ELSEVIER

FEBS Letters 343 (1994) 200–204

FEBS LETTERS

FEBS 13926

A well resolved ODMR triplet minus singlet spectrum of P680 from PSII particles

Donatella Carbonera[a], Giovanni Giacometti[a,*], Giancarlo Agostini[b]

[a]*Department of Physical Chemistry, University of Padua, Via Loredan 2, I-35131 Padua, Italy*
[b]*Centro Studi Stati Molecolari Radicalici ed Eccitati, CNR, Via Loredan 2, I-35131 Padua, Italy*

Received 24 February 1994; revised version received 17 March 1994

Abstract

An ADMR T–S spectrum of the primary donor (P680) of photosystem II (PSII) was obtained from anaerobically photoreduced particles. The spectrum is the best resolved obtained so far having a main bleaching band at 684 nm with a linewidth of only 100 cm^{-1}. The view that this spectrum is produced by native homogeneous P680 unlike those obtained before is defended. A small bleaching observed at 678 nm is discussed in terms of the reaction center structure. One possible interpretation of the observations is that P680 is a very loose dimer with an exciton splitting of only 144 cm^{-1} corresponding to a dimer center-to-center distance of roughly 11.5 Å.

Key words: Photosystem II; P680; T–S spectrum

1. Introduction

Optical spectroscopy, and optically detected magnetic resonance (ODMR) on purple bacteria photosynthetic reaction centers have been studied in recent years and the results were interpreted on the basis of the well-known X-ray diffraction structure [1–3]. This task is now more interesting for reaction centers of higher organisms for which direct X-ray structural information is still lacking. Although the similarities between the bacterial and plant reaction centers have been widely discussed, the T–S spectra seem to indicate differences in the interactions between pigments and in the structure of the primary donor [4]. The primary electron donor of PSII is associated with a spectral feature of 680 nm and is referred to as P680 [5,6]. A pheophytin molecule is known to act as an electron acceptor [7,8] in a light-induced charge separation which produces the radical pair $P680^{+}Ph^{-}$. In large PSII particles, just as in the bacterial centers, this charge separation is subsequently further stabilized by secondary electron transfer reactions. Double reduction of the Qa acceptor in PSII, or its absence as in isolated reaction centers, increases the radical pair lifetime and allows charge recombination giving a high P680 triplet yield [9,11]. This double reduction can be obtained by long exposure of the particles (several hours at room temperature) to reductant action. It has also been shown that during strong illumination under anaerobic conditions, the PSII centers undergo processes which facilitate light-induced formation of the P680 triplet [9,12].

^{3}P680 is the interesting species which can be observed by ODMR. This can be done either by observing the fluorescence (FDMR) or the absorption (ADMR) changes upon microwave resonance. In the absorption case, in particular, it is possible to record the Triplet minus Singlet (T–S) microwave induced absorption spectrum (MIA) of the species. We have recently demonstrated that ^{3}P680 observed by FDMR in isolated PSII reaction centers is a quite different spectroscopic object from that existing in larger particles [13]. In the latter the ^{3}P680 signal is quite narrow while in the isolated centers the corresponding signal is much wider and indicates the presence of heterogeneity clearly produced during the isolation procedures of the complex. The MIA spectrum of P680 was obtained earlier for isolated centers [4] but its analysis is complicated by this heterogeneity. For BBY particles ADMR was performed by den Blanken et al. [14] in 1983 but the results are difficult to reconcile with the most recent ones probably due to the prepara-

*Corresponding author. Fax: (39) (49) 831 328.

Abbreviations: PSI, photosystem I; PSII, photosystem II; BBY, photosystem II enriched membranes, known as BBY particles; D1D2, isolated reaction center of photosystem II known as D1D2-Cyt *b*-559 complex; P680, primary electron donor to photosystem II; RC, reaction center; ODMR, optically detected magnetic resonance; ADMR, absorption detected magnetic resonance; FDMR, fluorescence detected magnetic resonance; MIA, Microwaves induced absorption; LD-ADMR, linear dichroic ADMR; ZFS, zero field splitting; Qa, primary quinone acceptor of PSII; Chla, chlorophyll *a*; Ph, Pheophytin; MES, 4-morpholinecthanesulfonic acid; Tris, Tris(hydroxymethyl)aminomethane.

SSDI 0014-5793(94)00311-I

tion and to the scarce knowledge, at that time, concerning the production of the P680 triplet.

In this paper we reexamine the ADMR spectroscopy of BBY particles and verify the intactness of P680 in the latter as opposed to the presence of artificial heterogeneity in the isolated centers. We also investigate more deeply the origin of the heterogeneity by using the two different methods of population of P680 triplet (see above) and show that some damage also occurs for BBY when the particles are kept at room temperature for a long time. We show that, by using the photoreduction method, it is possible to obtain a highly resolved T–S spectrum of P680 containing spectral features which had hitherto escaped observation. Finally, we reexamine the possible interpretations of this T–S spectrum in terms of the structure of the primary donor of PSII.

2. Materials and methods

2.1. Apparatus

Our FDMR apparatus is described in [15]. The same apparatus as been adapted to ADMR experiments. The excitation light is also used to probe the absorbance: the beam, filtered by a 5-cm path of water, is focussed on a flat sample cell having an optimal path length of 1.5 mm, and after passing through the sample is refocussed on the slit of a monochromator Jobin Yvon HR250 equipped with DATA LINK. The intensity of the excitation beam is regulated by an iris diaphragm and a set of neutral filters. The transmitted light is dected by a hybrid photodiode OSI 5KM Centronic. The microwave apparatus is exactly the same as that used for FDMR experiments. The photodiode voltage is phase-sensitive detected by an EG&G 5210 lock-in amplifier at the frequency of the amplitude modulation of the microwaves and the signal is then divided by the photodiode voltage which is proportional to the total transmittance: monitoring this signal as a function of wavelength yields the T–S spectrum. Acquisition and plotting is controlled by PC software written in our laboratory.

2.2. Samples preparation

BBY particles were obtained from spinach leaves in the usual way [16]. The sample concentration was generally 40 μg/ml chlorophyll content. The medium was 0.4 M sucrose, 10 mM NaCl, 50 mM MES, pH 6. Glycerol was always added 66% vol/vol to the samples to avoid matrix cracking and heterogeneity. Oxygen was removed from the samples using a glucose/glucose oxydase system as described in [17]. 10 mM dithionite was added under nitrogen when required together with 30 μM methyl viologen and the sample incubated in darkness for the time necessary to insure double reduction of Qa. Anaerobic photoreduction was performed directly in the sample cell just before freezing in the cryostat for ADMR measurements. The samples were illuminated at room temperature with white light (150 W lamp) through a 5-cm-long water filter and an hot mirror (Ealing 35-6865) at 2000 μE $m^{-2} \cdot s^{-1}$ intensity, for different periods of time. Isolated RCs were prepared according to [18].

3. Results

Fig. 1 shows the normalized ADMR spectra (|D|–|E| and |D|+|E| transitions) obtained from BBY particles under steady-state illumination at 1.8 K and 683 nm detection wavelength. The spectra were obtained after the particles had been incubated for five hours with dithionite at room temperature, or after they had been photoreduced for five minutes at the same temperature. The reduced particles show resonances that are clearly shifted a few MHz towards higher frequencies and that are broader. No 2E transitions were detected.

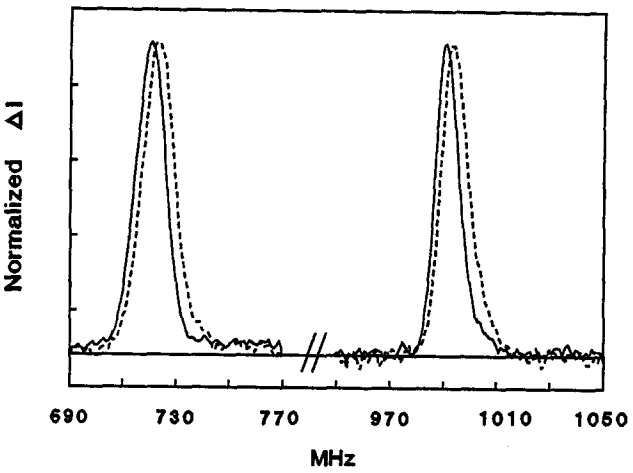

Fig. 1. ADMR spectra from 690 to 1050 MHz (all peaks are normalized to unity). Solid line: PSII particles after 5 min photoreduction; Dashed line: PSII particles after 5 h incubation with dithionite. Detection wavelength, 683 nm; modulation frequency, 33 Hz; microwave power, 13 mW; sweep rate, 3 MHz/s; time const., 300 ms; temp., 1.8K; 9 scans.

The differences in the resonance frequencies are even more evident on monitoring the absorbance at various wavelengths around the maximum: the |D|–|E| transitions detected in the range 680–688 nm are shown in Fig. 2. A similar distribution has been monitored for the |D|+|E| transition (not shown). We note that, for dithionite-treated samples, species absorbing at shorter wavelengths contribute more to the linewidth.

Our ADMR spectrum of the isolated RC is identical to that published by Van der Vos et al. [4], much broader than those of BBY and shifted to higher microwave frequencies and to shorter wavelengths with respect to singlet–singlet absorption (not shown). Fig. 3 shows the T–S spectrum of photoreduced PSII particles between 600 and 700 nm. In Fig. 4a spectra are also displayed for dithionite-treated PSII particles and for isolated RC, in the region where significant differences occur (between 660 and 700 nm). The recording was made at the maxima of the respective |D|–|E| transitions of ADMR spectra, with 1 nm resolution, and is normalized to emphasize the specific shape.

All of the spectra exhibit a strong bleaching at 684.5 nm (photoreduced PSII particles), 683.5 nm (dithionite treated PSII particles), 680 nm (RC), and with positive peaks at about 673 nm (PSII particles) or 671 nm (RC). Spectra from PSII particles show two small negative bands at 639 and 630 nm which are well reproducible and clearly not noisy. Interestingly the spectrum from the photoreduced sample also exhibits a small bleaching at about 678 nm. The preillumination time does not affect

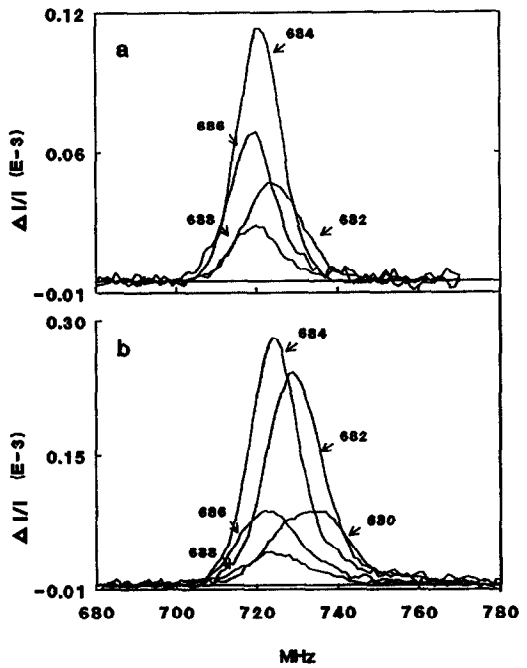

Fig. 2. ADMR spectra of PSII particles (|D|−|E| transitions) recorded at different wavelengths with 1 nm resolution; (a) after 5 min photoreduction; (b) after 5 h incubation with dithionite. Conditions as in Fig. 1.

the shape of the T−S signal but only its intensity which reaches the highest value after about 15 min and then decreases (data not shown), in agreement with the results of Vass et al. [12]. On the other hand, we have observed a sensitive dependence of the lineshape on the incubation time of the samples with reductant (ranging from 3 to 48 h) namely a progressive broadening of all the peaks and shift of the main bleaching at shorter wavelengths. In the case of reduced particles the triplet yield was highest after about 5 h of incubation and then slowly decreased (data not shown).

4. Discussion

It has been shown recently [4,13] that, during the isolation procedure, PSII reaction centers undergo modifications in the pigment's environment which produce a heterogeneity in the magnetic spectral properties of ^{3}P680, that is different ZFS values and a broadening of the zero field resonances, compared to those found for large PSII particles. From the T−S spectra of Fig. 3 it is clear that optical spectral changes also occur. The main bleaching, to be assigned to the primary donor, peaks 680 nm while for large particles it does so at about 684 nm. In view of the previous P680$^+$−P680 absorption difference spectra [8,19,20], it may be surprising to find P680 absorbing at such a long wavelength. However, most of the spectra mentioned are steady-state spectra which can be altered by the presence of oxidized antenna chlorophylls and they have been obtained when the preparations were not as good as they are today (note that experiments done on chloroplasts [21] give a red-shifted P680 absorption band). Moreover, the optical spectral bands are about three times as wide as for the T−S spectra because of the site selection of the magnetic resonance frequency in the ADMR experiment and because of the difference in temperature at which the experiments were carried out. It must also be noted that absorption of P680 extending up to 684 nm was observed even in isolated reaction centers [22,23].

In principle the difference between the absorption of P680 in isolated RCs and in large PSII particles could be due to the absence of Q_a, or to the removal of the Mn-cluster and antenna complexes, if these are able to influence directly the P680 'environment'. However, we have observed a blue-shift of the main bleaching together with band broadening even in PSII particles when left at room temperature for several hours, so that it seems likely that most of the shift in the isolated RC comes from protein alteration in the course of the isolation procedure. On the other hand Tris washing of the particles before photoreduction does not cause any blue shift in the main bleaching.

When comparing the ADMR and T−S spectra of dithionite treated and photoreduced samples (Figs. 2 and 4) it is evident that minor differences do exist. The latter probably come from alterations occurring during the long chemical treatment at room temperature which is used to double reduce Q_a rather than from some specific effect due to the photoreduction. Since double reduction and double protonation of Q_a takes place during chemical reduction as well as during prolonged photoreduction [12], we do not expect differences in the influence of

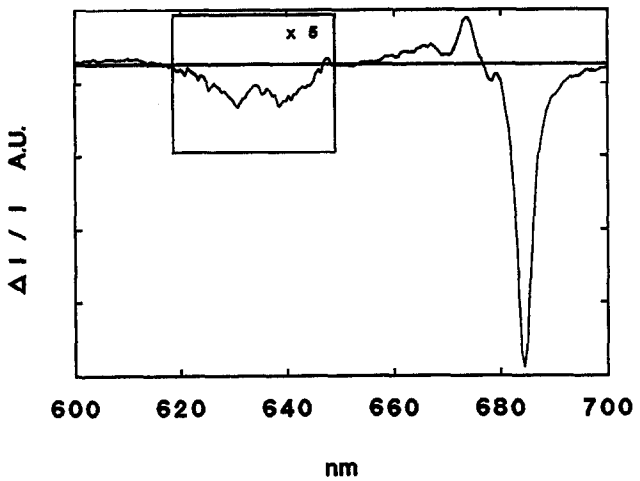

Fig. 3. T−S spectrum of photoreduced PSII particles from 600 to 700 nm. Microwave frequency, 721 MHz; sweep time, 0.13 nm/s; temp., 1.8K. Other conditions as in Fig. 1.

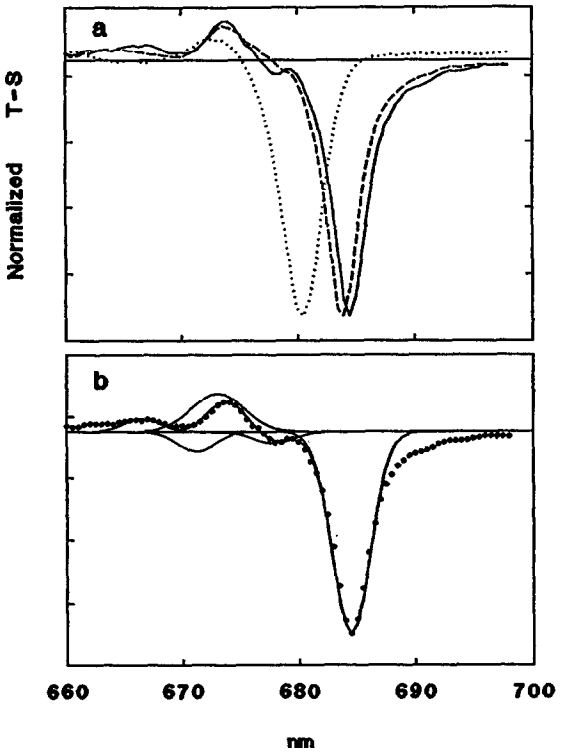

Fig. 4. (a) T–S spectra recorded, at the maximum of the |D|–|E| transition, from 660 to 700 nm: (1) PSII particles after 5 min photoreduction, detect. freq. 721 MHz (solid line); (2) PSII particles after 5 h incubation with dithionite, detect. freq. 724 MHz (dashed line); (3) RC complex, detect. freq. 735 MHz (dotted line). (b) fit of spectrum (1) of (a). (see Table)

the acceptor side to the PSII particles T–S spectra of Fig. 3.

On the basis of these arguments and guided by our results we believe that the spectrum obtained by the photoreduction procedure contains new information concerning PSII. We also think that the previously published T–S spectrum of PSII particles [14], having bleachings at 682 and 635 nm, and a small positive peak at 665, was affected by artefacts. Note that the ZFS parameters calculated from our ADMR spectrum are $|D| = 0.02855$ cm^{-1} and $|E| = 0.00450$ cm^{-1}, while in [14] two sets of values were given: $|D| = 0.02855$, 0.02888 and $|E| = 0.00388$, 0.00422 cm^{-1}, respectively. The small bleaching at 678 nm, which is lost for the samples treated with dithionite, is certainly not due to an artefact as it was perfectly reproduced in many experiments, hence it must be considered in analyzing the T–S spectrum in terms of the RC structure. Moreover the linewidth of the principal bleaching band which is of the order of 100 cm^{-1}, the smallest obtained to date, puts an upper limit to any splitting contained within the lineshape of this band.

Both of these novel features of the spectrum are related to some of the interesting questions about the structure of P680 [5,6]. The most recent discussions are those of Braun et al. [24], who have analyzed the optical absorption and the CD spectra of the D1D2 complex, and of van der Vos et al. [4] who have analyzed the T–S ADMR spectra of the same complex. The first paper concludes that there is a Chla dimer with a separation between the centers of the two pigments of about 10 Å with an exciton splitting in the Q_y band of about 250 cm^{-1}. The intensity ratio between the transitions assigned to the two exciton couplets is consistent with an angle of about 150° between the N_1–N_3 axes of the two chlorophyll molecules. The second paper concludes that no evidence can be extracted from ADMR T–S spectrum for any exciton splitting. This is clearly related to the very heterogeneous nature of the D1D2 complex and means that the analysis of Braun et al. cannot be considered definitive.

It is clear that our ADMR T–S spectrum of P680 obtained from photoreduced BBY particles is more easily amenable to analysis because of the intrinsic smaller linewidths. The upper limit of any exciton splitting or vibrational feature contained within the main bleaching band is less than 100 cm^{-1} (the linewidth of the band), less than the splitting invoked by Braun et al. and much less than the smallest vibrational interval ever invoked in any spectral analysis of monomeric chlorophyll systems (300 cm^{-1} [24]).

Our tentative interpretation of the new features is based on the hypothesis that the negative peak at 678 nm is the low intensity exciton component of the P680 Q_y absorption. A possible decomposition of the ADMR T–S spectrum, shown in Fig. 3, with a minimum number of gaussians to reproduce the blue side of the main bleaching is shown in Fig. 4b (660–700 nm region) and the components and amplitudes are reported in Table 1. Band 1 is assigned to the low energy, high intensity component of an exciton couplet originating in the Chla dimer constituting P680 whose high energy, low intensity component is band 2. This is an assignment of the type invoked by Braun et al. [24]. If band 2 is indeed a 'loose' exciton partner, its low intensity (one twentieth of the low frequency partner) is an indication of the almost antiparallel direction of the two transition moments and

Table 1
Parameters for the gaussian components of the fitting curve: $\Sigma_i A_i \exp - [(\lambda - \lambda_{0i})/(\Delta\lambda_i/2)]^2$

	λ_0 (nm)	$\Delta\lambda$ (nm)	A
(1)	684.5	4.6	−100
(2)	677.8	4.8	−6
(3)	673.0	6.4	+19
(4)	671.2	4.4	−9
(5)	666.5	4.6	+6
(6)	640.0	7.0	−2.5
(7)	631.0	7.0	−2.5

the splitting of only 144 cm^{-1} corresponds (within the point dipole approximation [25]) to a dimer center to center distance of about 11.5 Å. Band 3 is assigned to the transition corresponding to the $S_1 \leftarrow S_0$ transition of one component of the dimer when the other component is in the triplet state; bands 4 and 5 are possibly due to a blue shift of the Q_y band of an accessory Chla molecule when the neighbouring P680 Chla component is in the triplet state. An assignment based essentially on the main bleaching being due to one exciton component and the main absorption being due to the 'monomeric' component of the triplet spectrum, was used as early as 1983 both for P680 and P700 [14,26]. In the discussion of the latter the question was raised as to the apparently anomalously low intensities of the 'monomeric' components of the spectrum (bands 3–6 in our case) and it was left unanswered as it remains today, calling for theoretical reappraisal. Bands 6 and 7 (not shown in Fig. 4b, but see the enlarged scale inset of Fig. 3) could be assigned to a vibrational component of the main band and to a Q_x transition. These features would be in keeping with the classical assignment made by Shipman et al. for hexacoordinated Chla [27] where the vibration coupled with the main Q_y transition is 1160–1300 cm^{-1} and the difference between $Q_y(0,0)$ and $Q_x(0,0)$ is between 700 and 1000 cm^{-1} depending on the axial substituents. Bleachings of similar intensity have also been observed in T–S spectra of long wavelength absorbing chlorophylls present in CP43, CP47 complexes [28].

A second interpretative scheme is also possible, similar to that used for P700 T–S spectrum [26]. In this scheme the negative feature at 678 nm could be assigned to an accessory chlorophyll shifting toward the red when the 'dimer' is in the triplet state. The red positive component is not immediately seen in the spectrum but results from an 'ad hoc' fitting (not shown). In this case the blue exciton component may be one of the two negative features at 630–640 nm or it may be contained within the linewidth of the main band. In one case the exciton splitting would be significantly larger than in the former interpretation (1030 cm^{-1} corresponding to an interdimer distance of about 5–6 Å, at the limit of the point dipole approximation), in the other case the dimer interaction becomes almost negligible. At present we have no way to choose between these two interpretations but certainly help could be provided by LD-ADMR experiments [2] which we intend to carry out in the future.

Acknowledgements: We thank Roberto Barbato, Giulia Friso and Fernando Rigoni, from Prof. Giorgio M. Giacometti's laboratory at the Department of Biology of the University of Padova, for preparing all of our samples. This work was supported in part by MPI 40% Program 'Liveprotein'.

References

[1] Dijkman, G.A., den Blanken, H.J. and Hoff, A.J. (1988) Isr. J. Chem. 28, 141–148.
[2] Hoff, A.J. (1989) in: Advanced EPR: Applications in Biology and Biochemistry (Hoff, A.J., Ed.) pp. 633–680, Elsevier, Amsterdam.
[3] Budil, D.E., Gast, P., Chang, C.H., Schiffer, M. and Norris, J.R. (1987) Annu. Rev. Phys. Chem. 38, 561–583.
[4] van der Vos, R., van Leeuwen, P.J., Braun, P. and Hoff, A.J. (1992) Biochim. Biophys. Acta 1140, 184.
[5] Van Gorkum, H.J. and Schelvis, J.P.M. (1993) Photosynth. Res. 38, 297–301.
[6] Renger, G. (1992) in: The Photosystems: Structure, Function and Molecular Biology (Barber, J., Ed.) pp. 45–99, Elsevier, Amsterdam.
[7] Klimov, V.V., Klevanik, A.V., Shuvalov, V.A. and Krasnovsky, A.A. (1977) FEBS Lett. 82, 183–186.
[8] Nuijs, A.M., van Gorkom, H.J., Plijter, J.J. and Duysens, L.N.M. (1986) Biochim. Biophys. Acta 848, 167–175.
[9] van Mieghem, F.J.E., Nitschke, W., Mathis, P. and Rutherford, A.W. (1989) Biochim. Biophys. Acta 1015, 269–278.
[10] Okamura, M.Y., Satoh, K., Isaacson, R.A. and Feher, G. (1987) in: Progress in Photosynthesis Research (Biggins, J. Ed.) vol. I, pp. 379–381, Dordrecht.
[11] Durrant, J.R., Giorgi, L.B., Barber, J., Klug, D.R. and Porter, G. (1990) Biochim. Biophys. Acta 1017, 167–175.
[12] Vass, I. and Styring, S. (1993) Biochemistry 32, 3334–3341.
[13] Carbonera, D., Di Valentin, M., Giacometti, G. and Agostini, G. (1994) Biochim. Biophys. Acta, in press.
[14] den Blanken, H.J., Hoff, A.J., Jongenelis, A.P.J.M. and Diner, B.A. (1983) FEBS Lett. 157, 21–27.
[15] Carbonera, D., Giacometti, G. and Agostini, G. (1992) Appl. Magn. Res. 3, 859–872.
[16] Berthold, D.A., Babcock, G.T. and Yokum, C.F. (1981) FEBS Lett. 139, 231–234.
[17] Crystall, B., Booth, P.Y., Klug, D.R., Barber, J. and Porter, G. (1989) FEBS Lett. 260, 138–140.
[18] Chapman, D.J., Gounaris, K. and Barber, J. (1990) in: Methods in Plant Biochemistry, Vol. V (Rogers, L.J. Ed.) pp. 171–193, Academic Press, London.
[19] van Gorkum, H.J., Pulles, M.P.J. and Wessels, J.S.C. (1975) Biochim. Biophys. Acta 408, 331–339.
[20] van Gorkum, H.J., Tamminga, J.J., Hareman, J. and van der Linden, J.K. (1974) Biochim. Biophys. Acta 347, 417–438.
[21] Doering, G., Renger, G., Vater, J. and Witt, H.T. (1969) Zeit. Naturforsch. 24b, 1134–1143.
[22] Otte, S.C.M., van der Vos, R. and van Gorkom, H.J. (1992) J. Photochem. Photobiol. B: Biol. 15, 5–14.
[23] van Kan, P.J.M., Otte, S.C.M., Kleiherenbrink, F.A.M., Nieveen, M.C., Aartsma, T.J. and van Gorkom, H.J. (1990) Biochim. Biophys. Acta 1020, 146–152.
[24] Braun, P., Greenberg, B.M. and Scherz, A. (1990) Biochemistry 29, 10376–10387.
[25] Shipman, L.L., Norris, J.R. and Katz, J.J. (1976) J. Chem. Phys. 80, 877.
[26] den Blanken, H.J. and Hoff, A.J. (1983) Biochim. Biophys. Acta 724, 52–61.
[27] Shipman, L.L., Cotton, T.M., Norris, J.R. and Katz, J.J. (1976) J. Am. Chem. Soc. 98, 8222–8230.
[28] Carbonera, D., Giacometti, G., Agostini, G., Angerhofer, A. and Aust, V. (1992) Chem. Phys. Lett. 194, 275–281.