $\Delta\lambda_{kj}$ that minimize the square dispersion S of the calculated versus the experimental spectra:

$$S = \sum_{k=1}^{3}\sum_{i=1}^{n}[B_k(\lambda_i) - \sum_{j=1}^{m}C_j(\lambda_i)\Delta\lambda_{kj}]^2 \quad (3)$$

From a mathematical point of view it is the usual case of an optimization problem with mixed types of variables: band shifts $\Delta\lambda_{kj}$ enter linearly in this expression, whereas peak positions λ_j^0 and their band widths w_j enter nonlinearly. The optimum relative to the linear parameters $\Delta\lambda_{kj}$ results from the usual "minimal squares" procedure [see, for example, Seber (1977)]:

$$\frac{\partial S}{\partial \Delta\lambda_{kj}} = 0 \quad (4)$$

For each $k = 1-3$, eq 4 yields a system of m linear equations:

$$\begin{cases} \sum_{j=1}^{m}\sum_{i=1}^{n}C_1(\lambda_i)C_j(\lambda_i)\Delta\lambda_{kj} = \sum_{i=1}^{n}C_1(\lambda_i)B_k(\lambda_i) \\ \sum_{j=1}^{m}\sum_{i=1}^{n}C_2(\lambda_i)C_j(\lambda_i)\Delta\lambda_{kj} = \sum_{i=1}^{n}C_2(\lambda_i)B_k(\lambda_i) \\ \vdots \qquad\qquad \vdots \\ \sum_{j=1}^{m}\sum_{i=1}^{n}C_m(\lambda_i)C_j(\lambda_i)\Delta\lambda_{kj} = \sum_{i=1}^{n}C_m(\lambda_i)B_k(\lambda_i) \end{cases} \quad (5)$$

For each $k = 1-3$, the values of $\Delta\lambda_{k1}$, $\Delta\lambda_{k2}$, ..., $\Delta\lambda_{km}$ are determined as functions of λ_j^0 and w_j by sequential exclusion of the variables. After this step, the square dispersion is minimized further by varying λ_1^0/w_1, λ_2^0/w_2, ..., λ_m^0/w_m. For this purpose, we chose the nonlinear searching, nongradient procedure known as Powell's algorithm (Powell, 1970). This method converges rapidly even for a large number of variables (Gill et al., 1981). A working FORTRAN program of this algorithm has been published (Himmelblau, 1972). We installed it with modifications on an IBM-compatible PC-486.

Sequence Comparison. For the alignment of amino acid sequences and predictions of the secondary structure, we used PREDICTPROT mail server of EMBL (Heidelberg, Germany) described in Rost et al. (1994). The algorithms of the secondary structure prediction are described in Rost and Sander (1993, 1994). The nucleotide sequences were aligned with the program PileUp, which is based on an algorithm described in Feng and Doolitle (1987). The program is a part of the commercially available Wisconsin Sequence Analysis Package from Genetic Computer Group described in Devereux et al. (1984). Version 8 of the package was used.

RESULTS

Electrochromic Difference Spectra in PSII Core Particles. The spectra of electrochromic absorbance transients attributable to the reactions $Q_A \rightleftharpoons Q_A^-$, $Y_Z \rightleftharpoons Y_Z^+$, and $S_1 \rightleftharpoons S_2$ are given in Figure 2a−c. The following gross features are immediately obvious: (1) the response to Q_A^- formation is composed of a red shift centered at about 670 nm and a blue shift centered at about 685 nm; (2) the response to Y_Z^+ formation is a red shift centered at about 678 nm; and (3) the response to the $S_1 \rightleftharpoons S_2$ transition is mainly a blue shift at about 681 nm.

Spectral Properties of the Innermost Chlorins of PSII. In photosynthetic bacteria, the relevant electrochromic shifts have been recorded in response to the deposition of a negative charge on the bacteriopheophytin molecule on the L subunit that corresponds to branch A of PSII (further named $BPheo_A$ for simplicity) (Shuvalov et al., 1976) and on the primary and secondary quinone acceptors (denoted as BQ_A and BQ_B) (Vermeglio & Clayton, 1977; Vermeglio, 1982; Shopes & Wraight, 1985; Tiede & Hanson, 1992; Steffen et al., 1994). One remarkable feature in our context is the good agreement between the spectra obtained in isolated reaction centers and in chromatophores that contained the antennae complement (Vermeglio, 1982; D. Tiede, personal communication). Their similarity implies that the electrochromic response is predominantly attributable to the innermost pigments and that the contribution from antennae pigments, as well as the electrostatic distortion by the protein periphery, is negligible. We assume that the same holds true for the more complicated structure of PSII.

In PSII the number of these innermost pigment molecules and the assignment of the respective peak wavelengths are both points of contention. The minimal system, D1D2 complex, seems to be less well characterized than bacterial reaction centers. Heterogeneities of the preparations between different laboratories might explain some of the differences between the reported results. However, gross features seemed to be found consistently in D1D2 complexes, as in core complexes and thylakoids: (1) The peak wavelength of the accessory chlorophyll *a* moiety in D1D2 complexes is expected in the spectral range 666−670 nm, according to spectral deconvolution of absorption spectra of the D1D2 complexes (Shuvalov et al., 1989; Tetenkin et al., 1989; Otte et al., 1992). (2) The pheophytin in branch A ($Pheo_A$) could be expected to contribute in the range 682−685 nm. Its photoreduction in native thylakoid membranes (Ganago et al., 1982), oxygen-evolving PSII core complexes (Hillmann et al., 1995), and a D1D2 preparation (Barber & Melis, 1990) was accompanied by bleaching around 685 nm. By polarized spectroscopy of native thylakoid membranes (Ganago et al., 1982), the absorption transients were deconvoluted into the bleaching of a component with its Q_y axis perpendicular to the membrane plane ($Pheo_A$ with $\lambda_{max} \sim 681$ nm) and the blue shift of a component peaking at about 680 nm with Q_y

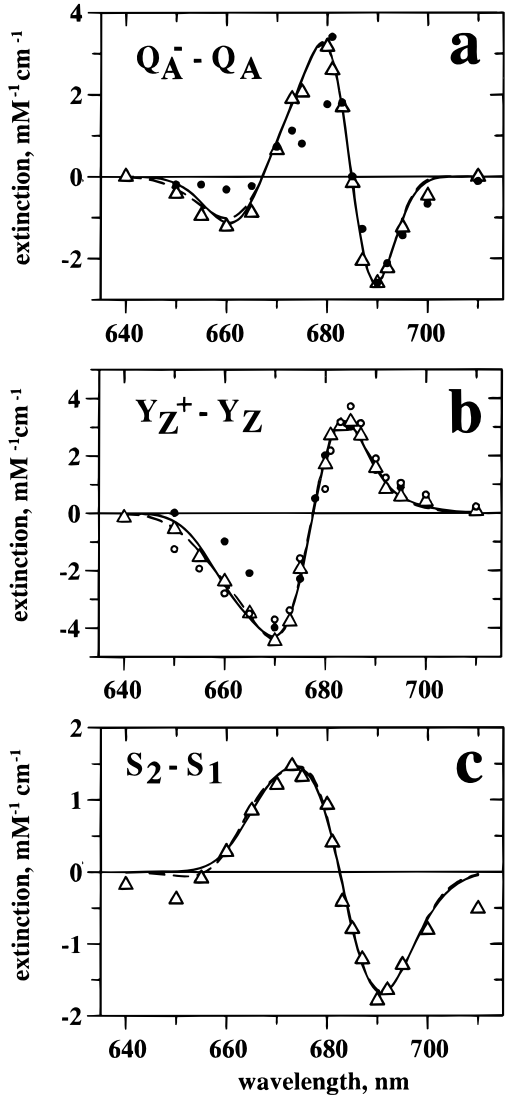

FIGURE 2: Electrochromic difference spectra obtained in core particles of PSII. Triangles are experimental data. They were fitted by three and four spectral components (dashed and solid lines, respectively). The three-component-fit curves were composed of Gaussians peaking at 667.3, 676.3, and 684.6 nm that could be tentatively attributed to the accessory chlorophyll, P, and $Pheo_A$, respectively. The four-component fit was composed of Gaussians peaking at 667.3, 677.1, 680.5, and 684.7 nm and attributed to the accessory chlorophyll, P_B, P_A, and $Pheo_A$ (see Table 1). (a) $Q_A^- - Q_A$ (△): Our data measured in the presence of 50 μM DCBQ as the electron acceptor and at the same time as the fluorescence quencher plus 50 μM DCMU. The spectrum was stable against higher DCBQ concentrations. The data from Velthuys (1988) were plotted for comparison (●, normalized at 690 nm). The corresponding spectrum in the blue region was also obtained. It was characterized by maxima at 415 and 445 nm and by a minimum at 430 nm (not shown), in good correspondence with the previously published values (Van Gorkom et al., 1982; Dekker et al., 1984a; Velthuys, 1988; Lavergne, 1991). (b) $Y_Z^+ - Y_Z$ (△): Our data with 100 μM DCBQ plus 1 mM hexacyanoferrate. (●): Spectrum obtained by Dekker (1985). (○): Our data with core particles [prepared according to van Leeuwen et al. (1991), see Materials and Methods; normalized at 665 nm]. The corresponding spectrum in the blue region (not shown) was characterized by a minimum at 430 nm and a maximum at 440 nm, in good correspondence with the previously reported values (Dekker et al., 1984a; Schatz & van Gorkom, 1985). (c) $S_2 - S_1$ (△): Our data with 100 μM DCBQ plus 1 mM hexacyanoferrate. The corresponding spectrum in the blue region (not shown) was characterized by a main minimum at 425 nm and a maximum at 440 nm, in good correspondence with the previously reported values (Dekker et al., 1984a,b; Velthuys, 1988; Lavergne, 1991).

oriented in the membrane plane. The latter component was later attributed to P (Schlodder & Brettel, 1988; Breton, 1990). The unexplained asymmetry of this blue shift leaves room for the following alternative interpretation: $Pheo_A$ is somewhat inclined to the membrane plane (Breton, 1990) and causes the asymmetry of the spectral shift at 680 nm. The peak position of $Pheo_A$ can then be reestimated as ~683 nm. This value equals the peak position of pheophytin in chemical bleaching experiments with a D1D2 preparation (Tetenkin et al., 1989). (3) The primary donor P could be expected to contribute around 680 nm at room temperature, as its oxidation causes photobleaching at this wavelength (Doering et al., 1969; Schlodder et al., 1984). Hillmann et al. (1995) recently have shown that the 680 nm bleaching band observed at room temperature splits at cryogenic temperatures. These authors considered the possibility that P is formed from two chlorophyll *a* molecules: one that peaks at 685 nm and houses the triplet state, and thus the positive charge in P^+, at 25 K [P_A; see also Carbonera et al. (1994)] and its counterpart, P_B, that peaks at 676 nm at this temperature.

Fit of the Electrochromic Difference Spectra by Three and Four Gaussian Components. By analogy with the electrochromic difference spectra in photosynthetic bacteria (Vermeglio & Clayton, 1977; Vermeglio, 1982; Shopes & Wraight, 1985; Tiede & Hanson, 1992), we assumed that the electrochromic band shifts were caused only by four chlorins that are bound to the D1D2 dimer, namely, by two chlorophyll *a* molecules forming P, the accessory chlorophyll *a* and pheophytin *a*. Fitting of k difference spectra with m Gaussians requires $2m$ nonlinear (namely, λ_j^0, w_j) plus km linear (namely, $\Delta\lambda_{kj}$) free fit parameters, totaling $N = (k + 2)m$. To narrow the field of search, we started by fitting the spectra with only three Gaussians. The results of this fit were singular (average dispersion = 0.238 mM^{-1} cm^{-1}); they are shown as dashed lines in Figure 2. The fitting curves were contributed by Gaussian components peaking at 667.3, 676.3, and 684.6 nm, which could be tentatively attributed to the accessory chlorophyll *a*, P, and $Pheo_A$, respectively. Although the fit was fair numerically, it was not satisfactory for two reasons. (1) There was a component peaking at 676 nm that could be tentatively attributed to P, but the expected electrochromic contribution at 680 nm, which was described elsewhere (Ganago et al., 1982), was lacking. (2) The positive charge on the Mn cluster induced a 3 times larger electrochromic band shift of $Pheo_A$ at 684.6 nm ($\Delta\lambda = -0.169$ nm) than of the assumed P component at 676 nm ($\Delta\lambda = -0.065$ nm) that was rather unlikely, as the Q_x transition of $Pheo_A$ at 545 nm was shown to be completely insensitive to the electric charges in the water-splitting system (Dekker, 1985). Therefore, we reiterated the fit routine but with four Gaussian components. This improved the quality of the fit, but the singularity was lost (9 nonlinear and 12 linear parameters were varied simultaneously; not shown). To reduce the number of parameters, we used a two-step approach that was based on the finding that, according to the results of the three-component fit, the response to the formation of Q_A^- was due to only two components. They were tentatively attributed to the accessory chlorophyll *a* and pheophytin *a*; the contribution from the third component (P) was negligibly small ($\Delta\lambda = +0.001$ nm). So on the first step the $Q_A^- - Q_A$ spectrum in Figure 2a was analyzed under the assumption that it was composed of only two components. The results of this fit were singular with an average

Table 1: Fit Parameter Set Resulting from the Deconvolution of the Electrochromic Difference Spectra in Figure 2a−c into Four Components

		relative contribution to spectra			
peak position λ_0 (nm)	bandwidth w (nm)	$Q_A^- - Q_A$ $\Delta\lambda$ (nm)	$Y_Z^+ - Y_Z$ $\Delta\lambda$ (nm), [P^+ (%)][a]	$S_2 - S_1$ $\Delta\lambda$ (nm)	attributed to
667.3	15.7	+0.088	+0.141	+0.015	Chl_A
677.1	14.4	0	+0.252	+0.045	P_B
680.5	20.9	0	+0.125, [1.44]	−0.269	P_A
684.6	11.7	−0.233	+0.006	−0.002	$Pheo_A$

[a] The admixture of a bleaching of P was chosen to account for the asymmetry of the difference spectrum in Figure 2b.

dispersion of 0.210 mM^{-1} cm^{-1}; they are shown as solid line in Figure 2a and the fit parameters are included in Table 1. The peak wavelength and width of these two components were used as fixed starting values for the simultaneous analysis of two remaining difference spectra ($Y_Z^+ - Y_Z$ and $S_1 - S_2$). The results of this second fit were also singular with an average dispersion of 0.171 mM^{-1} cm^{-1}. The fit curves are shown as solid lines in Figure 2b,c and the fit parameters are given in Table 1.

DISCUSSION

Electrochromic Band Shifts in PSII

The electrochromic difference spectrum of the reaction $Q_A \Rightarrow Q_A^-$ in the red is shown in Figure 2a (triangles). Its shape is in line with previously reported ones (van Gorkom, 1974; Klimov et al., 1977; Dekker, 1985; Schatz & van Gorkom, 1985). Only one published spectrum was slightly different (Velthuys, 1988). It was obtained with BBY membranes from spinach thylakoids and is replotted in Figure 2a (dots) for comparison. The major difference is a local minimum at 676 nm instead of a shoulder. This local minimum was readily simulated by our fitting routine by slightly varying the relative contributions of the blue shift at 684.7 nm and the red shift at 667.3 nm (not shown). The variability of the relative contributions of various pigments paralleled the situation in PSI where the electrochromic response to $P700^+$ formation varied between different preparations [see Figure 3 in Schaffernicht and Junge (1982)]. Despite these differences, all literature spectra [including Velthuys's (1988)] and our work showed that deposition of an electron on Q_A caused a blue shift of $Pheo_A$ and a smaller red shift of the accessory chlorophyll *a* (Figure 2a). This behavior drastically differed from that observed in the bacterial RCs. $BPheo_A$ was red shifted in the RC of *Rps. viridis* (Shopes & Wraight, 1985), *Rb. sphaeroides* (Vermeglio & Clayton, 1977; Vermeglio, 1982), *Rb. capsulatus* (Tiede & Hanson, 1992), *Rhodospirillum rubrum* (Vermeglio, 1982), and even *Chloroflexus aurantiacus* (Vasmel & Amesz, 1983). This is indicative of a similar mutual arrangement of $BPheo_A$ and BQ_A in all of these bacteria and a different one in PSII.

The difference spectrum attributable to the reaction $Y_Z \Rightarrow Y_Z^+$ in the Tris-washed material is shown in Figure 2b (triangles). We obtained very much the same spectrum (Figure 2b, open circles) with oxygen-evolving core particles, 2 ms after the third flash (see Materials and Methods). The shapes of both are similar to that obtained by Dekker (1985) with thylakoid membranes (Figure 2b, dots). The asymmetry of the $Y_Z \Rightarrow Y_Z^+$ spectrum (Figure 2b, triangles) is readily understood as a composite from red shifts centered at 667.3, 677.1, and 680.5 nm plus an admixture of 1.44% bleaching of P at 680.5 nm (Table 1). The latter contribution is expected if the midpoint potentials of P and Y_Z differ by 110 mV, a figure that agrees well with the reported estimate of 100 mV for this potential gap (Metz et al., 1989). The resulting red shifts of the spectral components that we attributed to P (677.1 and 680.5 nm) were opposite the expectations based on the analogy with bacterial RC. L-Arg135, which takes the position of Y_Z in the bacterial RC, lies almost in the line of the Q_y transitions of both bacteriochlorophylls of the special pair (Deisenhofer et al., 1995). If two chlorophyll *a* molecules were placed similar to their bacterial counterparts and a positive charge was placed at the position of L-Arg135, the latter should induce a strong blue shift of the nearest chlorophyll molecule (P_A) and a weaker red shift of P_B. It is noteworthy that a blue electrochromic band shift could be predicted from the structure of bacterial RC independently on the basis of the extent of excitonic interaction between two monomers in P (see Appendix 1). The sign inversion demonstrates that the internal structure of P strongly differs from that of its bacterial counterpart.

The difference spectrum attributable to the reaction $S_1 \Rightarrow S_2$ in the PSII core particles is shown in Figure 2c. The spectrum in Figure 2c resembles that published by Velthuys (1988) and also that from *Synechococcus* by Saygin and Witt (1985), except that the isobestic point is at 681 nm in the former two but at 676 nm in the latter. The difference may be attributable to the materials, namely, preparations from plant thylakoids [Velthuys (1988) and this work] versus core particles from *Synechococcus* (Saygin & Witt, 1985). The common feature of these difference spectra is the dominant blue shift of a band at 680 nm, in contrast to the red shift in this domain that was observed upon $Y_Z \Rightarrow Y_Z^+$ transition (Figure 2b). This alone excludes an in-line position of the manganese cluster relative to Y_Z and P.

Electrochemical Implications for the Structure of PSII

A unique property of the primary donor in PSII is its extremely positive midpoint potential (E_m) of >1.1 V (Klimov et al., 1979). It is ~700 mV greater than that in the bacterial RCs (Dutton & Prince, 1979) and ~650 mV greater than that in PSI (Parson & Ke, 1982). This imposes severe limitations on the modeling of the structure of PSII. As detailed in Appendix 2, the first oxidation potential E_{ox}^1 of chlorophyll *a* in a nonpolar environment, with correction for the junction potential, is <700 mV, which is ~500 mV lower than necessary for water oxidation [and more than 100 mV lower than the usually quoted, noncorrected results of electrochemical titrations in nonpolar media (Watanabe & Kobayashi, 1991)]. A point that is usually overlooked in this type of discussion is that not only must the E_m value of P be somehow increased to >1.1 V but also those of any other chlorophyll *a* in close vicinity, otherwise the more electronegative molecule (with $E_m \sim 700$ mV) will reduce P^+ without being able to subsequently oxidize Y_Z [$E_m \sim 1$ V (Metz et al., 1989)]. For this reason, the presence of accessory chlorophylls *a* in the positions of their bacterial

counterparts [as in the currently suggested structural models (Svensson et al., 1992; Ruffle et al., 1992)] is unlikely (additional arguments against their presence in these positions will be presented later). Indeed, the edge to edge distance between BP and the accessory bacteriochlorophyll in the bacterial RC is 5.5 Å. By taking at face value the kinetic ruler of electron transfer (Moser et al., 1992), a chlorophyll *a* molecule in the position of its bacterial counterpart is kinetically a thousandfold more potent reductant for P^+ than for Y_Z. On the basis of similar considerations, Van Gorkom and Schelvis (1993) have argued that antenna chlorophylls are to be placed more distantly from P. Their argument holds even more for the accessory chlorophylls *a* of PSII. As an alternative, one may speculate that the accessory chlorophylls *a* are not oxidized because their midpoint potentials are significantly higher than that of P. A 500−700 mV increase in the electrostatic potential over the space occupied by the primary donor and the putative accessory chlorophylls *a* in the positions of their bacterial counterparts could be achieved by the deposition of at least 6−8 positive charges from the lumenal side of the membrane. We consider a constellation with so many noncompensated positive charges unlikely.

Electrochemically more consistent is a scheme where P is formed from only two closely apposed, but excitonically almost uncoupled chlorophyll *a* molecules with high oxidizing potential. The high oxidizing potential could be contributed (1) by the hydrogen bonding of the keto and carbomethoxy oxygens of the chlorin ring [note from Figure 1 that chlorophyll *a* can form only *two* such bonds and that data on the mutant bacterial RCs showed that such shift hardly exceeded 0.1 V per bond (Lin et al., 1994)]; (2) by the fixed electric dipoles of α-helices (Hol et al., 1978) [the bacterial RC-type arrangement of the latter provides an electrostatic potential on P of +0.15 V (Parson et al. 1990)]; and (3) by 1−2 positive charges placed in close proximity to *both* chlorophyll *a* molecules of P. Such charges could be provided by arginine or histidine residues that are without counterparts in the bacterial structure and by Ca^{2+} atoms that are known to be bound in PSII [see Debus (1992) and references therein]. The other chlorophyll *a* molecules could be removed at a safe distance and interact with P via molecules of pheophytin *a* that cannot be oxidized because of a high E_{ox}^1 value (see Appendix 2).

Model Topology of Chromophores and Redox Cofactors as Derived from the Spectral Analysis

Under the above-described assumptions on the peak wavelength and excitonic coupling in PSII, a particular topology of chromophores and redox cofactors in PSII has emerged from our data. The tentative structure is illustrated in Figure 3b. Its corollary in the bacterial RC (Diesenhofer et al., 1984, 1995) is given in Figure 3a for comparison.

The peak at 667 nm revealed band shifts in response to the reactions $Q_A \rightrightarrows Q_A^-$ and $Y_Z \rightrightarrows Y_Z^+$. We attribute this peak to an accessory chlorophyll *a* molecule on the D1 subunit. The fact that these *opposite* charges both caused a red shift placed this molecule between Q_A and Y_Z. The current structural models place the accessory chlorophyll *a* in branch A of PSII between P and $Pheo_A$ in the position of the accessory bacteriochlorophyll of the bacterial RC (Svensson et al., 1992; Ruffle et al., 1992). In addition to the electrochemical considerations that were presented earlier, the following data favor the absence of a chlorophyll *a* molecule from this position [which has already been discussed by Shuvalov et al. (1989)]: (1) Michel and Deisenhofer (1988) pointed out that the histidines that coordinate the central magnesium atoms of the accessory chlorophylls in the bacterial RC are absent from the primary structures of D1 and D2. (2) In fact, the binding sites for $BChl_A$ and $BChl_B$ are formed not just by these histidines, but by a distinct sequence motif (Ix[S/T]H[L/I]xWx_3[F/T]) in the short α-helical regions (called CD helices) that connect C and D transmembrane helices in both the L and M subunits of the bacterial RC (Deisenhofer et al., 1984, 1995). This motif is conserved in the RCs of different bacteria and even in photosystem I of green plants, but it is absent from PSII (Kunh et al., 1994). Moreover, the absence of this motif from PSII correlates with a single deletion in the amino acid sequence of the CD loop relative to the bacterial sequence. As the amino acid sequences of CD loops of PSII show almost no similarity to their bacterial counterparts (see Appendix 3), we aligned the nucleotide sequences to localize this deletion. In spite of little sequence similarity even between the CD loops of D1 and D2, the deletion was localized in a similar place in both sequences (see Appendix 3). It corresponds to the substitution of a [Wx] amino acid doublet (corresponding, for example, to L-Trp156, L-Val157 and M-Trp183, M-Leu184 in *Rps. viridis*) by a single phenylalanine. The substituted tryptophan residues (a) belong to the conservative motif shown earlier, (b) form the binding pocket for accessory bacteriochlorophylls, and (c) apparently maintain the structural integrity of the special pair as the edge to edge distance between each of them and the primary donor is ∼5 Å (Deisenhofer et al., 1995; see Figure 3a). A deletion in this position was inevitably accompanied by a change in the orientation of the remaining residues of the CD loop. It is conceivable that the mutation that caused the loss of these tryptophan residues in both subunits of the still homodimeric primordial RC [see Blankenship (1992)] has started an evolutionary transition to a much more electropositive PSII. This might be occurring by the simultaneous distortion of both binding sites of the accessory chlorophylls *a* and of the configuration of the primary donor. (3) The formation of $BPheo_A^-$ in the bacterial RC caused a large blue shift of the Q_y peak of the accessory bacteriochlorophyll and a smaller blue shift of BP (Shuvalov et al., 1976). In PSII, the electrochromic shift in response to $Pheo_A^-$ lacks the contribution of accessory chlorophyll *a*; it has only one component at 680 nm (Ganago et al., 1982), which is attributable to P (Schlodder & Brettel, 1988; Breton, 1990).

Where then are the "accessory" chlorophyll *a* molecules of PSII? At least two of them are currently assumed to be bound by the strictly conserved histidines located somewhat closer to the lumenal ends of helices B (D1-His118 and D2-His118 in green plants) on the basis of structural and genetic data (Michel & Deisenhofer, 1988; Shuvalov et al., 1989; Pakrasi & Vermaas, 1992; Schelvis et al., 1994; Hutchison & Sayre, 1995; Vacha et al., 1995). This placed the one on D1 (Chl_A) roughly halfway between Q_A and Y_Z at the position that corresponds to L-Cys92 in the RC of *Rps. viridis* (Figure 3b). According to our analysis, this Chl_A responds to the charges on Q_A^- and Y_Z^+. The extent and polarity of electrogenic band shifts caused by both Q_A^- and Y_Z^+ argue in favor of a perpendicular orientation of its Q_y transition and, hence, of its chlorin plane in the membrane with ring V pointing to Q_A (see Appendix 1). The perpendicular

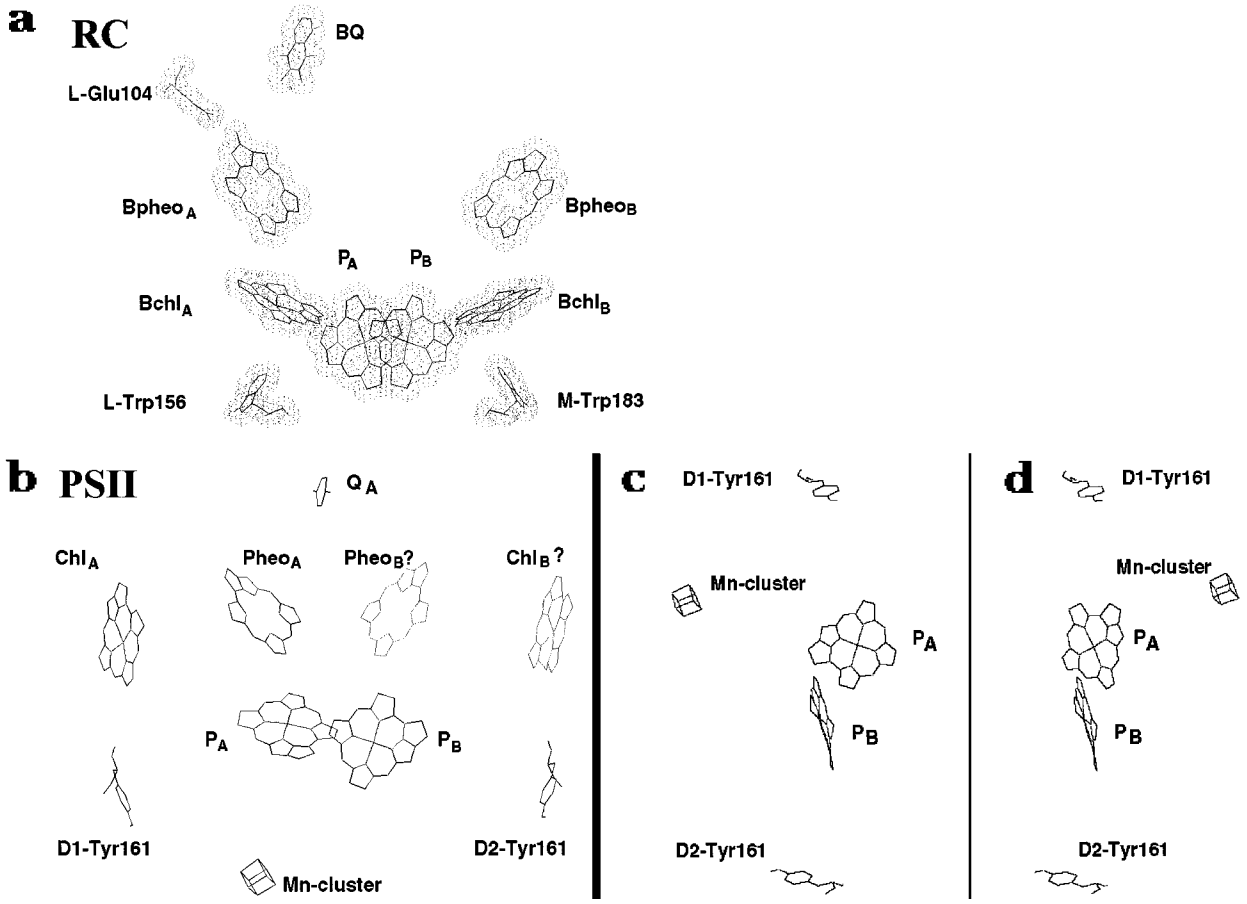

FIGURE 3: Pigments and redox cofactors in the RC of *Rps. viridis* and in PSII: (a) structural arrangement of cofactors and some amino acid residues in the RC of *Rps. viridis* (Deisenhofer et al., 1984, 1995); (b) proposed and tentative arrangements of chromophores and redox cofactors in PSII; (c and d) two possible arrangements of the Mn cluster relative to the Y_Z-P-Y_D line as viewed from Q_A. The arrangement in Figure 3b−d was derived by starting from the structure of the bacterial RC [Figure 3a of Deisenhofer et al. (1995)] and applying the following modifications: Bacteriochlorophyll *b*, bacteriopheophytin *b*, and menaquinone (BQ_A) molecules were substituted by chlorophyll *a*, pheophytin *a*, and plastoquinone (Q_A), respectively. The accessory bacteriochlorophyll molecules were removed and substituted by two chlorophyll *a* molecules coordinated by L-Cys92 and M-Phe119. The former was oriented as explained in the text. Within the framework of the kinetic ruler of Moser et al. (1992), we accommodated the faster temperature independent component of the $Q_A^- \Rightarrow P^+$ recombination in PSII [1.6 ms in PSII (Reinman & Mathis, 1981) but 20 ms in *Rb. sphaeroides* and 10 ms in *Rps. viridis* [see Shinkarev and Wraight (1993) and references therein]] with the same rate of $Pheo_A \Rightarrow Q_A$ electron transfer [about 200 ps in both systems (Renger, 1992)]: Q_A was moved closer to the primary donor and shifted parallel to the membrane plane (to preserve the $Pheo_A \Rightarrow Q_A$ distance). We chose a position where Q_A came on the top of rings IV and I of $Pheo_A$. In this case, a blue band shift of $Pheo_A$ in response to Q_A^- can be expected (Hanson et al., 1987a,b). The deeper immersion of Q_A in PSII relative to the bacterial RC corresponds to a relatively large electrogenic phase coupled with the protonation of non-heme iron that is close to Q_A [an amplitude of 30% relative to the $P \Rightarrow Q_A$ electrogenic step has been observed (Haumann et al., 1995a,b)]. $Pheo_A$ was left in the position of its bacterial counterpart for simplicity. P_A was tilted to a 30° angle to the membrane plane and reoriented in a way that is explained in the text. P_B was left in the position of its bacterial counterpart (although the tilting of its plane closer to the membrane plane cannot be excluded from our data). Y_Z (D1-Tyr161) and Y_D (D2-Tyr161) were placed in the position of L-Arg135 and M-His162, respectively. The Mn cluster was placed approximately in positions that correspond to the points with the following atomic coordinates in the structure of the *Rps. viridis* RC (Deisenhofer et al., 1995): $x = 102$, $y = 57$, $z = 25$ (panels b and c) and $x = 103$, $y = 45$, $z = 2$ (panel d). Chl_B was placed symmetric to Chl_A. $Pheo_B$ was placed symmetric to $Pheo_A$ quite arbitrarily, although a location close to the position of $BChl_B$ of the bacterial RC also seems attractive from data on the very small electrochromic band shift of its Q_x band in response to a charge on Q_B (Schatz & Van Gorkom, 1985) and a near to membrane plane orientation of its Q_x transition moment (Breton, 1990).

orientation is in line with the predictions of Michel and Deisenhofer (1988) based on structural grounds. The structure in Figure 3b is compatible with the fact that the electric field originating from $Pheo_A^-$ does not cause a noticeable band shift of Chl_A, as observed by Ganago et al. (1982), because the point charge of $Pheo_A^-$ is on the top of the chlorin ring of Chl_A. A chlorophyll *a* bound to His118 could still efficiently transfer the excitation energy to P via $Pheo_A$ [the center to center distance between Chl_A and $Pheo_A$ in Figure 3b is 17 Å; see also Shelvis et al. (1984)]. On the other hand, the chlorophyll *a* molecule in this position is sufficiently remote from P not to compete with Y_Z for the reduction of the former (Figure 3b).

The pheophytin of D1 ($Pheo_A$) was attributed to the pigment peaking at 685 nm, in accordance with the literature. The pronounced blue shift around 685 nm in response to the negative charge on Q_A^- is correlated with a blue shift around 545 nm, the position of the Q_x transition of pheophytin *a*, but not of chlorophyll *a* (Van Gorkom, 1974; Klimov et al., 1977). The much smaller electrochromic response to the $Y_Z \Rightarrow Y_Z^+$ and $S_1 \Rightarrow S_2$ transitions is in line with the data of Dekker (1985) cited earlier. It corroborates

the view that the water-splitting machinery is rather distant from $Pheo_A$. In the bacterial RC, L-Arg135 (corresponding to Y_Z in PSII) is placed at a distance of 26 Å from the nearest bacteriopheophytin *a* molecule (Deisenhofer et al., 1995). As $Pheo_A$ responds to Q_A^- with a blue shift of its Q_y and Q_x peaks (Van Gorkom, 1974; Klimov et al., 1977), opposite to those observed in the bacterial RC, we suggested in our preliminary report on this work that its *y*-axis is antiparallel to that of $BPheo_A$ in the bacterial RC (Cherepanov et al., 1995). Although this possibility cannot be completely ruled out, it now seems less likely for the following reason: The substitution of D1-Glu130 that is supposed to form a hydrogen bond with $Pheo_A$ (Lubitz et al., 1989; Nabedryk et al., 1990) for a glutamine causes a blue shift of the Q_x absorbance band of $Pheo_A$ (Giorgi et al., 1995; P. Nixon, personal communication). The homologue in the bacterial RC, L-Glu104, forms a hydrogen bond with the 13-keto group in ring V of $BPheo_A$ [Deisenhofer et al. (1984, 1995), Figure 3a)]. Its substitution by glutamine and leucine caused qualitatively similar blue shifts of the Q_x absorbance band of $BPheo_A$ (Bylina et al., 1988). The blue shift of $Pheo_A$ in response to Q_A^- instead may be explained by a less dramatic alteration of the mutual arrangement of $Pheo_A$ and Q_A. Whether Q_A^- is more deeply embedded in the membrane, shifted parallel to the membrane plane relative to the bacterial counterpart (see Figure 3b and the corresponding legend), or whether the $Pheo_A$ ring is tilted remains to be determined.

The primary donor P was attributed to the spectral components centered at 677 and 681 nm for four reasons: (1) These wavelengths are at the peak of the difference spectrum of P (Doering et al., 1969; Schlodder et al., 1984; Jankowiak & Small, 1993). (2) Neither of these bands revealed an electrochromic shift in response to Q_A^-. This is in line with the proposed distance of ~25 Å between Q_A and P based on structural modeling (Michel & Deisenhofer, 1988) and with the parallel orientation of the *y*-axis(es) of P to the membrane (Mathis et al., 1976; Ganago et al., 1982). (3) The components centered at 677 and 681 nm dominated the electrochromic response to the positive charges on Y_Z (Figure 2b) and the Mn cluster (Figure 2c). (4) As outlined earlier, it was unlikely that other chlorophyll *a* molecules were located close to P, Y_Z, and the Mn cluster.

The interpretation of electrochromic band shifts of P to yield structural information depends on the extent of the distortion of the Q_y transitions of monomers by the excitonic interaction. On the one hand, excitonic interactions in the range of <100 cm^{-1} cannot be ruled out *a priori* for any neighboring chlorins in the RC [see, for example, Durrant et al. (1995)]. On the other hand, the extent of the excitonic band splittings depends on the intrinsic peak positions (Pearlstein, 1982). Hillmann et al. (1995) have recently deconvoluted the ^3P and $P^+Q_A^-$ spectra obtained at 25 K in oxygen-evolving core particles. They propose that P is formed from two chlorophyll *a* molecules that differ in their intrinsic peak positions, one with its maximum at 685 nm, accommodating the triplet state and the positive charge, and the other one absorbing at 676 nm. For the 676 nm component, the extent of excitonic shift has been estimated as 1 nm; a similar or smaller shift could be estimated for the 685 nm one from the presented data. Such small shifts cannot cause a dramatic reorientation of the Q_y transition moments and, hence, hardly influence our structural consideration.

The results of our analysis are compatible with the presence of two chlorophyll *a* molecules in P peaking at 677 and 681 nm. This is in line with the presence of two molecules in the position of the primary donor of PSII, which was suggested on the basis of structural (Michel & Deisenhofer, 1988; Pakrasi & Vermaas, 1992) and spectroscopic grounds (Van Mieghem et al., 1991; Noguchi et al., 1993; Van Gorkom & Schelvis, 1993; Hillmann et al., 1995; Breton et al., 1995). Slight differences between the peak positions of P, as determined by Hillmann et al. (1995) and resulting from our work, may be attributed to different temperatures (25 K *versus* room temperature). An orthogonal arrangement of the Q_y transition moments of P_A and P_B has been suggested previously (Tetenkin et al., 1989; Noguchi et al., 1993) to explain the absence of a strong exciton interaction in PSII (van der Vos et al., 1992). This feature plus the very selective response of the spectral components that peaked at 677 and 681 nm to a positive charge on either Y_Z or the manganese cluster led us to propose the topology that is shown in Figure 3b. The molecules of P_A and P_B (peaking at 681 and 677 nm) are anchored to His198 residues in both D1 and D2 (homologous to the ones that anchor the special pair in the bacterial RC). The *y*-axis of P_B is oriented more or less parallel to the membrane to account for the red shift in response to Y_Z^+ (D1-Tyr161). The *y*-axis of P_A, which is also essentially parallel to the membrane plane (Mathis et al., 1976; Ganago et al., 1982; Breton, 1990), is arranged perpendicular to the $Y_Z \Rightarrow P_B$ line and the assumed *y*-axis of P_B. This accounts for the small electrochromic response of P_A to Y_Z^+ and for the low excitonic coupling in P (van der Vos et al., 1992). To accommodate the 30° angle between the planes of ^3P (in our notion P_A) and the membrane (Van Mieghem et al., 1991; van der Vos et al., 1992), we chose a ring orientation as shown in Figure 3b−d. This orientation also accounts for the blue band shift at 680 nm in response to $Pheo_A^-$ (Ganago et al., 1982; Breton, 1990). The blue band shift in response to a positive charge in the Mn cluster then implies that the Mn cluster is located in the plane of the P_A ring and close to the line extending its *y*-axis (Figure 3b−d, see the following). The proposed topology in Figure 3b−d is quantitatively compatible with the observation of Hillmann et al. (1995) that a positive charge on P_A (685 nm at 25 K) causes a red shift of P_B (from 676 to 683 nm at 25 K, see Appendix 1). Another observation of Hillmann and Schlodder (1995) is also compatible with the proposed structure: At 77 K, when electron transfer from Y_Z to P^+ is blocked, a chlorophyll *a* molecule peaking at 667 nm is oxidized in tens of milliseconds [see also Visser et al. (1977)]. This is accompanied by the blue shift of a pigment peaking at 685 nm (electrochromic shift of $Pheo_A$ in response to Q_A^-) and by an unidentified red shift of a pigment peaking at 677 nm. If the oxidized chlorophyll *a* molecule is bound to D1-His118 (Chl_A), our structural model (Figure 3b) accounts for their observation. It predicts a red electrochromic shift of P_B.

The Mn-containing catalytic center was tentatively located as shown in Figure 3b. This position resulted from the combination of our data with estimates on the distances between the redox groups in PSII, namely, (1) a $Mn \Rightarrow Y_Z$ distance of 15 Å (Rutherford & Boussac, 1992; Vermaas et al., 1993), (2) a $Mn \Rightarrow Y_D$ distance of 28−30 Å (Kodera et al., 1994), and (3) a $Mn \Rightarrow P$ distance of 21−26 Å (Kodera et al., 1992). (An estimate of the $Mn \Rightarrow P$ distance as <19−23 nm, as calculated from the extent of the electrochromic

band shift, is presented in Appendix 1.) Depending on the direction of the $\Delta\boldsymbol{\mu}$ vector of P_A, two different arrangements of the Mn cluster relative to the axis that connects Y_Z, P, and Y_D are possible, which are shown in Figure 3c,d. The arrangement in Figure 3c is more likely, as in this case the Mn cluster is located in a domain that in the bacterial RC contains L-His144, the homologue to Asp170 of the D1 subunit. The latter may participate in the assembling and/or binding of the Mn cluster (Nixon & Diner, 1992; Whitelegge et al., 1995). In line with the models of Svensson et al. (1991) and Ruffle et al. (1992), we assume that the residues of the loop that connects the C and D helices of D1 participate in the binding of the Mn cluster. Our model places the Mn cluster far out of the axis formed by Y_Z, P, and Y_D.

CONCLUSIONS

The following conclusions on the structure of PSII emerged from the analysis of the electrochromic difference spectra. (1) The accessory chlorophyll *a* molecules in PSII are placed at different positions from their bacterial counterparts: farther away from P and presumably anchored to His118 on D1 and D2. The chlorin plane of the accessory chlorophyll on D1 (Chl_A, λ_{max} = 667 nm) is roughly perpendicular to the membrane plane; its ring V is pointing to Q_A. (2) The blue electrochromic shift of pheophytin *a* on D1 ($Pheo_A$, λ_{max} = 685 nm) in response to Q_A^- is in sharp contrast with the red shift of its counterpart in the bacterial RC. Whether this is due to a major reorientation of $Pheo_A$ or to a different position of Q_A relative to their bacterial counterparts remains to be established. (3) P is composed of two chlorophyll *a* molecules, P_A and P_B, with intrinsically different peak positions at 681 and 677 nm. Their *y*-axes are oriented in the plane of the membrane. The orientation of the *y*-axis of P_B conforms with that of the *y*-axis of its bacterial counterpart; however, the *y*-axis of P_A is orthogonal to the former (in contrast with the situation in the bacterial RC). (4) The intermediate electron carrier Y_Z (Tyr161 on the D1 subunit) is located approximately coaxially with the *y*-axis of P_B. (5) The manganese cluster is located out of the line connecting Y_Z, P, and Y_D.

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APPENDIX 1

Electrochromism of Chlorins and Estimation of Dielectric Distances in PSII

Electrochromism of Chlorins. Here we recall some physical principles of electrochromism with emphasis on chlorophyll *a* and pheophytin *a*. For simplicity we use the classical description. The quantum mechanical approach can be found elsewhere (Liptay, 1974; Reich, 1979).

When exposed to an electric field, a chromophore changes the energy levels of its ground and excited states according to

$$U_g(\mathbf{E}) = U_g^0 - (\mu_g + {}^1/_2\mathbf{a}_g\mathbf{E})\mathbf{E} \qquad \text{(A1a)}$$

$$U_e(\mathbf{E}) = U_e^0 - (\mu_e + {}^1/_2\mathbf{a}_e\mathbf{E})\mathbf{E} \qquad \text{(A1b)}$$

Here $U_g(\mathbf{E})$ and $U_e(\mathbf{E})$ are the energies of the ground and excited states in the presence and U_g^0 and U_e^0 are the energies in the absence of the electric field vector $\mathbf{E}$, μ_g and μ_e are the permanent dipole moments of the ground and excited states, and $\mathbf{a}_g$ and $\mathbf{a}_e$ are the respective tensors of polarizability. The electric field $\mathbf{E}$ shifts the wavelength, λ_0, of the absorption maximum by an amount:

$$\Delta\lambda = \frac{\lambda_0^2}{hc}(\Delta\boldsymbol{\mu} + {}^1/_2\Delta\mathbf{a}\mathbf{E})\mathbf{E} \qquad \text{(A2)}$$

Some higher order effects may occur but are not considered further: the polarizability tensor may be influenced by the electric field $\mathbf{E}$ (saturation effect) and also by the transition moment, which alters the peak heights (hypo- or hyperchromism). The change in the polarizability tensor $\Delta\mathbf{a}$ of monomeric chlorophyll *a* is about 2 Å^3 for the Q_y band. Thus, the field-induced dipole moment $\Delta\mathbf{aE}$ is smaller than the permanent one [$\Delta\boldsymbol{\mu} \sim 1$ D (Krawczyk, 1994)] for field strength below 10^8 V/m.

Visible spectra of chlorophyll *a* and pheophytin *a* in various solvents consist of an intense band at approximately 660−670 nm, a few smaller bands at 500−615 nm, and the Soret band (Goedheer, 1966). The vector of the $Q_y(0,0)$ transition moment that is responsible for the former band is almost parallel to the *y*-axis of the chlorin (see Figure 1). The transition shifts the electron density along this axis from pyrrole ring III to ring I (Scherer & Fischer, 1989; Warshel & Parson, 1987). This is paralleled by a large positive dipole moment of the ground state, μ_g, and a smaller one, μ_e, of the first excited state (Fajer et al., 1992). The experimentally determined values of $\Delta\boldsymbol{\mu}$ were ∼1 D for chlorophyll *a* (Krawczyk, 1994) and 1.8−2.3 D for bacteriopheophytin *a* and bacteriochlorophyll *a* (Steffen et al., 1994). The absolute values of $\Delta\boldsymbol{\mu}$, which were calculated by the INDO/s method in Fajer et al. (1992), were 5 times greater, namely, ∼10 D for bacteriochlorophyll *a* and 5 D for chlorophyll *a*. This difference is readily explained by neglecting the screening effects in these calculations. The orientation of $\Delta\boldsymbol{\mu}$ is similar in both cases: the angle between $\Delta\boldsymbol{\mu}$ and the *y*-axis of chlorins is 150−170°, whereas that between $\Delta\boldsymbol{\mu}$ and the Q_y transition moment is 10−20° (Lockhart & Boxer, 1987; Krawczyk, 1994). We failed to find such information for pheophytin *a* in the literature. As the values of $\Delta\boldsymbol{\mu}$ are similar for bacteriochlorophyll *a* and bacteriopheophytin *a*, and as the spectral properties of pheophytin *a* are similar to those of chlorophyll *a*, we used a value of 1 D for pheophytin *a* (the same as for chlorophyll *a*) in our quantitative estimates.

Electrochromism of Excitonically Coupled Chlorins. Although the exciton coupling between pigments in PSII is essentially weaker than in the case of bacterial RC, it is worthwhile to analyze the possible influence of such coupling on the electrochromic effects. Let us consider two identical chromophores a and b in the local electric field of magnitudes E_a and E_b. Without a loss of generality, we assume that in the ground states the permanent dipoles of these pigments

are absent, whereas in the first excited states they are μ_a and μ_b. Let us denote by p_a and p_b the transition dipole moments of these pigments and by $\hbar\omega_0$ the energy of excitation of the unperturbed monomers. According to the quantum mechanical perturbation theory, the interaction of pigments leads to the splitting of the doubly degenerate excited state [see, for example, Shipman et al. (1976b)], which in the presence of the electric field can be described as

$$\hbar\omega_{1,2} = \hbar\omega_0 + \frac{1}{2}(\mu_a E_a + \mu_b E_b \pm \sqrt{(\mu_a E_a - \mu_b E_b)^2 + 4\chi^2 p_a^2 p_b^2})$$

Here χ is a geometrical factor that depends on the distance and the mutual orientation of the chromophores. The term $J = \chi|p_a||p_b|$ is the energy of the dipole−dipole coupling (for the sake of simplicity, the small exchange term as well as other types of interaction between pigments have been omitted in this consideration).

To obtain an upper estimate for the extents of the possible field-induced shifts of excitonic bands in PSII, we considered for P the geometry of the strongly coupled bacterial special pair. The dipole strength p^2 of the Q_y band of the Chl *a* molecule is about 23 D^2 (Shipman et al., 1986a). Then the magnitude of the term *J* would be about 400 cm^{-1}, i.e., about 3 times larger than the published upper estimates of excitonic coupling in PSII [see Durrant et al. (1995) and references therein]. Let us assume that a point charge is placed on L-Arg135 at a distance of 14 Å from the nearest pigment of the special pair, that the change in the permanent dipole during excitation is 1 D, and that the effective dielectric constant (ϵ_{eff}) is 8. Then the energy $\mu_a E_a - \mu_b E_b$ is 20 cm^{-1}, which is essentially lower than the exciton energy *J*. By this reasoning we neglect the first term under the root compared to the second, the square of *J*. Thus, if P resembled the strongly coupled bacterial special pair, both exciton bands would be shifted by the electric field (induced by Y_Z^+) in the same direction by a value of $^1/_2(\mu_a E_a + \mu_b E_b)$. In this arrangement, the observed opposite band shift caused by a charge on the Mn cluster could be explained only by placing the latter on the other side of P, opposite Y_Z. This configuration is neither kinetically nor structurally attractive. Therefore, the pseudobacterial structure with excitonic coupling does not explain the opposite electrochromic shifts caused by the $Y_Z^+-Y_Z$ and S_2-S_1 transitions. If P is formed from two orthogonally arranged molecules of chlorophyll *a* (Tetenkin et al., 1989; Nogushi et al., 1993; Hillmann et al., 1995; this work) with different absorption peaks, the electrochromic spectra are readily simulated. In this case, however, small excitonic coupling is expected, and the excitonic effects in the steady state spectra are wiped out due to the larger energy difference between the monomers (Pearlstein, 1982).

Estimation of Dielectric Distances in PSII. We assume that the electric field of a local charge is described by the Coulomb's law in homogeneous medium:

$$\vec{E} = \frac{Q\vec{r}}{\epsilon r^3} \qquad \text{(A3)}$$

Then the distance between this charge and a chromophore molecule can be calculated from the electrochromic band shift $\Delta\lambda$ according to

$$r = \sqrt{1119\left(\frac{\Delta\boldsymbol{\mu}\cos\theta}{\epsilon_{eff}\Delta\lambda}\right)} \qquad \text{(A4)}$$

wherein *r* denotes the distance in angstroms, $\Delta\boldsymbol{\mu}$ the difference between the permanent dipole moments in D, ϵ_{eff} the relative permitivity (effective dielectric constant), $\Delta\lambda$ the electrochromic shift in nanometers at wavelength 680 nm, and θ the angle between the electric field and the difference vector of the dipole moments $\Delta\boldsymbol{\mu}$.

The rough estimates of the dielectric distances in PSII could be obtained by introducing the extents of electrochromic band shifts into eq A4. To do so we needed values of $\Delta\boldsymbol{\mu}$ and ϵ_{eff}. We assumed $\Delta\boldsymbol{\mu} \cong 1$ D for both chlorophyll *a* and pheophytin *a*. The value of ϵ_{eff} is difficult to predict *ad hoc*, because of protein inhomogeneity and electrostatic screening. Broadly speaking, we are interested in the value of ϵ_{eff} in the inner part of a bundle of α-helical polypeptides that contains detergent and is surrounded by water. The following experimental estimates are, in our opinion, relevant in this case: (1) In the RC of *Rb. sphaeroides*, the values of ϵ_{eff} were determined at 1.5 K from the Stark spectra and electrochromic band shifts as 1.5−2.5 and 5−6 for the M and L electron transfer branches, respectively, and are estimated to be about 2 times greater at room temperature (Steffen et al., 1994). The latter corroborates the finding by Tiede and Hanson (1992) that the initial extent of electrochromic band shifts in *Rb. capsulatus* decreased to one-half because of the relaxation of the protein matrix (which is prevented at 1.5 K). (2) From the atomic structure of the RC of *Rps. viridis* (Deisenhofer et al., 1995) and the extents of electrochromic spectra that were measured in this RC (Shopes & Wraight, 1985), we obtained a rough estimate of ϵ_{ff} with the help of eq A4 [the values of A_0 and w_0 for bacteriochlorophyll *b* and bacteriopheophytin *b* from Oelze (1985) were used]. The resulting values of ϵ_{eff} were 12.5 for the interaction between BQ_A^- and $BPheo_A$ and 11 for the interaction between BQ_A^- and the accessory bacteriochlorophyll. Thus, ϵ_{eff} for the operative L branch can be estimated as 10−12 at room temperature. (3) By taking into account the determination of the electric field strength inside an α-helix in water at 273 K giving $\epsilon_{eff} = 8$ (Lockhart & Kim, 1992), we arrived at $\epsilon_{eff} = 8-12$ as a reasonable estimate for the inner part of the PSII core particle.

As the upper limit of the cosine function in eq A4 ($\cos\theta$) was 1, we obtained the upper estimates for the distances. They are presented in Table 2. These estimates are compared with the respective distances in the PSII model (Figure 3b), the EPR-obtained estimates, and the distances in the RC of *Rps. viridis* (where possible). A correspondence between our estimate for the distance between the Mn cluster and P_A and the respective EPR-obtained value (Kodera et al., 1992) points to a location of the Mn cluster in the plane of the P_A ring ($\cos\theta \sim 1$). Further, we estimated the angle θ between the vectors $\Delta\boldsymbol{\mu}$ and the electrical field **E** by comparing the estimated distances that were obtained under the assumption $\cos\theta = 1$ in eq A4 with the distances in the model in Figure 3b. These estimates are also presented in Table 2, along with the values of the corresponding angles in the chimeric bacterial RC that retained the orientation of its cofactors, but also contained Chl_A and Y_Z in the positions of L-Cys92 and L-Arg135, respectively. (As the Mn cluster cannot be attributed to a distinct amino acid residue, it was not possible to include it in this chimera.) It can be seen that certain

Table 2: Estimation of the Center−Center Distances and Angles between the Cofactors of PSII (Dielectric Constant ϵ_{eff} Was Varied in the Range 8−12)

		distance (Å)				angle between $\Delta\mathbf{u}$ and E[d] (deg)	
interaction	$\Delta\lambda$ (nm)	our estimate	EPR	PSII model (Figure 3b)	*Rps. viridis* (Figure 3a)	our estimate	chimeric RC of *Rps. viridis*[e]
$Q_A \rightarrow Pheo_A$	−0.23	<20−25		14	14	109−119	49
$Q_A \rightarrow Chl_A$	+0.09	<33−40		30		32−55	44
$Y_Z \rightarrow P_R$	+0.25	<19−24		19		13−44	44
$Y_Z \rightarrow P_A$	+0.13	<27−34	12[a]	13		77−81	166
$Y_Z \rightarrow Chl_A$	+0.14	<26−32		24		30−55	60
$Mn \rightarrow P_A$	−0.27	<19−23	21−26	20−22			

[a] Rutherford and Boussac (1992). [b] Kodera et al. (1992). [c] Deisenhofer et al. (1995). [d] $\Delta\mathbf{u}$ was assumed to be oriented along the axis that connects the nitrogen atoms NI and NIII of the chlorin ring (Hanson, 1991; Fajer et al., 1992). [e] Y_Z and Chl_A were modeled for the chimeric RC of Rps. viridis by L-Arg135 and a chlorophyll *a* ring coordinated by L-Cys92 and placed perpendicular to the membrane, respectively.

values in the last two rows are similar, thus confirming that P_B, Y_Z, and Chl_A can be shaped satisfactorily by BP_B, L-Arg135, and a chlorine ring coordinated by L-Cys92 and placed perpendicular to the membrane, respectively. On the other hand, the drastic differences between the angles relevant to $Pheo_A$ and P_A compared to the bacterial counterparts point to a different orientation of these two chlorins in PSII relative to the bacterial RC.

APPENDIX 2

Electrochemistry of Chlorins

The direct application of the electrochemically determined values of the first oxidation potential (E_{ox}^1) of chlorins to the systems *in vivo* is complicated by the problem of scaling the potential of the reference electrode (usually saturated calomel). The value of E_{ox}^1 is measured in nonaqueous solvents [the only way to dissolve chlorins *in vitro*; see Watanabe and Kobayashi (1991) for a review], whereas the midpoint potentials of the biological redox carriers are determined relative to the normal hydrogen electrode in aqueous solution in the presence of redox mediators [see Dutton and Wilson (1974)]. The liquid junction potential between water and nonaqueous solvent must be taken into account if these two sets of data are compared. This potential is connected with different solvation energies in these solvents and cannot be eliminated by using salt bridges (Diggle & Parker, 1974). An accurate estimate of the junction potential has been obtained by measuring the free energies of transferring tetraphenylarsonium tetraphenylborate (TATB) from water to various nonaqueous solvents (Parker, 1976). Because both the anion and the cation have the same absolute charge and similar radii, their solvation energies are similar in the same solvent. This allowed a comparison between various solvents. Table 3 contains the E_{ox}^1 values for chlorophyll *a*, pheophytin *a*, and bacteriochlorophyll *a* and the respective TATB-corrected estimates. The table includes only the solvents for which the TATB correction was determined.

Table 3: First Oxidation Redox Potentials E_{ox}^1 of Chlorophyll *a*, Bacteriochlorophyll *a*, and Pheophytin *a* in Different Aprotic Solvents *in Vitro* (According to Khanova and Tarasevich (1988) with Modifications and Additions)[a]

			E_{ox}^1 potential (V) vs NHE		
compound	solvent[b]	electrolyte[c]	without correction	with TATB correction	source ref
Chl *a*	AN	$LiClO_4$	0.76	0.67	1
	AN	$NaClO_4$	0.81	0.72	2
	DMF	TBAP	0.83	0.66	3
	DMF	TBAP	0.84	0.67	4
	DMF	$NaClO_4$	0.84	0.67	5
Pheo *a*	AN	$NaClO_4$	1.20	1.11	2
	DMF	TBAP	1.22	1.05	4
BChl *a*	AN	TBAF	0.66	0.57	6
	AN	LiClO	0.69	0.60	7
	MeOH	TBAP	0.56	0.54	8

[a] Source references: 1, Stanienda, 1963; 2, Kutyurin & Solov'ev, 1976; 3, Saji & Bard, 1977; 4, Wasielewski et al., 1980; 5, Miyasaka & Honda, 1981; 6, Cotton & van Duyne, 1979; 7, Drozdova et al., 1977; 8, Cotton & Head, 1987. [b] AN, acetonitrile; DMF, dimethylformamide. [c] TBAP, tetrabutylammonium perchlorate; TBAF, tetrabutylammonium tetrafluoroborate.

APPENDIX 3

Alignment of the Primary Sequences of the CD Loops of the L and M Subunits of Bacterial Reaction Centers with the Corresponding Domains of the D1 and D2 Subunits of PSII

Figure 4a shows the alignment of the protein sequences from several bacterial RCs, which includes the C and D transmembrane helices and the connecting CD helix (loop) with the presumably homologous parts of the D1 and D2 subunits of PSII. There are a few, but important similarities and identities: proline residues that mark the ends of α-helices and histidine residues that bind the chlorophylls of the primary donor and the non-heme iron in the acceptor part of the RCs, and, less evident, the conservation of the aromatic nature of the amino acid residues in several positions (highlighted). In the domain between D2-Gly175 and D2-Asn191 (underlined) the similarity between the PSII proteins and the subunits of bacterial RC is negligible, whereas the similarity is apparent among bacteria. Therefore, the single deletion that occurs in both D1 and D2 in this region cannot be positioned reliably from the comparison with the bacterial amino acid sequences. Another peculiarity of this region is a different prediction for the secondary structure of the CD loops of D1 and D2 (not shown; see Materials and Methods). Whereas the probability index for an α-helical structure (which varies from 0 to 9) in this region of D2 was about 9, the probability of an α-helical structure in the corresponding region of D1 was smaller (2−5).

To locate the deletion in the CD loops of D1 and D2, we have aligned the corresponding nucleotide sequences with the program PileUp. To obtain an alignment with a single 3-nucleotide gap in the PSII sequences, the gap weight had to be increased to >1.5, otherwise single nucleotide gaps were introduced by the program into the bacterial sequences as well. The gap length weight was kept standard at 0.3.

a

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                    ---------C-helix--------          --CD-helix--        ----------D-helix-----------
                  116                                                                              198
Rps. viridis        L   HVPLAFCVPIFMFCVLQVFRPLLLGSWGHAFPYGILSHLDWVNNFGYQYLNWHYNPGHMSSVSFLFVNAMALGLHGGLILSVA
Rb.  spaeroides     L   HIPFAFAFAILAYLTLVLFRPVMMGAWGYAFPYGIWTHLDWVSNTGYTYGNFHYNPAHMIAISFFFTNALALALHGALVLSAA
Chl. aurantiacus    L   HVPIAFGVAFSAWLVLQVIRPIALGMWHEGFVLGIMPHLDWVSNFGYRYNNFFYNPFHAIGITGLFASTWLLACHGSLILSAA
Rps. viridis        M   HIAWNFAAAIFFVLCIGCIHPTLVGSWSEGVPFGIWPHIDWLTAFSIRYGNFYYCPWHGFSIGFAYGCGLLFAAHGATILAVA
Rb.  spaeroides     M   HTAWAFLSAIWLWMVLGFIRPILMGSWSEAVPYGIFSHLDWTNNFSLVHGNLFYNPFHGLSIAFLYGSALLFAMHGATILAVS
Chl. aurantiacus    M   YLAYGFTGAIALYLVIYIIRPVWMGDWSEAPAHGIKALLDWTNNVSVRYGNFYYNPFHMLSIFFLLGSTLLLAMHAGTIWALE
Pisum sativum (Pea) D1  WIAVAYSAPVAAATAVFLIYPIGQGSFSDGMPLGISGTFN-FMIVFQAEHNILMHPFHMLGVAGVFGGSLFSAMHGSLVTSSL
Pisum sativum (Pea) D2  YNAISFSGPIAVFVSVFLIYPLGQSDWFFPPDFGVAAIFR-FILFFQGFHNWTLNPFHMMGVAGVLGAALLCAIHGATVENTL
                  142                                                                              223
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b

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                        149                                                                 166
Rps. viridis        L   G  I  L  S  H  L  D  W  V  N  N  F  G  Y  Q  Y  L  N

Chl. aurantiacus    L   ggcattatgccccacctcgattgggtgtcgaattttggctatcgctacaataat
Rps. viridis        L   ggcatcctgagccatctcgattgggtgaacaacttcgggtatcagtaccttaac
Rb.  spaeroides     L   gggatctggacgcacctcgactgggtgtcgaacacgggctacacctacggcaac
Rb.  spaeroides     M   ggcatcttctcgcacctcgactggacgaacaacttctcgctcgtccacggcaac
Rps. viridis        M   ggcatctggccgcacatcgactggctgactgcgttctcgatccgctacggcaac
Chl. aurantiacus    M   ggcatcaaagcgctgctcgactggacgaataatgtgagcgtgcgttacggcaat
Pisum sativum (Pea) D1  ggaatctctggtactttcaact...ttatgattgtattccaggcagagcacaac
Pisum sativum (Pea) D2  ggcgtagcagcgatatttcgat...tcatccttttcttccaaggatttcataat

                                                  ☝
Pisum sativum (Pea) D2    G  V  A  A  I  F  R     F  I  L  F  F  Q  G  F  H  N
                        175                                                 191
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FIGURE 4: Partial primary sequences of the L and M subunits from the purple bacteria *Rps. viridis* (Michel et al., 1986) and *Rb. sphaeroides* (Williams et al., 1986) and the green non-sulfur bacterium *C. aurantiacus* (Ovchinnikov et al., 1988a,b) compared with those of the D1 (Oishi et al., 1984) and D2 (Rasmussen et al., 1984) subunits from pea chloroplasts. The numbering for the L subunit of *Rps. viridis* and the D2 subunit of pea is indicated. The position of a single deletion in the D1 and D2 amino acid sequences in panel a was chosen according to the alignment of nucleotide sequences in panel b. (a) Amino acid sequences; (b) nucleotide sequences.

The low road alignment mode was used. The resulting alignment is shown in Figure 4b. In spite of low similarity between the D1 and D2 sequences in this region (30% identity of amino acid residues, <50% identity of nucleotide sequences), the gap was inserted in a similar place in both subunits (indicated by an arrow).

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