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HIGH-RESOLUTION ABSORBANCE-DIFFERENCE SPECTRA OF THE TRIPLET STATE OF THE PRIMARY DONOR P-700 IN PHOTOSYSTEM I SUBCHLOROPLAST PARTICLES MEASURED WITH ABSORBANCE-DETECTED MAGNETIC RESONANCE AT 1.2 K

EVIDENCE THAT P-700 IS A DIMERIC CHLOROPHYLL COMPLEX

H.J. DEN BLANKEN and A.J. HOFF

Center for the Study of the Excited States of Molecules and Department of Biophysics, Huygens Laboratory, State University of Leiden, Leiden (The Netherlands)

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ESR transitions in zero field of the triplet state of the primary donor (P-700^T) of Photosystem (PS) I subchloroplast particles of spinach have been measured by monitoring the absorbance at 697 nm at 1.2 K (absorbance-detected magnetic resonance (ADMR) spectra). Using the ADMR technique, we have recorded the triplet absorbance-difference spectrum (triplet-minus-singlet spectrum) of P-700^T minus P-700 of the primary donor of PS I at 1.2 K. This method is considerably more sensitive and selective than conventional flash absorption spectroscopy. The ADMR-monitored triplet-minus-singlet spectra of fresh and aged PS I particles are compared to published triplet-minus-singlet spectra of monomeric and dimeric chlorophyll (Chl) *a* in vitro. The triplet-minus-singlet spectrum of P-700 can be explained as a superposition of the triplet-minus-singlet spectrum of a dimeric Chl *a* complex and an additional red shift at about 689 nm, attributed to an adjacent Chl *a* molecule. We conclude that the primary donor of PS I is a dimeric Chl *a* complex with its long-wavelength absorption band peaking at 697 nm.

Introduction

The question as to whether the primary donor, P-700, of PS I of plants is a monomeric or a dimeric Chl *a* species is controversial. The bands of the light-induced absorbance-difference spectrum of P-700$^+$ minus P-700 [1] have been interpreted in several ways (see Ref. 2 and references therein), but no consensus has been reached with regard to the number of chlorophyll molecules making up P-700. Schaffernicht and Junge [3,4] could fit the absorbance-difference spectrum and the linear dichroism spectrum of photoinduced absorbance changes on the basis of the assumption that P-700 is a dimer. ESR and proton ENDOR data of P-700$^+$ were interpreted by Norris et al. [5,6] as proof that the odd electron of P-700$^+$ is delocalized over two chlorophyll molecules, the so-called 'special pair'. In contrast, Wasielewski et al. [7] recently proposed that P-700 is a monomeric Chl *a* enol. Support for this hypothesis was obtained by Norris et al. [8] who carried out a second-moment analysis of the line shape of the ESR signal of P-700 in fully (greater than 90%) ^{13}C ($I = 1/2$) -substituted algae and that of ^{13}C-labeled Chl a^+ in vitro. They found that the ratio of the second moment of the ESR line in vitro to

Abbreviations: ADMR, absorbance-detected magnetic resonance; PS, photosystem; Chl, chlorophyll; LDS, lithium dodecyl sulfate; BChl, bacteriochlorophyll.

that in vivo was close to unity, indicating that the odd electron is localized on one chlorophyll molecule. The results of the earlier ESR and ENDOR work on ^{12}C-P-700 were explained by assuming that the spin density on carbon atoms adjacent to the β-protons is not representative of the average carbon spin density in P-700$^+$.

Another probe of the structure of P-700 is the absorbance-difference spectrum of PS I reaction centers with P-700 in the light-induced triplet state minus that with P-700 in the singlet ground state. We will call this type of difference spectrum a triplet-minus-singlet spectrum. The triplet-minus-singlet spectrum has in common with the more generally studied P-700 oxidized-minus-reduced difference spectrum of PS I reaction centers the bleaching(s) of the singlet absorption bands of the pigment that is excited into the triplet state or photooxidized. The triplet-minus-singlet spectrum differs from the oxidized – reduced spectrum in that it exhibits the appearance of triplet-triplet absorption bands instead of the absorption spectrum of the cation. Band shifts of neighboring pigments caused by the introduction of the triplet state or of the cation will in general be different for the two types of optical difference spectra. It has recently been shown [9,10] that triplet-minus-singlet spectra of the (dimeric) primary donor in bacterial reaction centers taken at low temperature show detailed structure in the near-infrared region that contains information on the bleaching of exciton bands and the appearance of a monomer band, and on shifts of bands of accessory pigments. In particular, the technique of ESR of the triplet state detected optically via the singlet ground-state absorbance (ADMR) has been shown to permit the recording of high-resolution triplet-minus-singlet spectra with very good sensitivity [10]. This has prompted us to apply the ADMR technique to subchloroplast particles containing the P-700 reaction center, under conditions that the triplet state of P-700 is generated via the back-reaction between P-700$^+$ and the reduced 'first' electron acceptor (for reviews on triplet formation in photosynthesis by radical pair recombination, see Refs. 11 and 12).

In this communication we first report on the ESR signals of P-700^T obtained with the ADMR technique in various subchloroplast particles (the ADMR spectrum). We then present the ADMR-monitored absorbance-difference spectra of P-700^T – P-700 of these PS I particles and compare them to spectra obtained with flash spectroscopy of subchloroplast particles [13,14] and of monomeric and dimeric Chl *a* in vitro [15–17]. We will show that the triplet-minus-singlet spectra of a covalently linked pyro-Chl *a* dimer [16] in benzene/0.5 M methanol and of a Chl *a* dimer [17] in methylcyclohexane/0.01 M methanol show a striking resemblance to the ADMR-monitored triplet-minus-singlet spectrum of P-700. From this we conclude that P-700 is a dimeric Chl *a* species.

Materials and Methods

PS I particles of spinach chloroplasts containing about 100 Chl/P-700, so-called Triton (TSF 1) particles, were prepared according to Ref. 18. PS I particles containing about 40 Chl/P-700, so-called LDS particles *, were prepared according to a modification of the method of Ref. 19 as described in Ref. 20, with 2% LDS. The LDS particles were depleted of the ferredoxin-type acceptors F_X, F_A and F_B [21,20] as checked with ESR. Reduction of the Triton particles was carried out as described in Ref. 20. The samples were diluted to contain 65% (v/v) ethylene glycol or glycerol to obtain optically clear samples. The samples were free of cracks when slowly cooled to 77 K. The absorbance of the sample, contained in 3 mm inner diameter quartz tubes, was 0.7–1.3 per 3 mm at 680 nm.

Measurements of absorbance-detected electron spin resonance in zero magnetic field (ADMR) were carried out as described previously [22]. The excitation source was an iodine-tungsten 250 W projector lamp filtered by a Schott KG 3 (4 mm) glass filter. Kinetic measurements were performed with the pulse method [23–26] under excitation via Schott KG 3 (4 mm) and RG 695 (3 mm) glass filters and a Balzer AL 696 interference filter. ADMR-monitored absorbance-difference spectra of the triplet state minus the singlet ground state were recorded as detailed in Ref. 10.

* To avoid confusion with other membrane preparations we do not employ the label CP 1 as used by other authors for this type of particle [28,14].

Results

Absorbance-detected ESR spectra in zero field of P-700^T

LDS particles do not have the ferrodoxin acceptor complement, and at cryogenic temperatures a triplet state is observed by ESR when the particles that had been frozen in the dark are illuminated [19]. The special polarization pattern of the high-field ESR spectrum is indicative of triplet formation by radical recombination [27]:

$$P\text{-}700^+A^- \rightarrow P\text{-}700^TA$$

where A is an acceptor complex consisting most probably of two chlorophyll-type acceptors, A_0 and A_1 [20]. Fig. 1 shows the ADMR triplet spectrum of freshly prepared LDS particles. The transition frequencies correspond to values of the zero-field splitting parameters $|D|$ and $|E|$, that are equal to those measured with high-field ESR (Table I). The sign of the transitions corresponds to a decrease in transmittance at 697.5 nm, i.e., an increase in the singlet ground-state population of P-700. The ADMR spectrum of fresh Triton particles was identical to that of fresh LDS particles. When aged Triton and LDS particles were used, which had been repeatedly freeze-thawed, the ADMR spectrum showed an extra set of resonances, the intensity of which depended on the ageing procedure. Fig. 2 shows the ADMR triplet spectrum of aged Triton particles. Apparently, ageing produces P-700 centers with slightly different values of $|D|$ and $|E|$ caused by a slightly different configuration and/or environment of the pigments. We will call these structurally different centers 'altered P-700 centers', although it should be kept in mind that we have no indication that the photochemical properties of these centers are changed. Samples prepared with ethylene glycol and with glycerol gave identical spectra.

The ADMR spectrum of Fig. 1 bears much resemblance to those found for bacterial reaction center triplets [29,30]. The transition lines are comparatively narrow, and only the $|D| \pm |E|$ transitions are observed. Presumably, the $2|E|$ transition has a low intensity because of almost equal steady-state population of the two levels connected by the resonant microwaves.

The kinetics of the triplet state in intact P-700 centers were measured as a function of incident light flux by monitoring the change in transmittance after a brief pulse of microwaves with frequency corresponding to the $|D|+|E|$ or the $|D|-|E|$ transition of Fig. 1. At the lower light intensities the kinetics were independent of light flux, hence in this region the true molecular decay rates k_x, k_y and k_z are measured [23,25]. The values of the decay rates (Table I) correspond with those previously measured with high-field ESR

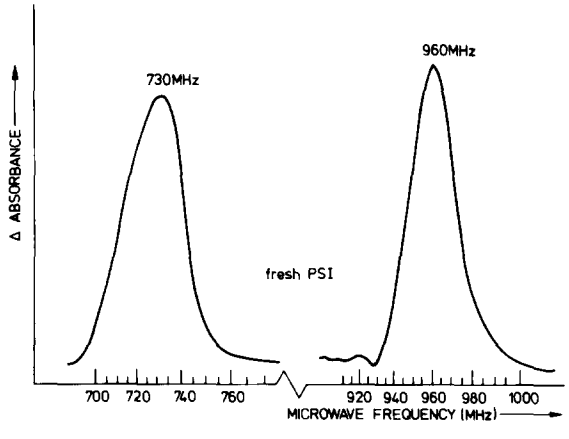

Fig. 1. ADMR transitions of fresh PS I LDS subchloroplast particles at 1.2 K. The detection monochromator was set at 697 nm with 3.2 nm resolution. The spectra are single scans with amplitude modulation of the microwaves at 35 Hz and lock-in detection. Total scan time 100 s, response time 1 s.

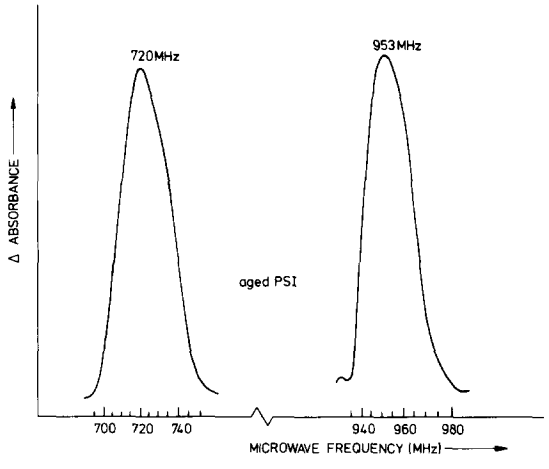

Fig. 2. ADMR transitions of aged PS I Triton subchloroplast particles at 1.2 K. The detection monochromator was set at 702 nm with 3.2 nm resolution; scale and other parameters as in Fig. 1.

[27,19]. Our values of the decay rates are more accurate, because, in contrast to the high-field method [32], with the pulsed ADMR method in zero magnetic field one does not have to extrapolate the microwave power to zero.

ADMR-monitored absorbance difference spectra P-700^T – P-700

Fig. 3 displays the triplet-minus-singlet spectrum of P-700 of fresh LDS particles, monitored with the microwave frequency set at the triplet $|D|+|E|$ ADMR transition of Fig. 1. Also shown in Fig. 3 is the flash-induced triplet-minus-singlet spectrum of a Chl *a* dimer in methylcyclohexane/0.01 M methanol measured by Periasamy and Linschitz [17] and the triplet-minus-singlet spectrum of monomeric Chl *a* in ethanol measured by Hurley et al. [15]. For ease of comparison we have reversed the sign of the ADMR-monitored triplet-minus-singlet spectrum of P-700 (the resonant microwaves cause a decrease in triplet concentration, whereas the flash technique measures an increase). It is seen that the triplet-minus-singlet spectra of P-700 and the Chl *a* dimer in vitro [17] are very similar, and that the triplet-minus-singlet spectrum of monomeric Chl *a* in vitro [15] deviates considerably from either of the other spectra. Fig. 4 displays the 620–700 nm region of the triplet-minus-singlet spectrum of P-700 in greater detail. Fig. 5 shows the ADMR-monitored P-700^T – P-700 spectrum of altered P-700 centers.

Discussion

ADMR spectra, zero-field splitting parameters and decay rates

For photosynthetic preparations the ADMR

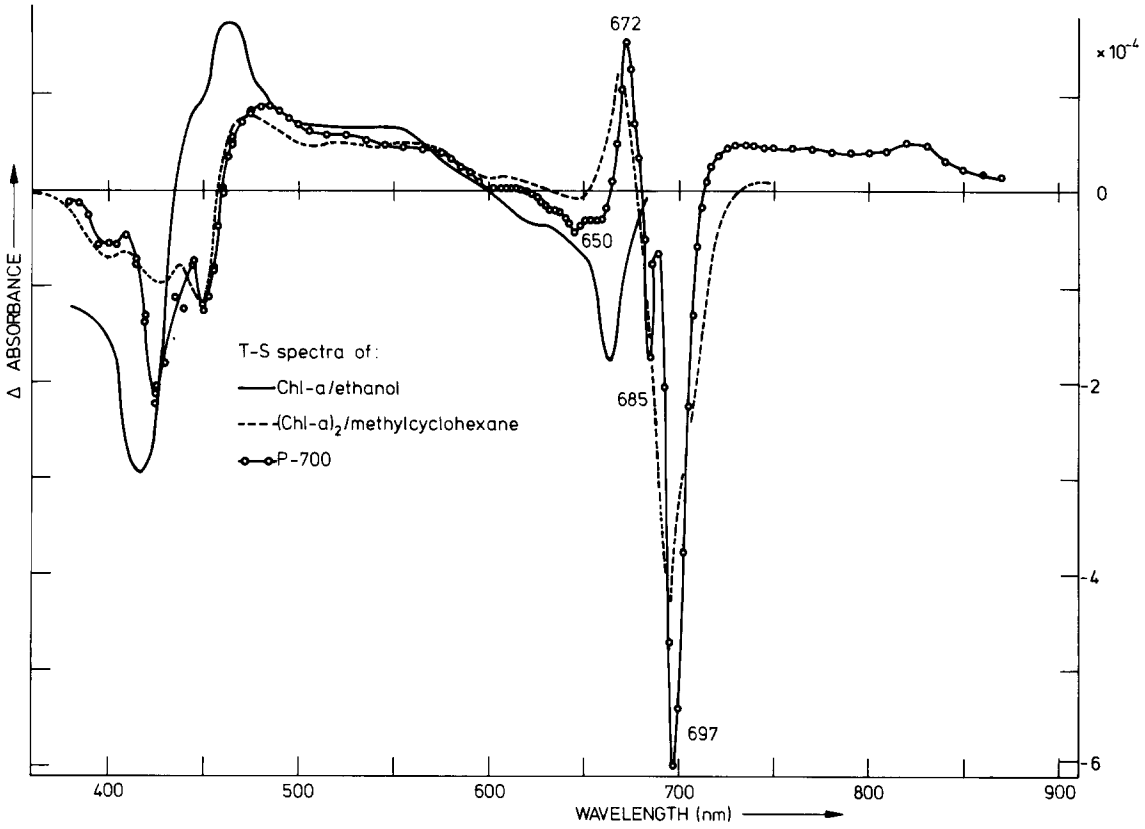

Fig. 3. The ADMR-monitored P-700^T-minus-P-700 absorbance-difference spectrum of fresh PS I LDS subchloroplast particles (triplet-minus-singlet spectrum) at 1.2 K (O——O). The resonant microwaves were set at 960 MHz; the optical resolution was 3.2 nm. Solid line: the flash-induced triplet-minus-singlet spectrum of monomeric Chl *a* in vitro at approx. 25 °C [15]; dashed line: the flash-induced triplet-minus-singlet spectrum of a Chl *a* dimer in vitro at −78 °C [17]. For the other parameters see Fig. 1.

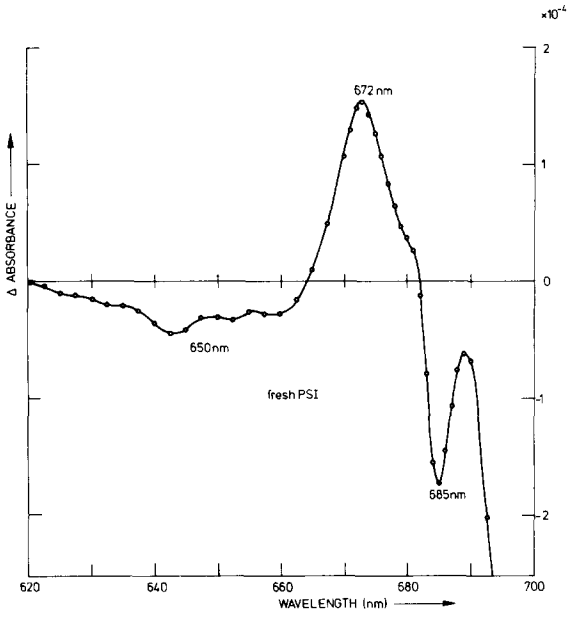

Fig. 4. Enlargement of the P-700^T – P-700 spectrum of fresh PS I LDS subchloroplast particles of Fig. 3.

technique has a number of advantages over the previously employed fluorescence-detected ESR of triplet states in zero field (FDMR):

(i) The pigment on which the triplet state giving rise to the ESR transitions in zero field is located is more easily identified, because the absorbance spectrum usually contains a number of identified bands as opposed to a paucity of bands in the fluorescence spectrum (because of excitation energy transfer to fluorescence bands at longer wavelength).

(ii) The sign of the ADMR signals directly reflects the change in triplet concentration, whereas in the FDMR technique sign reversal because of energy transfer may take place [22,24,33]. Additional experiments are then needed to ascertain the presence or absence of energy transfer in order to permit conclusions as to the identity of the pigment on which the triplet state resides (e.g., antenna or reaction center chlorophyll).

(iii) With ADMR it is usually a simple matter to determine which fraction of pigments absorbing at a given wavelength is reactive to microwaves, in contrast to FDMR where a very small concentration of impurities with high fluorescence quantum yield may be responsible for all, or a large part, of

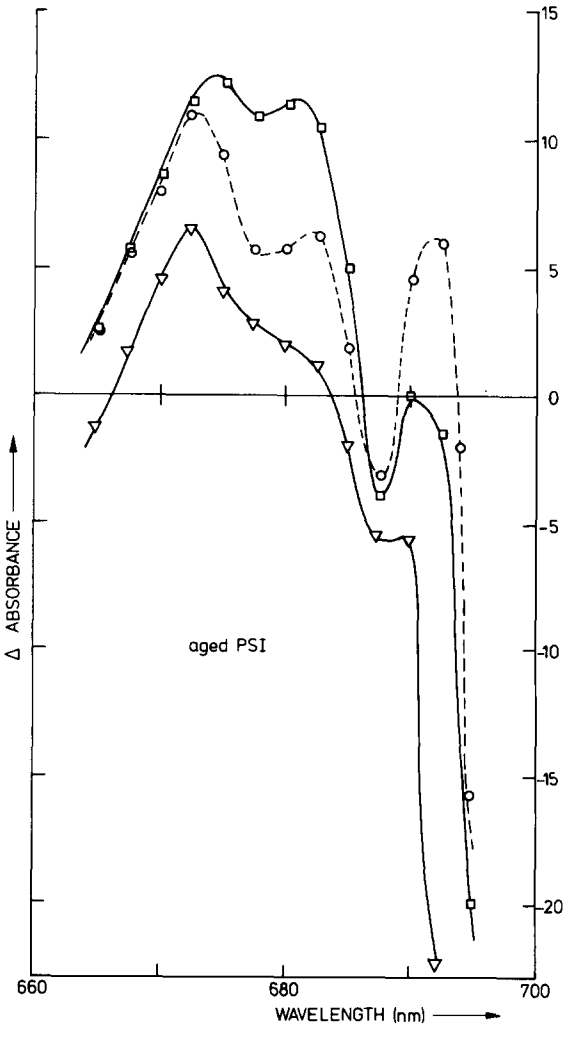

Fig.5. AMDR-monitored P-700^T – P-700 spectra of two differently aged PS I LDS subchloroplast particles at 1.2 K. The microwaves were set at different positions within the $|D|+|E|$ ADMR transitions, the optical resolution was 3.2 nm. The vertical scale is in arbitrary units.

the microwave-induced change in fluorescence. This is especially true for pigments that transfer excitation energy to a photochemical trap, such as the photosynthetic reaction center. Closing the trap by formation of a triplet state may enhance the fluorescence quantum yield of such pigments by several orders of magnitude (for a discussion of this effect, see Ref. 33).

(iv) For pigments with low fluorescence yield, such as commonly found in photosynthetic preparations, the ADMR technique has far greater

sensitivity than the FDMR method, mainly because of the reduction of photodetector quantum noise.

Our ADMR spectra can be unambiguously assigned to P-700^T: (i) we see no transitions when the bulk antenna chlorophylls are monitored at 680 nm, (ii) the $|D|$ and $|E|$ values correspond to those of the spin-polarized triplet signals measured with high-field ESR [27]. Furthermore, we have determined that the application of resonant microwaves with frequencies corresponding to the $|D| \pm |E|$ transitions of P-700^T, i.e., at 730 and 960 MHz for intact particles and 720 and 753 MHz for altered reaction centers, causes a decrease in triplet concentration. These two findings aid in the interpretation of the often complex FDMR spectra obtained in chloroplasts, algae and PS I particles [34–39]. For example, only those FDMR transitions which occur at the correct microwave frequency can be due to P-700^T, regardless of the fluorescence band on which they are detected. As P-700 itself will show only very little fluorescence or none at all (either the trap is open, or it is in the triplet state), the fluorescence which in FDMR experiments is reactive to microwaves resonant with the P-700^T triplet transitions is due to antenna or accessory pigments. The sign should then be negative, i.e., a decrease in fluorescence, because of energy transfer [22,24,33]. If the sign of the microwave-induced change in fluorescence is positive as is sometimes observed [36–39], it then follows that the fluorescence of the pigment under consideration is quenched by P-700^T. Further detailed discussion of the ensuing interpretation of published FDMR experiments in plant photosynthetic material is outside the scope of the present paper.

The $|D|$ and $|E|$ values of P-700^T are close to those found for monomeric and dimeric Chl *a* in vitro [40,41]. Attempts have been made to correlate the magnitude of the zero-field splitting parameters of reaction center triplets to structural properties of the primary donor, i.e., dimer vs. monomer, geometry of a dimeric complex, etc. [42–44] (see, for an extensive discussion, Ref. 12). Such attempts would have limited validity, were it only for the fact that variation of the ligands to the central magnesium of Chl *a* may change the values of $|D|$ and $|E|$ by as much as 10% [41]. In addition, admixture of relatively small charge-transfer contributions to the wave function of a triplet state in a dimeric complex may strongly vary the magnitude of the zero-field parameters [45]. Hence, the close correspondence of the $|D|$ and $|E|$ values of P-700^T to those of monomeric or dimeric Chl *a* in a particular solvent may not be taken as evidence that P-700 is a monomer, or that the macrocycles of a purported Chl *a* dimer are parallel.

The kinetics of P-700^T measured with the ADMR technique agree well with those measured with high-field ESR (Table I). The values of k_u ($u = x,y,z$) reported by Van Brakel [37] for an FDMR-monitored triplet state in PS I particles deviate from those collected in Table I. This is probably caused by the use of the equilibrium method for the kinetic measurements. As demonstrated in Refs. 24–26, this method does not give the correct decay rates.

Comparison of our decay rates with those measured for ^{3}Chl *a* in various solvents might, in

TABLE I

CHARACTERISTICS OF THE TRIPLET STATE OF THE PRIMARY DONOR OF PS I

PS I particles	D (cm^{-1}) ($\times 10^{-4}$)	E (cm^{-1}) ($\times 10^{-4}$)	k_x (s^{-1})	k_y (s^{-1})	k_z (s^{-1})	Ref.
Digitonin	278 ±9	39 ±9				27
Triton	278 ±2	38 ±2				61
LDS	283	40				19
SDS (CP I)	280 ±5	38 ±2	1150±350	1050±350	⩽130	31
Triton			1100±110	1300±130	83±16	21
Fresh Triton	281.7±0.7	38.3±0.7	990±100	1010±100	92± 5	this work
Fresh LDS	281.7±0.7	38.3±0.7				this work
Aged Triton	278.9±0.7	38.8±0.7				this work
Aged LDS	278.9±0.7	38.8±0.7				this work

principle, yield information on the aggregation state of the chlorophyll(s) in P-700. Again, however, the nature of the ligand and the coordination number of the central magnesium influence considerably the magnitude of the decay rates [41] and no conclusions as to the structure of P-700 can be drawn from the decay rates, alone or in combination with the values of the zero-field splitting parameters.

The triplet-minus-singlet ground-state absorbance-difference spectrum P-700^T – P-700

The primary electron donor in photosynthesis is a (bacterio)chlorophyll molecule or complex with very special status: among the many (bacterio)chlorophylls present in a photosynthetic unit, it alone is capable of very fast charge separation upon photoexcitation. The nature of its special character is still an open question. A more or less trivial explanation would be that it is the only chlorophyll in the unit that is closely coupled to a suitable electron acceptor. More likely, however, the special character of the primary donor arises because either it consists of a special arrangement of more than one pigment, or interaction with the protein matrix conveys to it special electron-donating properties. Alternatively, the donor is not a common chlorophyll but a derivative tailored to function as a photochemical trap [46].

In bacterial photosynthesis it is now generally accepted on the basis of ESR and ENDOR experiments on the oxidized primary donor that this donor is a BChl *a* dimer [47,48]. Additional evidence for its dimeric character is provided by the absorbance-difference spectra of the photooxidized donor [49] and of its triplet state [9,10]. It is therefore likely that in the bacterial reaction center fast electron donation is made possible by pairing of two BChl molecules [50].

In plant PS I the situation is much less clearcut. Initially, ESR and ENDOR data were interpreted as evidence that P-700 is a dimer [5,6]. This view was supported by the deconvolution of the absorbance-difference spectrum P-700$^+$ – P-700 and of the photoselected linear dichroism spectrum [3,4]. Recently, however, Norris et al. [8] showed that the line shape of P-700$^+$ in ^{13}C-substituted algae could only be explained on the assumption that the odd electron is localized on one Chl *a* molecule. Concurrently, Wasielewski et al. [7] proposed that P-700 is a monomeric Chl *a*-enol. The spin density distribution on the carbons of the enol is such that in ^{12}C-Chl *a*-enol the ESR linewidth of the cation (mainly determined by proton hyperfine interaction) is about 1.7-times less than that of ^{12}C-Chl *a*, whereas for ^{13}C-labeled Chl *a*-enol (where the linewidth is governed by hyperfine interaction with the $I = 1/2$ ^{13}C nuclei) the linewidth (or more accurately the second moment) is practically equal to that of monomeric ^{13}C-labeled Chl a^+ in vitro.

The study of the triplet-state spectrum of P-700 provides an independent assessment of the structure of P-700. The ADMR-monitored triplet-minus-singlet spectrum displayed in Fig. 3 shows a striking resemblance to the triplet-minus-singlet spectrum obtained by Periasamy and Linschitz [17] of the flash-induced triplet state of a Chl *a* dimer in methylcyclohexane + 0.01 M methanol at −78°C. This spectrum is similar to the triplet-minus-singlet spectrum of a covalently linked pyro-Chl dimer in the 'folded' form [16]. The triplet-state spectra of both the in vitro dimer systems were interpreted to result from the formation of a triplet state that becomes localized on one of the constituent monomers. In this interpretation [16,17] the 695 and 648 nm bands in the red and the 449, 427 and 400 nm bands in the Soret region represent the bleaching of the singlet absorbance spectrum of the dimer. The bands between 460 and 620 nm were ascribed to the appearance of the triplet absorbance spectrum of a Chl *a* monomer and the band at 670 nm to the appearance of the singlet absorbance spectrum of the second Chl *a* monomer.

Since the triplet-minus-singlet spectrum of P-700 is so similar to the triplet-minus-singlet spectrum of the Chl *a* dimers, we interpret the triplet-minus-singlet spectrum of P-700 in the same way: we ascribe the bands at 697 and about 650 nm in the red and those at 450, 425 and 400 nm in the Soret region to the bleaching of the singlet spectrum of a dimeric complex, the bands appearing between 460 and 620 nm and 715–870 nm to the triplet spectrum of a Chl *a* monomer and the band appearing at 672 nm to the singlet spectrum of the other Chl *a* monomer that forms part of the dimeric complex. Thus, the strong reduction of the

exciton interaction in the dimeric P-700 complex by the formation of the triplet state leads to localization of this state on one member of the dimeric Chl *a* complex, whereas the second member absorbs at a wavelength close to that of monomeric Chl *a* in vitro. The integrated intensity in the triplet-minus-singlet spectrum of the band assigned to the singlet monomer, which appears at 670 nm in the in vitro complexes [16,17] and at 672 nm in the P-700 preparation, is less than expected for a noninteracting Chl *a* monomer. The same phenomenon was observed in the triplet-minus-singlet spectra of the primary donor in bacterial photosynthesis [10]. At present, we have no ready explanation for this effect. In porphyrins the Q_y transition is only weakly allowed; in chlorophyll it acquires intensity because of the lesser symmetry. Possibly, interaction of the singlet monomer with the triplet state on its partner in the dimer leads to an intensity redistribution between the bands of the singlet manifold.

The triplet-minus-singlet spectrum of Fig. 3 shows a broad absorption band between 720 and 850 nm, peaking at 820 nm. This band parallels the spectral features in that wavelength region observed by Setif et al. [14], albeit that in our spectrum the intensity at 820 nm is somewhat more pronounced. In the absorbance difference spectrum of ^{3}Chl *a* in vitro, only little intensity at 820 nm is found [51]; however, a clear increase in absorbance at 820 nm is observed in the absorbance-difference spectrum of oxidized-minus-reduced P-700 [14]. In conjunction with the observation that the 672 nm band of the triplet-minus-singlet spectrum is close to a Chl a^- absorption band [52,53], one might be tempted to ascribe both bands to a charge-transfer state mixed with the triplet state P-700^T. We note, however, that the Chl a^- absorption band and that of P-700$^+$ have been measured under conditions that only one charged species was present and thus do not reflect a Chl a^+ Chl a^- charge-transfer state. In addition, it is unlikely that all the intensity at 672 nm in the triplet-minus-singlet spectrum can be ascribed to a charge-transfer contribution, because (i) such a band is also seen in synthetic dimers, which in the triplet state are in an unfolded configuration [17], (ii) in PS II particles, the analog to the 672 nm band at 665 nm, but not the 820 nm is present (Den Blanken, H.J. and Hoff, A.J., unpublished observations). We therefore prefer to ascribe the 672 nm band to monomeric Chl *a*, without excluding the possibility that the 820 nm band is (partly) due to some charge-transfer contribution to the triplet manifold of P-700^T.

The triplet-minus-singlet spectrum of P-700 shows a somewhat higher resolution than that of the in vitro dimers, partly because the triplet-minus-singlet spectrum of P-700 was recorded at much lower temperature and partly because the ADMR technique selects sites with a particular set of $|D|$ and $|E|$ values, which translates in a distribution of optical sites that is somewhat narrower than the optical singlet absorption band [54].

The amplitude and location of the 685 nm band and other features in the 680–695 nm region are rather variable with preparation (Fig. 5). In fresh samples the 672 and 685 nm bands are well separated (Fig. 4) and have quite different widths. In aged particles the triplet-minus-singlet spectrum of P-700 shows a less pronounced band at 685 nm (Fig. 5) and, in addition, negative bands at 682 and 692 nm. The intensity variations of the bands at 682, 685 and 692 nm can be well explained by the combination of a red shift around 689 nm and a blue shift around 683 nm, both of which are dependent on the state of the sample. We think it unlikely that the band at 672 nm is part of a blue shift. It is tempting to correlate the band shifts around 683 and 689 nm with corresponding ones observed in triplet-minus-singlet spectra of bacterial reaction centers [10]. These were attributed to shifts of bands due to the two accessory BChl pigments present in the purified reaction centers. Thus, also in PS I reaction centers, two Chl *a* may be closely associated with the primary donor P-700.

The intensity of the band around 650 nm is also dependent on sample preparation, becoming more intense with ageing. We provisionally attribute this band to a weak blue exciton partner of the red-most band at 697 nm. In intact P-700 reaction centers the intensity of the band is very low, in aged reaction centers this band is growing, possibly because of slight change in the geometry of the P-700 dimer (less parallel transition moments).

Our absorbance difference spectra monitored by magnetic resonance of the triplet state (Figs.

3–5) correspond closely to the absorbance-difference spectra reported by Shuvalov et al. [13] and Setif et al. [14]. This constitutes decisive evidence that the former spectrum is not due to the reduction of an intermediate electron acceptor as suggested by Shuvalov et al. [13], but to a triplet-minus-singlet difference spectrum as proposed by Hoff [55] and Setif et al. [14].

If the 650 nm band constitutes the blue exciton partner of the 697 nm band, it possesses little oscillator strength in intact P-700 reaction centers. This agrees with the models for P-700 proposed by Shipman et al. [56] (two parallel partly overlapping macrocycles, with parallel transition moments), but clearly contrasts with the model proposed by Fong et al. [57,58] (two parallel macrocycles with transition moments making an angle of 60°). Simple exciton theory [59] predicts that in the latter model the intensities of the two exciton band should be in a ratio of 3 : 1 with the larger oscillator strength in the blue component.

The proposition by Wasielewski et al. that P-700 is a monomer Chl *a* enol appears not to be compatible with the P-700$^+$ – P-700 difference spectrum [60]. Comparison of the electronic absorption spectrum of the Chl *a* enol (Fig. 2 in Ref. 7) with that of the spectrum in Fig. 19 in Ref. 60 shows that the characteristic enol splitting at 360 and 440 nm is not reproduced in the experimental P-700$^+$ – P-700 spectrum. Moreover, the ratio of the intensity in the Soret to that of the Q-band region in the enol spectrum does not agree with that ratio in the oxidized-reduced spectrum.

In conclusion, we find that a dimeric P-700 complex fits our ADMR data very well. The exciton interaction between the two Chl *a* molecules of P-700 is strongly reduced by the formation of the triplet state. This is because the exciton interaction of a dimer in the triplet state is generally much weaker than that in the singlet state as the transition dipole moment of a singlet-triplet transition is small (it involves a 'forbidden' spin flip). As a result, the 700 nm band in the singlet-singlet absorbance spectrum is bleached, the triplet state is localized and a singlet and triplet absorbance spectrum of monomeric Chl *a* appears. The feature at 685 nm of the in vivo difference spectrum is not present in the in vitro difference spectra; it is well explained by a band shift of one or more accessory pigments in the reaction center of PS I.

The above interpretation of our triplet-minus-singlet spectrum of P-700 is in agreement with the interpretation by Schaffernicht and Junge [3,4] of the oxidized – reduced absorbance difference spectrum of P-700. The result of Norris et al. [8], that in P-700$^+$ the odd electron is not shared down to a time scale of 10–20 MHz, can be explained by assuming that the electronic coupling between the two Chl *a* molecules in the cationic complex is much weaker than in the unperturbed native state. This is not unrealistic, as the optical absorption spectra, and consequently the electronic structures, of Chl *a* and Chl *a*$^+$ are quite different.

Note added in proof (Received May 31st, 1983)

A recent paper [62] discusses the possibility that P-700 is a monomeric Chl *a* molecule in which the highest occupied and lowest unoccupied electron orbitals are mixed because of an (unspecified) interaction with the protein matrix. In P-700$^+$, this mixing would account for the deviation of the spin densities as measured by ENDOR with respect to those found for monomeric Chl *a*$^+$ in vitro. The validity of this proposal can obviously be further tested by a detailed calculation of the absorbance difference spectra (oxidized-minus-reduced and triplet-minus-singlet) as a function of mixing coefficient, and comparison with the experimental spectra.

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