Species-specific Differences of the Spectroscopic Properties of P700

ANALYSIS OF THE INFLUENCE OF NON-CONSERVED AMINO ACID RESIDUES BY SITE-DIRECTED MUTAGENESIS OF PHOTOSYSTEM I FROM CHLAMYDOMonas REINHARDTII

Heike Witt‡, Enrica Bordignon‡, Donatella Carbonera*, Jan P. Dekker‡, Navassard Karapetyan**, Christian Teutloff‡, Andrew Webber‡‡, Wolfgang Lubitz‡§§, and Eberhard Schlodder‡¶¶

From the ‡Max-Planck-Laboratorium für Biophysikalische Chemie, Technische Universität Berlin, Strasse des 17. Juni 135, 10623 Berlin, Germany, § Dipartimento di Chimica Fisica, Università di Padova, Via Loredan 2, 35131 Padova, Italy, *A. N. Bakh Institute of Biochemistry, Russian Academy of Sciences, Leninsky Prospect, 33, 119971 Moscow, Russia, the ¶Department of Physics and Astronomy, Vrije Universiteit, De Boelelaan 1081, 1081 HV Amsterdam, The Netherlands, ††Center for the Study of Early Events in Photosynthesis, the Department of Plant Biology, Arizona State University, Tempe, Arizona 85287-1601, and §§MPI für Bioanorganische Chemie, Stiftsstraß 34-36, 45470 Mülheim/Ruhr, Germany

We applied optical spectroscopy, magnetic resonance techniques, and redox titrations to investigate the properties of the primary electron donor P700 in photosystem I (PS I) core complexes from cyanobacteria (Thermosynechococcus elongatus, Spirulina platensis, and Synechocystis sp. PCC 6803), algae (Chlamydomonas reinhardtii CC2696), and higher plants (Spinacia oleracea). Remarkable species-specific differences of the optical properties of P700 were revealed monitoring the absorbance and CD difference spectra. The main bleaching band in the Q_b region differs in peak position and line width for the various species. In cyanobacteria the absorbance of P700 extends more to the red compared with algae and higher plants which is favorable for energy transfer from red core antenna chlorophylls to P700 in cyanobacteria. The amino acids in the environment of P700 are highly conserved with two distinct deviations. In C. reinhardtii a Tyr is found at position PsaB659 instead of a Trp present in all other organisms, whereas in Synechocystis a Phe is found instead of a Trp at the homologous position PsaA679. We constructed several mutants in C. reinhardtii CC2696. Strikingly, no PS I could be detected in the mutant YW B659 indicating steric constraints unique to this organism. In the mutants WA A679 and YA B659 significant changes of the spectral features in the absorbance and CD difference spectra are induced. The results indicate structural differences among PS I from higher plants, algae, and cyanobacteria and give further insight into specific protein-cofactor interactions contributing to the optical spectra.

** This work was supported by Sfb 498 TCP5 and TPA6 and the TMR Programme FMXR-CT98-0214. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence may be addressed: Max-Planck-Laboratorium für Biophysikalische Chemie, Tel.: 49-30-31429568; Fax: 49-30-31421122; E-mail: h.witt.berlin@web.de.

§ To whom correspondence may be addressed: Max-Planck-Laboratorium für Biophysikalische Chemie, Tel.: 49-30-31429688; Fax: 49-30-31421122; E-mail: eber@gmx.de.

1 The abbreviations used are: PS I (II), photosystem I (II); ADMR, absorption-detected magnetic resonance; B_p and B_m, accessory chlorophylls ligated by subunit PsA and PsB, respectively; CAPS, 3-cyclohexylaminol-1-propanesulfonic acid; Chl, chlorophyll; β-DM, n-dodecyl β-maltoside; ENDOR, electron nuclear double resonance; hfc, hyperfine coupling constant; LHC, light-harvesting complex; P700, primary electron donor of PS I; P700+, cation radical of P700; P700−, triplet state of P700; P_p and P_o, the two chlorophylls constituting P700 ligated by subunit PsA and PsB, respectively; T-S, triplet-minus-singlet; Tricine, N,N′-dihydroxy-1,1-bis(hydroxymethyl)methylglycine; TMPD, tetramethyl-p-phenylenediamine dihydrochloride; ZFS, zero-field splitting.
large subunits are highly conserved and the same holds for the primary photochemistry of the reaction center of PS I, although there is a discussion going on that in some species both branches participate in the primary charge separation whereas in other species only the A-branch seems to be active (9, 10).

After absorption of light by an antenna pigment, the excitation energy is transferred to the primary electron donor P700, a chlorophyll a dimer. P700 being in the lowest excited singlet state donates an electron to the primary electron acceptor $\Lambda_\alpha$, a chlorophyll a monomer. Charge stabilization is achieved by subsequent electron transfer to secondary acceptors, the phylloquinone A1 and Fx, a [4Fe-4S] iron-sulfur cluster and finally to FA and FB. The x-ray crystallographic analysis of cyanobacterial PS I (8) has revealed that the primary electron donor P700 of photosystem I is a dimer composed of one chlorophyll a and one chlorophyll a' that is the $13^\text{\textdegree}$ epimer of Chl a. The coordinating ligand of Chl a' ($P_\alpha$) is provided by a His from PsaA (His-A676) (numbering according to *Chlamydomonas* throughout the text), whereas the one of Chl a ($P_\beta$) is provided by PsaB (His-B656). Based on ENDOR spectra of P700$^{-}$, which exhibit nearly identical hyperfine coupling constants of the methyl protons of all investigated species, it has been concluded that the positive charge is mainly localized on $P_\beta$ (11–14).

Despite the remarkable similarities described above, optical spectroscopy of PS I complexes from different species indicates considerable differences. For example, although the flash-induced (P700$^{-}$–P700) absorbance difference spectra of *T. elongatus* and *Spinacia oleracea* (spinach) are rather similar (15, 16), those recorded for *Anabaena variabilis* (15) and *C. reinhardtii* (17, 18) look different.

To investigate the reasons for species-specific differences, we were looking for differences in the primary sequences. In all organisms sequenced so far, the amino acid residues around P700 are highly conserved. Based on the 2.5-Å structure of PS I from *T. elongatus*, we searched for all amino acids located within a distance of 10 Å with respect to the magnesium atoms of $P_\alpha$ and $P_\beta$. The sequence comparison shows that the respective amino acids are identical for *T. elongatus* and spinach. Two differences exist between *C. reinhardtii* and *T. elongatus*. A Ser is found at position A654 instead of an Ala in *T. elongatus*. The exchange of this Ala against Ser using the program Swiss PdbViewer indicates that the Ser could be located close to $P_\alpha$ between the vinyl and methyl side chains. Although the polarity of the binding pocket of $P_\alpha$ might be slightly higher for *C. reinhardtii*, we do not expect that this amino acid residue has a significant influence on the optical properties of P700. A striking difference is a Tyr at position B659 in *C. reinhardtii*, whereas a Trp is conserved in all other organisms. For *Synechocystis* three differences are found, namely Gly instead of Ala-A675, Phe instead of Leu-A622, and a Phe is present at position A679 which is the homologous position to B659 on PsaA where Trp is always found in the other organisms. We did not investigate further the differences found for *Synechocystis* due to the mutagenesis system chosen (see below). Unfortunately, the amino acid sequence of *Spirulina platensis* is not available so far. The amino acid residues at positions B659 and A679 are the most interesting ones due to their close vicinity to P700 and the accessory Chls (within 4 Å). An additional interesting aspect of these residues is their location one helix turn toward the stromal side with respect to the coordinating ligands, forming a roof-like structure orthogonal to P700 (Fig. 1).

In *C. reinhardtii*, the Tyr-B659 might be able to form a hydrogen bond to the keto group of the phytol side chain of $P_\beta$. This would be analogous to the proposal based on the crystal structure that the conserved Tyr-A731 might be able to form a
hydrogen bond with the phytol ester carbonyl oxygen of $P_a$. As the single amino acid deviations mentioned above are the only obvious differences between the different species, site-directed mutagenesis has been applied to clarify whether these differences are responsible for the spectroscopic peculiarities of the species. For $T. elongatus$, it has not been achieved to perform site-directed mutagenesis whereas such a system is established for $C. reinhardtii$ and this organism has been preferred as a model organism for the analysis of photosynthesis using (site-directed) mutagenesis (12, 17–19). $C. reinhardtii$ is able to grow heterotrophically on media with acetate as the carbon source (20) and is easily amenable to genetic manipulation with the advent of chloroplast genome transformation and selectable markers. Site-directed mutagenesis in turn is a powerful tool to identify the role of individual amino acids and to study the relationships between structure and function of photosynthetic reaction centers.

To get reliable information on species-specific differences, we measured and compared triplet-minus-singlet (T-S) difference, $(P700^-\rightarrow P700)$ absorbance difference, and CD difference spectra of PS I core complexes from various species all purified using $\beta$-dodecyl maltoside as detergent. To study the influence of the amino acid side chains described above on the spectroscopic properties of $P700$ and to gain further insight into protein cofactor interactions, we mutated Trp-A679 to Ala, Tyr, and His, Tyr-B659 to Trp, Leu, Ala, and His, and Tyr-A731 to His in $C. reinhardtii$. The effects of these mutations were analyzed using steady state and transient absorbance difference spectroscopy. Additionally, we applied redox titrations and electron nuclear double resonance (ENDOR) spectroscopy.

**Materials and Methods**

**Strains, Chloroplast Transformation, and Growth Conditions**—As the recipient of the donor plasmids, strain $C. reinhardtii$ CC2696 was used which in contrast to wild type carries the DS-521 nuclear mutation leading to a deficiency in the Cab proteins and a deletion in psaA causing the loss of PS II. Therefore, this strain is well suited for the preparation and analysis of PS I core complexes.

Chloroplast transformation and selection of the transformants on Tris-acetate-phosphate plates containing 150 $\mu$g/ml spectinomycin were carried out as described previously (17).

For enzyme purification, strains were grown heterotrophically in $C. reinhardtii$ media which were used in this work. It was calculated from the maximum flash-induced absorbance decrease in the $Q_r$ region due to photo-oxidation of $P700$ by using the molar extinction difference coefficients given under "Results" and from the flash-induced absorbance increase at 826 nm due to the formation of $P700^+$ using an extinction coefficient of 7500 $\text{M}^{-1} \cdot \text{cm}^{-1}$. For all PS I core complexes from all the species used in this work, the ratio was $100 \pm 15$. For the PS I preparations from $C. reinhardtii$ and spinach, this gives evidence that virtually no LHC I is present bearing in mind that the Chl $a/P700$ ratio for PS I holocomplexes is about 200–250.

**Preparation of PS I Core Complexes**—Isolation of native and mutated PS I core complexes from $C. reinhardtii$ CC2696 was performed according to Witt et al. (17) and Fromme and Witt (25) and from Synechocystis PCC 6803 (kind gift of M. Rogner) as described in Kruip et al. (24). Material from the isolated PS I core complex was subcloned into pAlter-1 to $PstI$ and $EcoRI$-PsI fragment that encodes the $psaB$ gene and part of $rbcL$ was subcloned into pAlter-1 to perform site-directed mutagenesis.

**Spectroscopic Properties of P700**

Transient Absorption Spectroscopy—Flash-induced absorbance difference spectra of $(P700^-\rightarrow P700)$ were measured at room temperature as described previously (17) with PS I core complexes diluted to 10 $\mu$M Chl in 20 mM Tricine (pH 7.5), 25 mM MgCl$_2$, 100 mM KCl, 0.02% $\beta$-DM, 5 mM ascorbate, and 10 $\mu$M phenazine methosulfate. The difference between the molar extinction coefficients of $P700^-$ and $P700$ at the peak wave-length was calculated from the flash-induced absorption change of $N,N,N',N'$-tetramethyl-p-phenylenediamine dihydrochloride (TMPD) according to Hiyama and Ke (15). TMPD is oxidized by the flash-induced $P700^+$. An extinction coefficient of 12,000 $\text{M}^{-1} \cdot \text{cm}^{-1}$ has been determined for oxidized TMPD (pH 8.0).

For the light-minus-dark absorbance difference spectra at 77 K, PS I core complexes were diluted to 10 $\mu$M Chl in 20 mM Tricine (pH 7.5), 25 mM MgCl$_2$, 0.02% $\beta$-DM, 5 mM ascorbate, and 60% glycerol. Measurements were performed as described previously (17).

To study the triple state of $P700$, flash-induced T-S spectra have been recorded at 5 K. PS I core complexes were diluted to 10–15 $\mu$M Chl in 100 mM CAPS (pH 10), 10 mM MgCl$_2$, 10 mM CaCl$_2$, and 0.02% $\beta$-DM.

**Circular Dichroism—CD Spectra** were recorded on a Jasco J-720 spectrometer at room temperature as described previously (17). PS I core complexes diluted to 13 $\mu$M Chl in 20 mM Tricine (pH 7.5), 25 mM MgCl$_2$, 100 mM KCl, 0.02% DM. For the measurements the solution was divided into two samples. Ferricyanide (1 mM) was added to one sample to oxidize $P700$, whereas 5 mM ascorbate and 10 $\mu$M phenazine methosulfate was added to the second sample to keep $P700$ in the reduced state. Spectra of the samples with $P700$ oxidized or reduced were measured alternating. Oxidation-minus-reduction was obtained by subtracting the average reduced from the average oxidized spectrum.

Redox Titration—To determine the oxidation midpoint potential of $P700$, the flash-induced absorbance change at 826 nm, associated with oxidation of $P700$, was measured as a function of the redox potential. Purified PS I core complexes were diluted to 20–30 $\mu$M Chl in 20 mM Tricine (pH 7.5), 100 mM KCl, 25 mM MgCl$_2$, 0.02% $\beta$-DM, and the redox potentials were adjusted by adding ferricyanide and ferrocyanide. After each experiment, the potential was measured using a combination Pt/Ag/AgCl electrode (Schott PT5004) which was calibrated against the Sorensen potential of a saturated solution of quinhydrone as a function of pH (26). A $\Delta E/\Delta pH$ of -62 was used to read out the redox potential. All redox potentials are given relative to the standard hydrogen electrode (normal hydrogen electrode).

**ENDOR—ENDOR measurements were performed on a Bruker ESP 300E X-band EPR spectrometer with home-built ENDOR accessories (29). ENDOR experiments were carried out on the cation radical $P700^-\rightarrow P700$ and from $P700^-\rightarrow P700$ $\Delta E/\Delta pH$ of -62 was used to read out the redox potential. All redox potentials are given relative to the standard hydrogen electrode (normal hydrogen electrode).
electron acceptor A₁ was essentially the same as described for the flash-induced T-S spectra. Measurements were performed at 1.8 K. The absorption-detected magnetic resonance and the T-S microwave-induced spectra were recorded using the laboratory-built apparatus described previously (30).

RESULTS

By applying standard site-directed mutagenesis techniques, we constructed several mutants in PsAA and PsAB of PS I from C. reinhardtii CC2696 to investigate the influence of Trp-A679 and Tyr-B659 on the properties of P700. No PS I could be detected in mutants where Tyr-B659 was replaced by Trp or Leu. This is very surprising for the Trp mutant because all other species have a Trp at this position. The native and the mutated PS I core complexes WA A679, WH A679, WY A679, YA B659, and YH B659 from C. reinhardtii were purified and spectroscopically characterized together with PS I core complexes from four other species (T. elongatus, S. platensis, Synechocystis sp. PCC 6803, and S. oleracea) by steady state and transient absorption spectroscopy, circular dichroism, ADMR, ENDOR, and redox titrations.

Mutant Phenotype—As the mutations were introduced into the Chlamydomonas strain CC2696, which in contrast to wild type contains the DS-521 nuclear mutation leading to a deficiency in the Cab proteins and a deletion in psbA causing the loss of PS II, PS I is the main chlorophyll-binding protein. It is therefore possible to detect consequences of the amino acid substitution on the amount of PS I by inspection of the phenotype. All mutants contain PS I amounts comparable with the native CC2696 except for YW B659 and YL B659 which show a yellow phenotype indicating the absence of PS I. The negative phenotype of YW B659 was confirmed by Western blots (not shown) using thylakoid membranes and antibodies directed against PsAA which did not show any detectable amount of PS I.

Triplet-minus-Singlet (T-S) Absorbance Difference Spectra—To study the triplet state of P700, measurements were performed with PS I complexes under reducing conditions with the secondary acceptor A₁ in the reduced state. Therefore, the electron transfer to A₁ is blocked and the primary radical pair, P700−·A₁, recombines to the triplet state of P700 with high yield. Fig. 2, A and B, shows the flash-induced T-S absorbance difference spectra of C. reinhardtii, T. elongatus, S. platensis, Synechocystis, and S. oleracea (spinach) detected at 5 K. These spectra reflect the absorbance difference between P700 in its triplet state and its singlet ground state. The spectra have been normalized between 660 and 726 nm to the same area assuming that the loss of oscillator strength upon triplet formation of P700 is the same in all species. The flash-induced absorbance difference spectra at 5 K attributed to ³P700 formation are virtually identical with the microwave-induced T-S spectra detected at 1.8 K (see Figs. 2A and 3A for C. reinhardtii; Schlodder et al. (28) for T. elongatus; and Fig. 2A and Carbonera et al. (30) for spinach). The same holds for Synechocystis and S. platensis (not shown). Because virtually identical T-S spectra of P700 are observed by both methods (flash-induced absorbance difference spectroscopy and ADMR), contributions of Chl triplets in the antenna can be excluded, except for the case when the resonance microwave frequency and the decay kinetics are identical for ³P700 and ³Chl. Despite some remarkable differences, all T-S spectra exhibit the following characteristic fingerprint-like features as follows: 1) a main bleaching around 700 nm, which is assigned to the disappearance of the low energy excitonic band of P700; 2) a second bleaching around 687 nm; and 3) positive bands around 682 and 672 nm. However, the spectra reveal some differences between the different species. The position of the main bleaching varies between 698 nm for C. reinhardtii, 701 nm for T. elongatus, and

Synechocystis, and spinach, and 705 nm for S. platensis. It should be noted that the T-S spectrum of P700 measured with wild type PS I holocomplexes from C. reinhardtii CC125 also exhibits the main bleaching band at 698 nm. Even more impressive is the variation of the line shape of the low energy exciton band (compare the T-S spectra of C. reinhardtii and those of Synechocystis and S. platensis). For PS I from cyanobacteria the line width of the main bleaching band is broader, and the zero-crossing is shifted much more to the red (up to 735 nm for Synechocystis) compared with the green algae. The positive and negative features at the short wavelength side of the main bleaching band (687 nm (−)), 682 (+) and 672 nm (++) are differently pronounced for the species presented. It should be noted that the features in this spectral region are sensitive to the isolation procedure, pH values, and pretreatment (30).² The positive and negative band on the blue side of the main bleaching are blue-shifted for the green algae by 2–3 nm compared with the other species.

For comparison, we have measured the T-S spectrum of monomeric Chl a in β-DM micelles using the same apparatus. In Fig. 2A we plotted the spectrum, shifted to the red by 28 nm to align the peak with the one of the ³P700−(P700) spectrum from spinach. It should be noted that the large line width of the Q₁ band of the monomeric Chl a, which is bleached upon triplet formation, is mainly due to inhomogeneous broadening (31), whereas the low energy exciton band of P700 is predominantly homogeneously broadened (16, 32). The T-S spectrum of Chl a completely lacks the second sharp negative and positive features at shorter wavelengths. Excitonic interactions between the six Chls in the reaction center can probably account for these additional spectral features (see under “Discussion”).

The effects of the replacement of Tyr B659, Trp-A679, and

² E. Bordignon, D. Carbonera, and E. Schlodder, unpublished results.
Methods.

Parameters of 3P700 for the mutants are almost unchanged. The modulation frequency was 33 Hz, the microwave power 800 milliwatts, the optical resolution 1.5 millidegrees (OD 678.5 nm 1.1) at the positive maximum. The main bleaching band is blue-shifted by about 2 nm for the mutant WA A679 and WY A679 (C. reinhardtii) and 716.5 MHz (YH B659). The modulation frequency was 33 Hz, the microwave power 800 milliwatts, the optical resolution 1.5 nm, and T = 1.8 K. For experimental details see “Materials and Methods.”

Tyr-A731 have been monitored by ADMR-detected T-S spectra. Fig. 3, A and B, shows the T-S absorbance difference spectra of native and mutated PS I from C. reinhardtii CC2696. The spectra are normalized to −1 at the minimum of the main bleaching band to allow better comparison. The position of the main bleaching band is blue-shifted by about 2 nm for the mutant WA A679 and the second bleaching at about 685 nm is slightly more pronounced compared with native PS I. The T-S spectrum for the mutant YA A679 is essentially the same as that of unmutated PS I (Fig. 3A). The main bleaching of YA B659 is blue-shifted by about 2 nm, and the second negative bleaching is completely absent in this mutant. Moreover, the positive band located at 670 nm in the unmutated PS I is blue-shifted to 665 nm for the mutant YA B659, whereas a negative band is appearing at 670 nm (Fig. 3B). The spectrum for YH B659 is essentially unchanged compared with native PS I (Fig. 3B).

The zero field-splitting (ZFS) parameters [D] and [E] reflect the magnetic field splitting between the triplet sublevels in a zero magnetic field. The ZFS parameters depend on the spatial distribution of the unpaired electrons in the triplet state over the chlorophyll PA and PB, and/or on the admixture of charge-transfer states. At low temperature (T = 1.8 K) the ZFS parameters of 3P700 for the mutants are almost unchanged in comparison to unmutated PS I (not shown). They are virtually identical to those of triplet states from monomeric Chl a (33, 34) or Chl a^3 in organic solvents. This has been taken as evidence that the triplet state of P700 is mainly localized on one of the chlorophylls constituting P700. The localization of the triplet state is thus not significantly altered in the mutants.

(P700^−−P700) CD Difference Spectra at Room Temperature.—The CD spectra of PS I core complexes from C. reinhardtii CC2696 and T. elongatus with P700 in the oxidized form are shown in Fig. 4A. In accordance with CD spectra of PS I complexes reported earlier in the literature (23, 35-37), the rotational strength of the positive band on the high energy side is smaller than that of the negative band on the low energy side. The CD spectrum of C. reinhardtii displays the negative band at 685 nm and the positive band at 669 nm, whereas the one of T. elongatus has its negative band at 686 nm and the positive band at 671 nm. In addition, a broad negative absorption is observed between 700 and 720 nm for the cyanobacterium which can be attributed to the long wavelength Chls. The excitonic interactions between all chlorophylls of PS I contribute predominantly to the CD intensity, although the unequal rotational strength of both bands (non-conservative spectra) clearly indicates other contributions.

In order to obtain information on the excitonic coupling between the chlorophylls constituting the primary donor in PS I, the (P700^−−P700) circular dichroism difference spectra were recorded (see Fig. 4B). The ΔCD spectra have been normalized to 1 at the positive maximum to allow a better comparison.

In C. reinhardtii, the ΔCD spectrum is dominated by two components located at 694 (positive rotational strength) and 678 nm (negative rotational strength). In T. elongatus, we observe a band of negative rotational strength at 679 nm and two bands of positive rotational strength at 668 nm and at about 702 nm.

In order to monitor changes of the excitonic coupling between the reaction center Chls induced by the mutations in C. reinhardtii, (P700^−−P700) circular dichroism difference spectra were recorded. The spectra for native PS I and PS I containing the mutations WA A679 and YA B659 are shown in Fig. 5. The spectrum of WA A679 is blue-shifted by 2−3 nm compared with

Fig. 3. ADMR measurements of triplet-minus-singlet spectra of P700 of native and mutated PS I from C. reinhardtii CC2696 WA A679 and WY A679 (A), and YA B659 and YH B659 (B), taken at the maximum of the intensity of the |D|=|E| transitions: 718.5 MHz (WT), 728.7 MHz (WA A679), 719 MHz (WY A679), 718.5 MHz (YA B659), and 716.5 MHz (YH B659). The modulation frequency was 33 Hz, the microwave power 800 milliwatts, the optical resolution 1.5 nm, and T = 1.8 K. For experimental details see “Materials and Methods.”

3 F. Lendzian, L. Fiedor, and W. Lubitz, unpublished results.

Fig. 4. A, CD spectra of PS I with P700 in the oxidized form; B, (P700^−−P700) circular dichroism spectra of PS I core complexes from C. reinhardtii CC2696 and T. elongatus measured at room temperature with a spectral bandwidth of 2 nm. The difference spectra were obtained by subtracting the CD spectra measured with P700 in the reduced state from those measured with P700 in the oxidized state. Oxidation was achieved with 1 nm ferricyanide. The difference spectra are normalized to 1 at the positive maximum to allow better comparison. The absolute ΔCD values for C. reinhardtii are 2.1 millidegrees (OD 677 nm 1.1) and for T. elongatus 3.8 millidegrees (OD 678.5 nm 1.1) at the positive maximum.
unmutated PS I. For YA B659, the shape and position of the band with the positive rotational strength is similar to unmutated PS I, whereas the one with negative rotational strength is slightly shifted. The CD difference spectrum of YH A731 is nearly the same as that of native PS I (not shown).

\[(P700^-\rightarrow P700)\] Absorbance Difference Spectra at Room Temperature and 77 K—The optical features of the primary electron donor were additionally investigated by recording oxidized-minus-reduced absorbance difference spectra. The absorbance changes due to the oxidation of P700 were monitored as a function of wavelength. The molar extinction difference coefficient for the maximum bleaching in the \(Q_r\) region has been measured as described by Hiyama and Ke (15). The values are 61,000 M\(^{-1}\) cm\(^{-1}\) for \(T.\ elongatus\) and 57,000 M\(^{-1}\) cm\(^{-1}\) for \(S.\ platensis\), 83,000 M\(^{-1}\) cm\(^{-1}\) for \(S.\ platensis\), and 64,000 M\(^{-1}\) cm\(^{-1}\) for spinach. For \(C.\ reinhardtii\) the following molar extinction difference coefficients have been determined: 100,000 M\(^{-1}\) cm\(^{-1}\) for native PS I, 73,000 M\(^{-1}\) cm\(^{-1}\) for WA A679, 98,000 M\(^{-1}\) cm\(^{-1}\) for YA B659, 88,000 M\(^{-1}\) cm\(^{-1}\) for YH A731, and 90,000 M\(^{-1}\) cm\(^{-1}\) for YH B659. The margin of error is about 5%.

The \((P700^+\rightarrow P700)\) spectra in the \(Q_r\) region obtained for \(C.\ reinhardtii\), \(T.\ elongatus\), \(S.\ platensis\), spinach, and \(S.\ platensis\) at room temperature are shown in Fig. 6A. Although the overall shape of the spectra displaying one main bleaching band and a second smaller negative band seems to be similar, several differences between the spectra from different species can be observed. The main bleaching band of \(C.\ reinhardtii\) is located at 698 nm which is the furthest to the blue. The position of the main bleaching band is at 702 nm for \(S.\ platensis\) and at 703 nm for \(T.\ elongatus\), \(S.\ platensis\), and spinach. A second smaller bleaching band appears for all species besides the \(C.\ reinhardtii\), \(T.\ elongatus\), \(S.\ platensis\), and spinach. A second smaller bleaching band appears for all species besides the green algae where it is only visible as a shoulder at about 682 nm. The positions of this band (and of a positive band (+)) between the two bleachings are 682 (−) and 691 nm (+) for \(T.\ elongatus\), 677.5 (−) and 687.5 nm (+) for \(S.\ platensis\), 682 (−) and 688 (+) for \(S.\ platensis\), and 685 (−) and 691 (+) for spinach. For \(S.\ platensis\) the positive band even exceeds the zero line and reaches positive values. Another difference is the zero crossing in the red region at 725 nm for \(C.\ reinhardtii\), at 728 nm for spinach, at about 730 nm for \(S.\ platensis\) and \(T.\ elongatus\), and at 738 nm for \(S.\ platensis\). In the Soret region, the position of the main bleaching band is at about 431 nm for \(C.\ reinhardtii\) and at about 434 nm for \(T.\ elongatus\) and \(S.\ platensis\) (Fig. 6B).

The room temperature \((P700^+\rightarrow P700)\) spectra of mutated and native PS I from \(C.\ reinhardtii\) are compared in Fig. 7. A blue shift of about 2–3 nm of the main bleaching band is observed for WA A679 coupled with a decrease of the oscillatory strength of this band, whereas the oscillatory strength of the band at 682 nm increases (Fig. 7A). For YA B659 the main bleaching band is also shifted to blue by about 2–3 nm and a rather broad additional bleaching is observed between 665 and 685 nm (Fig. 7B). A slight blue shift can also be seen for the mutant YH B659, and the molar extinction difference coefficient is slightly reduced.

The \((P700^+\rightarrow P700)\) spectra detected at 77 K are presented in Fig. 8. These spectra were obtained by subtracting the absorbance spectra of PS I in the dark adapted state (with P700 reduced) from those after illumination. After illumination by saturating flashes from a xenon flash lamp, an irreversible charge separation \(P700^-F_{AB}\) is induced at 77 K in a fraction of the PS I complexes (38, 39). The size of this fraction varies between the different organisms (not shown). The spectra exhibit strong contributions from electrochromic bandshifts induced by the positive charge localized on the oxidized P700. All spectra have been normalized to −1 at the minimum of the long wavelength bleaching band. The spectra for \(C.\ reinhardtii\), \(T.\ elongatus\), \(S.\ platensis\), \(S.\ platensis\), and spinach are shown in Fig. 8, A and B. The spectrum of \(C.\ reinhardtii\) is dominated by a strong bleaching at 700 nm and a strong absorbance increase at 689 nm. Minor absorbance difference bands are observed at 683 (−), 670 (+), and 660 (−) nm. The spectra of the other species display the following features: 702 (−), 689 (+), 684.8 (−), 677.9 (+), 672.8 (−), and 667.9 (±) nm for spinach; 703 (−), 689.2 (+), 696.5 (−), 690.2 (+), 684.8 (−), 680.8 (+), 679.6 (−), 677.6 (+), 674 (−), and 669.4 (±) nm for \(T.\ elongatus\); 701.3 (−), 694.7 (+), 689.3 (−), 683.1 (−), 678.1 (+), 673.7 (−), and 667.5 (±) nm for \(S.\ platensis\); and 704.3 (−), 691.1 (+), 682.9 (−), and 669 (±) nm for \(S.\ platensis\) (+).
indicates an absorbance increase or decrease induced by the oxidation of P700, respectively.

The (P700−/P700) spectra at 77 K of native and mutated PS I from C. reinhardtii CC2696 are shown in Fig. 9, A and B. The difference spectrum of the native PS I has been described before (Fig. 8A). For the mutant WA A679, the main bleaching band is blue-shifted to 696 nm, whereas the strong positive absorbance difference band at 670 nm remains unchanged, whereas this band is red-shifted by about 4 nm in the mutant YA B659. The main bleaching band of YA B659 is displaced 3 nm to the blue shift and the strong positive absorbance difference band is located at 688 nm (Fig. 9B). For the mutant YH B659, the main bleaching band is blue-shifted to 697 nm, whereas the two positive bands are only slightly affected.

Oxidation Midpoint Potential of P700

The midpoint potential (Em) of P700−/P700 is a very sensitive probe for changes of the environment of the primary donor. To determine the oxidation midpoint potential of P700, the flash-induced absorbance change at 826 nm, associated with the oxidation of P700, was measured as a function of the potential adjusted by adding varying amounts of ferricyanide and ferrocyanide. The dependence of the amplitude of the absorbance change could be satisfactorily fitted using the one-electron Nernst equation. In comparison to C. reinhardtii, the midpoint potentials of P700−/P700 are decreased for the cyanobacteria, whereas that for spinach is increased (see Table I, last column). The midpoint potentials of P700−/P700 of the mutants WA A679, WY A679, and YA B659 remain unchanged within the error limit. In contrast, the midpoint potential of P700−/P700 is decreased for YH B659, whereas the one of WH A679 is slightly lowered. The error limits for the Em values obtained by one set of measurements under identical conditions are about ±4 mV corresponding to the standard deviation derived from the fits. The absolute value for native PS I from C. reinhardtii CC2696 was determined to 478 ± 8 mV. In this case the error includes additionally the uncertainty of the calibration of the electrode. It should be noted that the midpoint potential of P700−/P700 determined with wild type PS I holocomplexes from C. reinhardtii CC125 is the same within the limits of error.
ENDOR—ENDOR spectroscopy was used to characterize the electronic structure of P700\(^{-}\) by resolving the individual proton hyperfine couplings (hfcs). The assignment of the obtained hfcs to specific hydrogens of P700 was based on the comparison of ENDOR data reported earlier for Chl \(\alpha\) in Synechocystis PCC 6803, S. platensis, and S. oleracea (spinach) measured under the same conditions. All PS I core complexes have been isolated using the mild detergent \(\beta\)-dodecyl maltoside. This is an important prerequisite as the spectra are also dependent on the purification procedure and the detergent used for the preparation (30, 40). Although common characteristic features exist for all species, significant differences can still be observed (Figs. 2, 6, and 8). They mainly concern the position and the line shape of the main bleaching band as well as the features around the second bleaching. Remarkably, the molar extinction difference coefficients for the maximum bleaching in the \(Q_e\) region of the (P700\(^{-}\)–P700) spectra at room temperature (Fig. 6) have been found to vary considerably. In good approximation, the area under the curves in the \(Q_e\) region is the same for all species indicating that the loss of oscillator strength upon oxidation of P700 is virtually identical for all of them. Whereas the \(\Delta\varepsilon\) value of spinach and \(T.\ elongatus\) is in good agreement with the value of 64000 M\(^{-1}\) cm\(^{-1}\) (15) which is widely used in the literature for all species, it becomes evident that the \(\Delta\varepsilon\) value has to be determined for each PS I sample. It should be noted that this value also depends on the oligomeric state of the PS I complex (41) and on the preparation procedure and the detergent (not shown). The spectra obtained with PS I complexes of \(C.\ reinhardtii\) display the largest differences compared with the others as the position of the main bleaching band is the furthest to the blue, the extinction difference coefficient is the largest and the second bleaching is only visible as a shoulder. A blue shift of the main bleaching band is also visible in the Soret region for \(C.\ reinhardtii\). The position of the main bleaching band in the \(Q_e\) region in the other four species is located between 701 and 703 nm with similar extinction difference coefficients besides S. platensis where this value is somewhat larger. Striking differences between the species are also observed for the position and shape of the smaller second bleaching at about 682 nm and the spectral region between the two bleachings (see Fig. 6). Only in \(S.\ platensis\) is an absorbance increase observed between the two negative bands, and the whole feature is blue-shifted compared with the other species.

The isotropic hyperfine couplings extracted from the spectra of native and mutated PS I from \(C.\ reinhardtii\) in frozen solution (not shown) are summarized in Table I. For comparison, the proton hfcs of Chl \(\alpha\) in organic solvents are given. On the basis of the analysis of ENDOR spectra of P700\(^{-}\) obtained in PS I single crystals, the second largest splittings (line pairs 12) belong to the CH\(_3\) protons at position 12 of the chlorine ring (13). Kass et al. (13) concluded from EPR/ENDOR experiments that the spin density is mainly localized (>85%) on one of the Chls constituting P700.

### Table I

<table>
<thead>
<tr>
<th>Methyl group</th>
<th>Oxidation midpoint potential of P700(^{-})/P700, ND, not done.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\Delta\varepsilon) (mV) (^a)</td>
</tr>
<tr>
<td></td>
<td>12</td>
</tr>
<tr>
<td>Native</td>
<td>5.3</td>
</tr>
<tr>
<td>WA A679</td>
<td>5.4</td>
</tr>
<tr>
<td>WH A679</td>
<td>5.2</td>
</tr>
<tr>
<td>WY A679</td>
<td>6.0</td>
</tr>
<tr>
<td>YH B659</td>
<td>5.6</td>
</tr>
<tr>
<td>T. elongatus(^b)</td>
<td>5.3</td>
</tr>
<tr>
<td>Synechocystis(^b)</td>
<td>5.6</td>
</tr>
<tr>
<td>Spinach(^b)</td>
<td>5.6</td>
</tr>
<tr>
<td>S. platensis</td>
<td>ND</td>
</tr>
<tr>
<td>Chl (\alpha) (^{+})</td>
<td>7.1</td>
</tr>
</tbody>
</table>

\(^a\) \(\Delta\varepsilon\) is the difference \(\varepsilon_{\text{ox}} - \varepsilon_{\text{red}}\). The absolute value for the oxidation midpoint potential for P700 of native PS I from \(C.\ reinhardtii\) was determined to be \(\varepsilon_{\text{red}} = (478 \pm 8)\) mV.

\(^b\) Values have been taken from Ref. 11.

The values of Chl \(\alpha\) \(^{+}\) in CH\(_2\)Cl\(_2\)/THF are taken from Ref. 50. The hfcs in the hfs is \(\approx 0.1\) MHz for the methyl groups. The \(A_{\text{iso}}\) values have been calculated from the \(A_{\text{h}}\) and \(A_{\text{c}}\) components with the equation \(A_{\text{iso}} = 1/3(3A_{\text{h}} + 2A_{\text{c}})\).

**DISCUSSION**

Species-specific Differences of Optical Properties of P700—To obtain reliable information on the spectroscopic properties of PS I from several species, we compared the flash-induced absorbance difference spectra of (P700\(^{-}\)–P700) and of (P700–P700) of PS I complexes from \(C.\ reinhardtii\), \(T.\ elongatus\), Synechocystis PCC 6803, S. platensis, and S. oleracea (spinach) measured under the same conditions. All PS I core complexes have been isolated using the mild detergent \(\beta\)-dodecyl maltoside. This is an important prerequisite as the spectra are dependent on the purification procedure and the detergent used for the preparation (30, 40). Although common characteristic features exist for all species, significant differences can still be observed (Figs. 2, 6, and 8). They mainly concern the position and the line shape of the main bleaching band as well as the features around the second bleaching. Remarkably, the molar extinction difference coefficients for the maximum bleaching in the \(Q_e\) region of the (P700\(^{-}\)–P700) spectra at room temperature (Fig. 6) have been found to vary considerably. In good approximation, the area under the curves in the \(Q_e\) region is the same for all species indicating that the loss of oscillator strength upon oxidation of P700 is virtually identical for all of them. Whereas the \(\Delta\varepsilon\) value of spinach and \(T.\ elongatus\) is in good agreement with the value of 64000 M\(^{-1}\) cm\(^{-1}\) (15) which is widely used in the literature for all species, it becomes evident that the \(\Delta\varepsilon\) value has to be determined for each PS I sample. It should be noted that this value also depends on the oligomeric state of the PS I complex (41) and on the preparation procedure and the detergent (not shown). The spectra obtained with PS I complexes of \(C.\ reinhardtii\) display the largest differences compared with the others as the position of the main bleaching band is the furthest to the blue, the extinction difference coefficient is the largest and the second bleaching is only visible as a shoulder. A blue shift of the main bleaching band is also visible in the Soret region for \(C.\ reinhardtii\). The position of the main bleaching band in the \(Q_e\) region in the other four species is located between 701 and 703 nm with similar extinction difference coefficients besides S. platensis where this value is somewhat larger. Striking differences between the species are also observed for the position and shape of the smaller second bleaching at about 682 nm and the spectral region between the two bleachings (see Fig. 6). Only in \(S.\ platensis\) is an absorbance increase observed between the two negative bands, and the whole feature is blue-shifted compared with the other species.

The isotropic hyperfine couplings extracted from the spectra of native and mutated PS I from \(C.\ reinhardtii\) in frozen solution (not shown) are summarized in Table I. For comparison, the proton hfcs of Chl \(\alpha\) in organic solvents are given. On the basis of the analysis of ENDOR spectra of P700\(^{-}\) obtained in PS I single crystals, the second largest splittings (line pairs 12) belong to the CH\(_3\) protons at position 12 of the chlorine ring (13). Kass et al. (13) concluded from EPR/ENDOR experiments that the spin density is mainly localized (>85%) on one of the Chls constituting P700.

- Lowering the temperature yields absorbance difference spectra that are better resolved due to the narrowing of the absorbance bands. On the other hand, electrochromic shifts of absorbance bands as a consequence of the formation of P700\(^{-}\) become more visible in the (P700\(^{-}\)–P700) spectra upon lowering the temperature leading to additional narrow positive and negative bands. This can be explained in part by the fact that at low temperatures the shielding of the electric field is weakened due to the temperature dependence of the dielectric relaxation. The long range electrostatic interactions due to the charge on P700 may not only affect Chls in close proximity of P700 but very likely also those located in the core antenna system. The dominant absorbance increase at about 690 nm probably caused by an electrochromic bandshift is of comparable size as the absorbance decrease due to the bleaching of the low energy exciton band in all the species. The only exception is the PS I complex of \(S.\ platensis\).

We also monitored T-S absorbance difference spectra of P700 at 5 K (Fig. 2). The main bleaching band can be assigned to the
disappearance of the low energy exciton band upon triplet formation whereby the excitonic interaction between the Chl carrying the triplet state and the neighboring Chls of the reaction center is lost. The spectra represent very likely pure absorbance difference spectra of the reaction center because contributions of electrochromic bandshifts are avoided upon formation of the triplet state of P700. As the T-S spectrum of monomeric Chl a in micelles lacks the narrow features on the short wavelength side of the main bleaching band, they probably reflect the altered excitonic interactions between the six reaction center Chls.

The (P700−−P700) absorbance difference spectra (Fig. 8) indicate that the low energy exciton band is very broad for all species even at low temperatures. A line width of 300 cm⁻¹ at 5 K has been determined by hole-burning experiments using PS I complexes from spinach (32). The low energy exciton band of P700 is predominantly homogeneously broadened whereas the large line width of the Qₙ band of monomeric Chl a in micelles, which is bleached upon triplet formation, is mainly due to inhomogeneous broadening (31). The remarkable homogeneous broadening of the low energy exciton band has been explained by an unusually strong electron-phonon coupling probably due to mixing of excited states with charge transfer states. If the electronic transition involves a substantial displacement of charge, the protein environment reacts stronger on an excitation thereby giving rise to stronger vibrational side bands. Interestingly, the line width of the low energy exciton band of P700 in PS I from cyanobacteria is even larger than that in PS I from higher plants (see Fig. 8). In addition, the strongly red-shifted zero crossings for the cyanobacteria (708 nm for C. reinhardtii up to 740 nm for Synechocystis; see Fig. 2) strongly indicate that the 0–0 transition energy is lowered in cyanobacteria. Taken together this means that the absorption of the primary donor extends much more to the red. Thereby the overlap between the P700 absorption and the fluorescence of the red Chls in the core antenna is enhanced and consequently the possibility of direct energy transfer from these red Chls to P700 itself. The 77 K absorption spectra of the PS I core complexes from all species under study (data not shown) show that all species contain red Chls absorbing between 705 and 708 nm whereby C. reinhardtii and spinach contain the smallest number of these red Chls. The trimeric PS I core complexes in cyanobacteria contain in addition Chls absorbing even further to the red at 714 (in Synechocystis), 719 (in T. elongatus), and 740 nm (in S. platensis) (42). Keeping this in mind, the presence of the broad and red-shifted low energy exciton transition in cyanobacteria might be of functional relevance increasing the efficiency of energy transfer from these far red antenna Chls to the reaction center. If antenna Chls are present, which are able to exploit the red region of the spectrum, the absorbance wavelength of the photochemical trap also has to be shifted to the red to facilitate the trapping of excitation energy localized on red chlorophylls present in the core antenna.

Interestingly, green algae and spinach, which possess their most red antenna Chls in the external LHCI complexes, do not display such a broad low energy exciton band. For these red Chls, the distance to P700 is too large for a direct energy transfer, i.e. the efficiency of trapping would not be increased if the absorption of the primary donor extends more to the red. The spectroscopic differences between the primary donor of PS I in cyanobacteria and that in green algae and higher plants are hard to explain keeping in mind that the amino acid residues in the vicinity of P700 are highly conserved and are even identical for T. elongatus and spinach. The differences could be caused by long range effects due to variations of amino acids in PsaA and PsaB located further away that might slightly modify the geometrical arrangement of the cofactors, thus changing, for example, the charge transfer character of the excited state of the primary donor.

To get further insight into these species-specific differences, we investigated PS I complexes also with circular dichroism spectroscopy. For the interpretation of the various (P700−−P700) CD difference spectra, it should be kept in mind that the spectral features cannot easily be explained by a model of an excitonically coupled dimer but, as has been shown before, that all excitonic interactions between the six reaction center Chls have to be taken into consideration (see Ref. 37 for the calculation of the excitonic couplings). This was shown by comparison of the measured with a calculated CD difference spectrum where only the excitonic interaction between P₆ and P₈ was considered (17). Moreover, the calculated CD difference spectrum taking into account the excitonic interactions between all six reaction center Chls (not shown) has the qualitative features of the measured one for T. elongatus (Fig. 4). Based on the measurement of the ΔCD of P700 in S. platensis, Karapetyan and co-workers (43) proposed that even contributions of antenna chlorophylls located closest to the reaction center are observed. Comparison of the CD difference spectra of T. elongatus and C. reinhardtii reveals several differences, and the spectrum for C. reinhardtii obviously cannot be simulated on the basis of the structural data of the cyanobacterium. This gives evidence that structural differences exist in the arrangement of the cofactors between the species, besides the fact that the amino acid sequence is highly conserved.

**Effects of the Mutations on the Optical Properties of P700**

The most challenging task is to clarify the origin of the species-specific differences. They seem to be quite surprising because it is generally assumed that the structures of PS I complexes from different species are rather similar. Furthermore, the amino acid sequences around the primary donor P700 are highly conserved with only few deviations. It has already been mentioned that in C. reinhardtii a Tyr is found at position B659 where a Trp is conserved in all other species. Another variation in the amino acid sequence is found in Synechocystis where in the homologous position in the A subunit A679 is a Phe instead of a Trp present in the other species. To investigate whether these amino acid variations might be responsible for the spectroscopic differences, we constructed mutants at these two positions in C. reinhardtii CC2696. Furthermore, we assume that Tyr-B659 might be able to form a hydrogen bond to the keto group of the phyltyl side chain of P₆. As Tyr-A731 present in all species is a putative hydrogen bond donor to the keto group of the phyltyl side chain of P₆A, we also constructed a mutant at this position⁴ to investigate further the influence of hydrogen bonds on P700 and to understand the influence of the protein environment on the cofactors in photosynthetic reaction centers. Although in this case the specific influence of the hydrogen bond might be negligible as the keto group of the phyltyl side chain does not belong to the conjugated π-orbital system, it might play a role in stabilizing the position of the phyltyl side chain with respect to the accessory Chls. It should be noted that the phyltyl side chain of P₆A is in close proximity to the accessory chlorophyll B₆ whereas the phyltyl side chain of P₇B is close to the accessory chlorophyll B₇ (8), whereas the excitonic coupling is strong between P₆ and P₈ and B₆ respectively (37).

Strikingly, the mutant YW B659 shows a negative phenotype which seems to be quite surprising. Because a Trp is present in

---

⁴ H. Witt, unpublished data.
all other organisms, it is quite reasonable to assume a similar three-dimensional structure considering the conservation of the amino acid sequence around P700. However, Tyr-B659 cannot be replaced by Trp in C. reinhardtii. Probably the assembly is dramatically affected, and it can be assumed that there are steric constraints unique to this organism. It seems as if the side chain of Trp impedes the assembly of PS I. It is well known that FsaA and FsaB play a critical role in the assembly of PS I in C. reinhardtii and that complexes assembled only partially are rapidly degraded in this organism (44).

The effects of the mutations on the optical properties have been characterized by steady state and transient absorbance difference spectroscopy. Replacement of Trp-A679 and Tyr-B659 by Ala alters significantly the optical properties of the PS I reaction center (see Figs. 3, 5, 7, and 9). For both mutants, we observe a blue shift of the main bleaching band in the triplet-minus-singlet absorbance difference spectra and (P700\textsuperscript{−}−P700) absorbance difference spectra at room temperature and 77 K. This also holds for WA A679 looking at the CD difference spectrum. The position of the main bleaching band is related to the strength of the excitonic coupling between the Chls of the dimer and the two accessory Chls. Therefore it can be assumed that the blue shift indicates a decrease of this excitonic interaction by the mutations. One reason for this might be that Ala destabilizes the protein due to the smaller size and a loss in hydrophobicity with respect to Tyr and Trp. In addition, removal of the aromatic π-systems in the surrounding of cofactors may impede the reorganization of the protein surrounding upon excitation which finally gives rise to higher site energies. As a consequence the low energy exciton band will also be blue-shifted. The spectral features between 660 and 690 nm are affected differently for both mutants. This spectral range very likely reflects excitonic interactions with other pigments in the reaction center and, in case of the (P700\textsuperscript{−}−P700) spectra, electrochromic bandshifts of pigments in close proximity. For WA A679 the peak maximum of the main bleaching band is decreased, whereas the peak maximum of the second bleaching is increased. In the T-S spectrum of YA B659, the second bleaching is completely absent, whereas the positive band around 670 nm is blue-shifted. As Tyr-B659 seems to stabilize the phytyl side chain of P\textsubscript{B} which is located in close proximity to the accessory Chl B\textsubscript{B}, these changes can be reasonably explained if we assume that the spectral properties of P\textsubscript{B} are altered due to structurally induced changes. The broad absorption between 665 and 685 nm in the (P700\textsuperscript{−}−P700) absorbance difference spectrum of YA B659 gives additional evidence for the contribution of the P\textsubscript{B} in this spectral region. As Trp-A679 might mainly affect P\textsubscript{A}, a mutation will exert its effect on Chls nearby only indirectly via the excitonic coupling. P\textsubscript{A} is excitonically coupled mainly with P\textsubscript{B} and B\textsubscript{B}, whereas the interaction of the dimer is reflected in the main bleaching band. So far, it is not known which spectral features represent the remaining interactions within the reaction center. It is therefore reasonable to assume that both mutations influence the interaction of P\textsubscript{A} and B\textsubscript{B} and that this interaction becomes at least partly visible in the second bleaching of the (T-S) absorbance difference spectra. Furthermore, there is strong evidence that the influence of the mutations induces not only changes in the excitonic interaction with accessory chlorophylls but that electrochromic bandshifts due to P700\textsuperscript{−} formation are altered.

The exchange of Tyr-B659 against His leads only to minor changes in the spectra which might indicate that, although the hydrogen bond is probably broken, this exchange is not destabilizing the protein scaffold to a larger extent. The same seems to be the case with the YH A731 mutant (not shown). By using the Swiss PdbViewer, we have estimated that the distance between the histidine and the keto group of the phytyl side chain is more than 4 Å, i.e. too large to form a hydrogen bond. 

Taken together the results give evidence that the few amino acid variations between the different species cannot account for all the differences between the spectral properties of P700 from higher plants, algae, and cyanobacteria.

**Effects of the Mutations on the Redox Properties of P700\textsuperscript{−}/P700**—The redox potential is a measure for the energy difference between the neutral ground state and the oxidized state of P700. The energy of the oxidized state can be influenced by electrostatic effects like permanent dipoles, charges, and the polarizability of the surrounding residues. Hydrogen bonding in turn can stabilize the neutral ground state. The influence of hydrogen bonds on the oxidation midpoint potential has been demonstrated for the primary donor of PS I in C. reinhardtii (17) and for bacterial reaction centers (45). It should be noted that in these cases the carbonyl groups involved in the hydrogen bonding interaction were part of the conjugated π-system.

Tyr-A731 and Tyr-B659 are putative hydrogen bond donors to the keto groups of the phytyl side chain of P\textsubscript{A} and P\textsubscript{B}, respectively. Substitution of Tyr-B659 by His leads to a decrease of the oxidation midpoint potential of P700\textsuperscript{−}/P700 (a similar effect is observed for Tyr-A731, not shown), but the redox potential remains unchanged in the mutant YA B659. Since in both cases the hydrogen bond has probably been broken, the results indicate that the effects cannot be correlated with a specific influence of the hydrogen bond; this was the case after the hydrogen bond to the 13\textsuperscript{1} keto group of P\textsubscript{A} had been removed. A possible explanation is that the keto groups of the phytyl side chains are not part of the conjugated π-system. However, it seems more likely that the histidine in close proximity exerts an influence on P700. A slight diminution of the midpoint potential was also observed for WH A679 where no hydrogen bond has been removed.

**Distribution of the Electron Spin in P700\textsuperscript{−}**—ENDOR spectroscopy was applied to get information on the localization of the spin. The electronic structure of Chl a\textsuperscript{−} is sensitive to environmental changes, for example, ligation, hydrogen bonding, and electrostatic interactions (46). Likewise, the spin density distribution in P700\textsuperscript{−} can be perturbed by environmental effects, conformational changes of the chlorine ring, or changes in the distance and relative orientation of the two Chls constituting P700. ENDORE spectroscopy applied to P700\textsuperscript{−} leads to an assignment of proton hfcs of the spin carrying Chl, P\textsubscript{B} (12, 13). Unfortunately, an assignment of the hfcs of P\textsubscript{A} has not been achieved so far. Compared with monomeric Chl a\textsuperscript{−} in organic solvents, the hfcs of the 12- and 2-methyl groups of P700\textsuperscript{−} are reduced, whereas the hfc of the 7-methyl group is increased (13). It has been argued that the all over decrease of spin density on P\textsubscript{B} as compared with monomeric Chl a\textsuperscript{−} in organic solvents reflects a delocalization of spin density between the two halves of P700, and it has been estimated that at least 85% of the spin density is localized on P\textsubscript{B} (13). Comparison of the hfcs of the methyl groups shows essentially no difference among the different species giving evidence that the electronic structure of P700\textsuperscript{−} is the same for all organisms. The same observation holds for different preparations from different C. reinhardtii strains (47) showing that the electronic structure of the P700\textsuperscript{−} is not susceptible to different preparation methods. However, studies on mutant reaction centers have shown that the spin density distribution can be specifically influenced by mutations that have a direct influence on the primary donor in bacterial reaction centers (48) or PS I (12, 17). ENDORE studies have been performed with mutants in which the axial His ligands to P\textsubscript{A} and P\textsubscript{B} were substituted by
several other amino acid residues. They revealed that only mutations to P\textsubscript{1} lead to changes in the spectra, whereby only changes of the hfc of the 12-methyl group were resolved. This increase of only one hfc has been interpreted as a modification of the local environment of this methyl group (12). In contrast, mutations where the hydrogen bond to the 13\textsuperscript{1} keto group of P\textsubscript{A} was removed led to changes of the hfc of the other half of the dimer P\textsubscript{B} which was explained by a spin density shift from P\textsubscript{B} toward P\textsubscript{A}. The observed redistribution could therefore be taken as evidence for an electronic coupling between the two halves of the dimer as this was the first time that a mutation specifically affecting P\textsubscript{A} showed an influence on the hfc attributed to P\textsubscript{B}. This could be rationalized within a dimer model that was originally proposed for the primary donor of the bacterial reaction center (17). The ENDOR results for the different mutants are also summarized in Table I. The changes in the hfc of the different methyl groups of the mutants WA, WY, and WH A679 are very close to the error limit. It seems that the substitutions of Trp-A679 do not significantly affect the electron density distribution and that the slight changes might be the result of only minor perturbations in the surrounding of P700, because the distance between this Trp residue and the 7-methyl group is only about 4 Å. In contrast, mutations of Tyr-B659 display a larger effect on the hfc of the 12-methyl group comparable with the ones observed for the mutation of the axial His ligands. Conformational changes of the chlorine ring of the spin carrying Chl P\textsubscript{B} have been proposed as an explanation for these results (12). Likewise, it can be speculated that substitution of Tyr-B659 by His or Ala induces a slight conformational change. On the other hand, the ZFS parameters for all the mutants under study calculated from the ADMR spectra (data not shown) are only slightly different (less than 1%) from the values previously obtained (17) for native PS I from C. reinhardtii (|D| = 0.0280 cm\textsuperscript{-1}, |E| = 0.0039 cm\textsuperscript{-1}). The small conformational distortions possibly caused by the mutations can probably not be revealed by changes of the ZFS parameters. The question of the triplet state location still remains to be clarified due to the lack of effects on these parameters induced by variations in the P700 protein environment either on the PsAa or PsAb subunit (12, 49). If we assume that Tyr-B659 donates a hydrogen bond to the keto group of the phytyl side chain of P\textsubscript{B}, mutating this residue will lead to the removal of the hydrogen bond. As the phytyl side chain does not belong to the conjugated π-orbital system, a specific effect of the hydrogen bond on this π-system can be excluded. However, the hydrogen bond might keep the phytyl side chain in a certain conformation. Upon breakage of the hydrogen bond the phytyl side chain might become more flexible leading to slight conformational changes of the chlorine ring and concomitantly to a redistribution of spin density within the macrocycle of P\textsubscript{B}. Interestingly, the hfc for the methyl groups of C. reinhardtii and T. elongatus are essentially the same, although it is exactly the residue at position B659 that is different for the two organisms. Although Trp is not a hydrogen bond donor, it might be able to keep the phytyl side chain in a certain position due to its size. This is in contrast to the Ala or His mutations in C. reinhardtii where not only the hydrogen bond is broken but both residues are smaller. This is also in line with the fact that Ala is the smallest residue showing the largest effect.

The relatively small differences between the hfc for the species can be rationalized by the fact that the cation is mainly localized on one of the two Chls constituting P700. In contrast, the delocalization of the excited singlet states over the six Chls of the P700 reaction center may explain the susceptibility of the optical properties to slight structural differences in the various species.

In summary, all the deviations observed in the optical spectra and in the reaction center properties clearly suggest the presence of significant differences in the properties and geometrical arrangement of the cofactors in PS I from different species despite the high degree of sequence homology. Furthermore, the results obtained with site-directed mutagenesis show that the deviations observed cannot be put down to single non-conserved amino acids in the vicinity of the cofactors. Such deviations could possibly be explained by long-range effects due to variations of amino acids on PsAa and PsAb located further away which might slightly modify the protein-cofactor and cofactor-cofactor interactions. Therefore, it should be emphasized that the 2.5-Å structure of PS I from T. elongatus cannot be directly used for structurally based simulations of spectra and functional properties of PS I from other species.

Acknowledgments—We are grateful to Marianne Çetin, Heidi Pannier, and Claudia Schulze (Technische Universität Berlin) for excellent technical assistance. We thank Hanno Kass for the ENDOR measurements of different species and Ludwig Krabben and Uwe Fink for help in the initial stage of this work. We thank Margitta Dathe (Max-Delbrück-Center, Berlin-Buch, Germany) for the opportunity to use the CD spectrometer in her laboratory and for help with the CD measurements. We are also indebted to Matthias Rogner, University of Bochum, for providing us from Synechocystis.

REFERENCES

Spectroscopic Properties of P700