Volume 292, number 1,2, 279–283 FEBS 10359 November 1991
© 1991 Federation of European Biochemical Societies 00145793/91/$3.50
ADONIS 0014579391010782

ESE relaxation measurements in photosystem II
The influence of the reaction center non-heme iron on the spin-lattice relaxation of Tyr D˙

M.K. Bosch[1], R.G. Evelo[1], S. Styring[2], A.W. Rutherford[3] and A.J. Hoff[1]

[1]*Department of Biophysics, Huygens Laboratory, Leiden University, P.O. Box 9504, 2300 RA Leiden, The Netherlands,*
[2]*Department of Biochemistry, Arrhenius Laboratories for Natural Sciences, Stockholm University, S-10691 Stockholm, Sweden,*
[3]*Service de Bioénergétique; Département de Biologie Cellulaire et Moleculaire, Centre d'Études Nucléaires de Saclay, 91191 Gif-Sur-Yvette, Cedex, France*

Received 8 July 1991

The spin-lattice relaxation of the tyrosine radical D˙ in Photosystem II particles was studied at 4.2 K in samples in which flash-induced oscillations of the oxidation state of the Mn-cluster of the oxygen evolving system were abolished by addition of ANT2P, leaving Fe^{2+}/Fe^{3+} oscillations intact. Samples subjected to 0, 1 or 2 light-flashes all showed the same relaxation kinetics. No period-2 oscillation in the spin-lattice relaxation corresponding to the Fe^{2+}/Fe^{3+}-oscillation was observed. Thus the T_1-oscillations of D˙ as a function of flash number in untreated samples are solely caused by the charge-oscillations of the Mn-cluster (1989, Biochim. Biophys. Acta 973, 428–442).

Photosynthesis; Photosystem II; Oxygen evolving complex; Manganese; ADRY-agent; Electron spin echo spectroscopy

1. INTRODUCTION

During the past decades investigations on plant photosynthesis have led to the conclusion that the oxidation of water to molecular oxygen takes place at the donor side of photosystem II (PS II) during a four-step cyclic process in a manganese-containing membrane-bound protein complex, called the oxygen evolving complex (OEC). The states involved in this cycle are denoted the S-states and are labelled S_0 to S_4 [1]. The chemical nature of the S-states has been subject to a large number of spectroscopic studies and, although these states have not yet been fully understood, it is now generally accepted that manganese ions in different oxidation states act as the accumulator of the oxidizing equivalents for some or possibly all S-states. (For a recent review of EPR-spectroscopy on PS II see Miller and Brudvig [2].)

Recently Evelo et al. [3] measured the spin lattice relaxation time (T_1) of the stable tyrosine radical D˙ (Signal II) in PS II particles using Electron Spin Echo (ESE) spectroscopy. It was found that the relaxation time T_1 of D˙ oscillated with the S-state. The relaxation of D˙ was slow in S_1 (dark), fast and similar in S_2 and S_3, and fastest in S_0. Similar results were obtained using cw-EPR [4]. From the S-state-dependent average relaxation time of D˙ and from considerations of data in the literature it was concluded that two Mn in the OEC form an antiferromagnetically coupled binuclear cluster, which is in the redox state $Mn^{2+}{\cdot}Mn^{3+}$, $Mn^{3+}{\cdot}Mn^{3+}$, $Mn^{3+}{\cdot}Mn^{4+}$ and $Mn^{3+}{\cdot}Mn^{4+}$ in the S_0, S_1, S_2 and S_3 states, respectively. The distance between the Mn-cluster and D˙ was found to be 30–40 A [3].

In [3] the biphasicity of the decay was suggested to be due to a frozen-in charge resonance between the two other Mn of the OEC. In later work it was proposed that the biphasicity in Mn-depleted material originates from a dipolar interaction with the acceptor-side iron [5–7]. In the presence of certain quinones added as electron acceptors, this iron ion oscillates between Fe^{2+} and Fe^{3+} with a period of 2 when cycling through the S-states with a series of flashes [8]. This oscillation could, through the magnetic dipole–dipole interaction between D˙ and $Fe^{2+/3+}$, influence the average relaxation in the various S-states. If this effect were severe, this could invalidate the above conclusion on the redox state of the binuclear Mn-cluster as a function of S-state and the calculation of the distance between the Mn-cluster and D˙.

In this report, the effect of the charge-oscillations of the iron ion on the relaxation of D˙ is investigated. PS II samples were prepared in different S-states and

Abbreviations: ADRY: acceleration of the deactivation reactions of the watersplitting enzyme system Y (OEC); ANT2p: 2-(3-chloro-4-trifluoromethyl)anilino-3,5-dinitrothiophene; BBY: thylakoid membrane fragments prepared according to [10]; DMSO: dimethylsulfoxide; EPR: electron paramagnetic resonance; ESE: electron spin echo; MES: 2-(N-morpholino)ethanesulfonic acid; OEC: oxygen evolving complex; PPBQ: phenyl-*p*-benzoquinone; PS II: photosystem II of higher plants.

Correspondence address: A.J. Hoff, Department of Biophysics, Huygens Laboratory, Leiden University, P.O. Box 9504, 2300 RA Leiden, The Netherlands.

Published by Elsevier Science Publishers B.V.

treated with ANT2p. This compound, a so-called ADRY-agent, causes the higher S-states to return to S_1 [9], while maintaining the redox state of the iron [8]. This makes it possible to investigate the influence of the redox state of iron on the relaxation behaviour of D˙, independent of the S-state.

2. MATERIALS AND METHODS

Photosystem II preparations (BBY-particles) were prepared as described by Berthold et al. [10] with the modifications of Ford and Evans [11]. These particles were suspended in a MES buffer containing 20 mM MES at pH 6.3, 0.4 M sucrose, 5 mM $MgCl_2$, 15 mM NaCl and 30% (v/v) ethyleneglycol to a final concentration of 2 mg Chl per ml.

To trap the BBY-particles in the desired S-states, the samples were first dark-adapted for 10 min at room temperature. A saturating laser preflash was given, using a Spectra Physics DCR3G Nd-YAG frequency- doubled laser (8 ns, 300 mJ at 532 nm). The samples were kept in the dark for 10 min in order to equilibrate. Then 0.5 mM PPBQ (in DMSO) was added and the desired number (0–3) of saturating laser flashes was given at 2 Hz frequency. After a waiting time of 10–15 s the samples were frozen rapidly in an ethanol/solid CO_2 bath (200 K) [4]. This series of samples is indicated as 'untreated'.

With the same batch of BBY-particles a second series of samples was prepared according to the protocol described above. Immediately after the last flash to a sample, 5 μM ANT2p was added and mixed very quickly in the dark. After a total time of ~10 s the samples were frozen in an ethanol/solid CO_2 bath. This series of samples is indicated as 'ANT2p'.

X-band cw-EPR measurements were recorded at a microwave frequency of 9.239 GHz with a Bruker ESP 300 spectrometer using the ESP 300 program. The spectrometer was equipped with an Oxford Instruments cryostat and temperature controller. Normalization of the EPR spectra for tube calibration factors and chlorophyll concentration was done with the computer.

Pulsed EPR experiments were carried out on a home-built X-band ESE-spectrometer [6], equipped with an Oxford Instruments helium flow cryostat and a home-built temperature controller. Temperature control was within 0.5 K; absolute temperature was accurate within 1 K. T_1 measurements were performed using a three-pulse echo sequence ($\pi - T - \pi/2 - \tau_p - \pi - \tau_p$ – echo, see inset Fig. 3), in which the echo intensity was recorded as a function of the time T between the first and the second microwave pulse. The first pulse (40 ns) perturbs the thermal equilibrium, while the subsequent 2-pulse echo sequence, with a constant spacing τ_p, serves as a monitoring echo sequence to record the recovery of the magnetization to equilibrium. For all T_1 measurements a Space Microwave 1 kW microwave power amplifier was used, which made it possible to realise a τ_p as short as 180 ns. The time resolution of the T_1 measurements was better than 1 μs. The repetition rate was 7 Hz for all ESE experiments. In previous experiments [3] it was found that the relaxation of D˙ was independent of the width of the first pulse up to 60 ns, indicating that spin diffusion effects are negligible (for pertinent discussions see [3,12]).

The iron oscillation was detected with cw-EPR at g=8 [13]; the S_2-state was monitored by the multiline signal. T_1-relaxation measurements were performed in the same samples on the ESE-spectrometer as described [3,14]. All relaxation curves were fitted to bi-exponential decay functions using the E04CGF fitting routine from the Fortran NAG library. The average relaxation time $\bar{\tau}$, which is defined as the area bounded by the relaxation curve and the steady state echo intensity [3,14], was approximated from the data using Simpson's rule.

3. RESULTS

To make sure that the new samples were comparable with the samples used for the measurements described in [3], relaxation measurements with the untreated samples were performed (data not shown). From this we conclude that, within the experimental error, the relaxation behaviour of the newly prepared samples was identical to that of the samples used for our earlier work.

To determine the miss factor in the experiment the amplitude of the multiline signal, which is a measure of the amount of S_2 present, was measured for all samples (Fig. 1, left hand side). Figure 2A shows this amplitude as a function of flash number. These data could be well fitted with a miss factor of 23%, assuming no double hits (Table I).

To determine the Fe^{3+} concentration at the acceptor-side of PS II we measured the intensity of the g=8 signal (Fig. 1, right hand side). The oxidized form of the acceptor-side iron (Fe^{3+}) gives rise to an EPR signal that is visible at g=8 while Fe^{2+} is not [13]. Therefore the intensity of this signal is proportional to the relative concentration of Fe^{3+}. Both the untreated and ANT2p samples displayed a period-2 charge-oscillation of Fe^{3+} observable at g=8 (Fig. 2B), although the oscillation in the ANT2p series is only 40% of its original amplitude (untreated sample, 1 flash: 68; ANT2p sample, 1 flash: 28; Fig. 2B). The reason for the lower amount of Fe^{3+} in the ANT2p series is not known. In BBY-particles at pH 6.3,

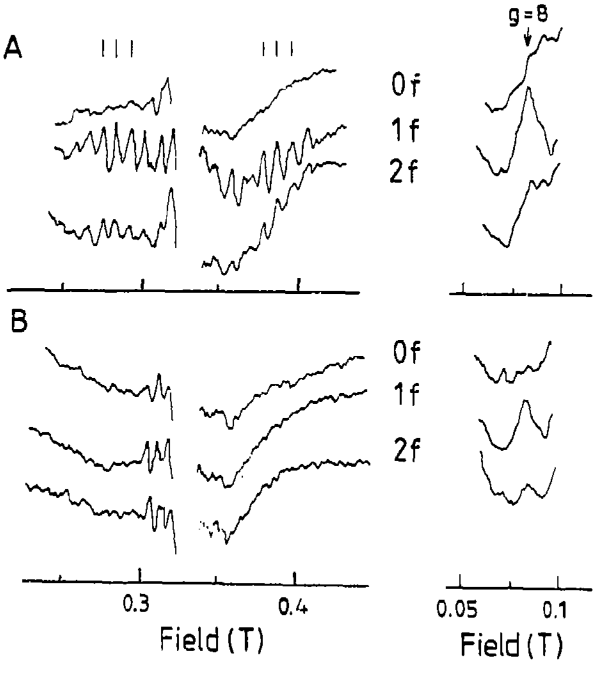

Fig. 1. The oscillation with flash number (0–2 flashes) of the S_2 state multiline EPR signal around g=2 (left hand spectra) and the EPR signal at g=8 from the oxidized form of the acceptor-side iron (right hand spectra). (A) Untreated samples. (B) Samples treated with the ADRY agent ANT2p immediately after the laser flashes. The spectra were recorded at 9.239 GHz with a modulation amplitude of 3.2 mT. The multiline EPR signal was recorded at 10 K with a microwave power of 20 mW and the g=8 Fe^{3+} signal at 4 K with 32 mW microwave power. The bars indicate the peaks used for the measurement of the amplitude of the multiline signal. In the middle of the multiline spectra (left) the large signal from Tyr_D˙(Signal II) has been omitted.

Table I

Calculated S-state distributions for 0, 1, 2 and 3 flashes assuming 100% S_1 from the start, 23% misses and no double hits

Flash number	S_0 (%)	S_1 (%)	S_2 (%)	S_3 (%)
0	0	100	0	0
1	0	23	77	0
2	0	5	35	59
3	45	1	12	40

the maximum oxidizable percentage of Fe^{2+} is approximately 50–75% on a reaction center basis [15,16]. Thus the maximum expected Fe^{2+}/Fe^{3+} oscillation in the ANT2p series involves 50–75% × 40% = 20–30% of the PS II centers.

The samples characterized by cw-EPR were subsequently used for our ESE relaxation measurements (Fig. 3). τ_p was chosen as 260 ns. Since the maximum Fe effect is expected to be about 20–30% we confined the temperatures to 4.2 K, as the signal-to-noise ratio diminishes at higher temperatures [3].

The differences between the different S-states (untreated samples) are expressed in the values of $\bar{\tau}$, as defined in section 2. For the untreated samples we determine $\bar{\tau}$ values of 15.4 ms, 11.4 ms and 10.8 ms for S_1 (0 flash), S_2 (1 flash) and S_3 (2 flashes), respectively, whereas for the ANT2p samples we find values of 14.4 ms, 14.9 ms and 15.2 ms. We see that the values of $\bar{\tau}$, for the three ANT2p samples are the same within 3%, and close to $\bar{\tau}$ of the 0-flash untreated sample (S_1). Thus, the relaxation of D˙ in ANT2p samples conforms to the expected action of ANT2p, which rapidly returns the higher S-states to S_1, while no period-2 T_1-oscillation larger than 3% is observed. Since the iron oscillation is about 20–30% of the total amount of Fe present (see above), the contribution of the acceptor side iron to the spin-lattice decay of the tyrosine radical D˙ is smaller than 15–10%.

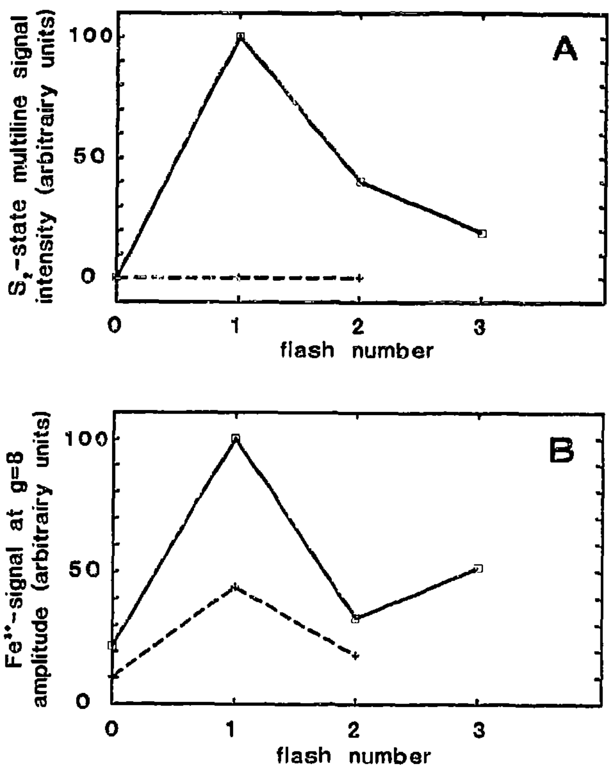

Fig. 2. Cw-EPR-detected flash dependence of (A) the multiline and (B) the g=8 EPR-signal intensity in ANT2p-treated (+) and untreated (☐) synchronized BBY-particles. The amplitude of the multiline signal was simulated assuming 0% double hits, 23% misses and 100% S_1 before the flashes.

The above conclusion is reinforced by fitting the spin-lattice relaxation curves to bi-exponential decay curves of the shape

$$A(t) = A_0 + W_f e^{t/\tau_f} + W_s e^{t/\tau_s} \quad (1)$$

All fits were satisfactory as seen from the residuals in Fig. 3. The fit parameters are reported in Table II. For the untreated samples, the slow decay time τ_s shows similar variation as $\bar{\tau}$, viz. τ_s is 33.1 ms for S_1; 18.9 ms

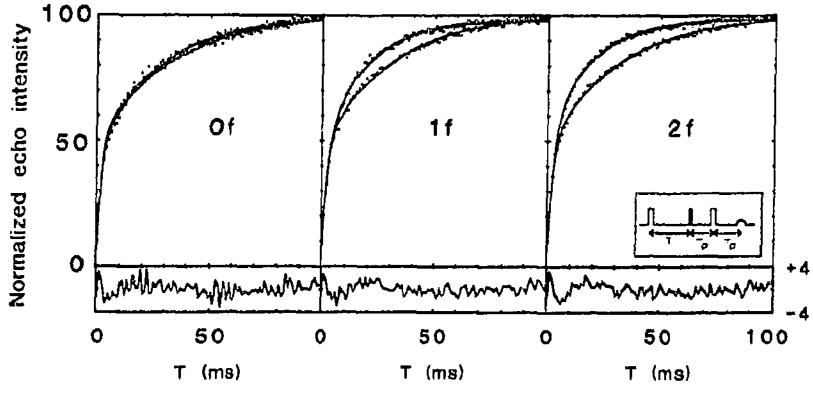

Fig. 3. ESE-detected T_1-kinetics of ANT2p-treated (+) and untreated (☐) synchronized BBY-particles for different flash numbers, measured at 4.2 K with a 3-pulse echo sequence $\pi - \pi/2 - \pi$ with variable spacing T between the first and the second pulse and with a constant delay τ_p of 260 ns between the second and the third pulse (see inset). The drawn lines are bi-exponential fits; the residuals of the ANT2p samples are shown at the bottom of each panel.

Table II

ESE-detected relaxation kinetics of Signal II (D). The fit parameters W_f, τ_f, W_s, τ_s and $\bar{\tau}$ are defined in formula (1).

	Flash number	State*	W_f (%)	τ_f (ms)	W_s (%)	τ_s (ms)	τ (ms)
Untreated	0	S_1 Fe^{2+}	47.4	2.2	51.0	33.1	15.4
	1	S_2 Fe^{3+}	46.7	2.6	50.5	18.9	11.4
	2	S_3 Fe^{2+}	51.3	2.5	46.4	19.0	10.8
ANT2p	0	S_1 Fe^{2+}	47.3	2.0	51.0	29.6	14.4
	1	S_1 Fe^{3+}	47.1	2.2	52.1	31.1	14.9
	2	S_1 Fe^{2+}	46.5	2.0	52.2	32.0	15.2

* Fe^{2+}: in almost all reaction centers ferrous iron; Fe^{3+}: in 40% of the reaction centers iron oxidized.

and 19.0 ms for S_2 and S_3, respectively. In the ANT2p-treated samples the values of τ_s are all similar and approach the same value as for S_1-untreated: 29.6 ms, 31.1 ms and 32.0 ms for 0, 1 and 2 flashes, respectively. The flash number dependence of the fast lifetime τ_f in the untreated samples is less dramatic than that for τ_s: 2.2 ms for the S_1-state; 2.6 ms and 2.5 ms for S_2 and S_3. In the ANT2p-treated samples the τ_f lifetimes all have become fast and within the accuracy of ~0.1 ms the same as for S_1-untreated: 2.0 ms, 2.2 ms and 2.0 ms for 0, 1 and 2 flashes, respectively. The same behaviour is found in the fitted weights W_f and W_s: in the untreated samples the weights differ for the different flash numbers; in the ANT2p samples, within the measuring accuracy the same value is found for all flash numbers, which is the same as for the untreated S_1 sample. Thus, not only the average relaxation $\bar{\tau}$, but also the shape of the relaxation curves as parametrized by the bi-exponential fit is within the measuring accuracy the same for all ANT2p samples, and agrees with that of the S_1 untreated sample.

4. DISCUSSION

Both the cw-EPR multiline data and the ESE T_1-relaxation measurements show that ANT2p addition after the flashes rapidly deactivates the S_2- and S_3-states in the OEC. However, after the ANT2p-treatment the Fe^{2+}/Fe^{3+} oscillation still occurs at a level of about 40% with respect to the untreated sample.

Comparing the relaxation behaviour of $D^{\cdot}$ in the ANT2p-treated samples and the untreated samples leads to the conclusion that $D^{\cdot}$ relaxes similarly in the ANT2p-treated flashed samples (in which no S-state oscillation occurs, but in which Fe-oscillation is still observed) and in the untreated S_1 sample (0-flash untreated). The Fe-oscillation influences the weight or lifetime of neither the fast nor the slow phase in the ANT2p-treated samples, nor does the Fe-oscillation alter the value of $\bar{\tau}$ by more than 15–10%. We therefore conclude that the T_1-oscillations as observed by Evelo et al. [3] are solely caused by the oscillation of the redox state of the four manganese ions in the oxygen evolving complex.

The absence of an influence of the $Fe^{2+} \rightarrow Fe^{3+}$ oxidation on the relaxation of $D^{\cdot}$ can be explained in 2 ways: either the Fe-ion is too far from $D^{\cdot}$ to cause an appreciable relaxation enhancement, or the spin-lattice relaxation times (which governs the enhancement of the relaxation [3]) of Fe^{2+} and Fe^{3+} in the PS II reaction center are very similar. The former possibility is unlikely as, judging from the crystal structure of the reaction center of *Rsp. viridis* [17], the D-tyrosyl residue is about 30–40 Å from the Fe-ion, a distance that is small enough for appreciable dipolar enhancement [3,6,7]. Supporting this conclusion is the observation that for PS II preparations without Mn-cluster (Tris-EDTA washed particles) the relaxation of $D^{\cdot}$ is still a factor of about 10 faster than that of the Tyr^+ cation in vitro [5, 12, 14].

Information on low temperature T_1's of high-spin Fe-ions is scant. Moreover spin lattice relaxation of such ions depends rather much on the precise crystal environment, which makes it hazardous to use literature data on T_1 of high-spin Fe in crystals in vitro for estimating T_1 of Fe^{2+}/Fe^{3+} in the PS II reaction center. Some idea, however, may be gleaned from power saturation measurements. Evans et al. reported a $P_{1/2}$ of about 18 mW at 4.5 K for $Q_A^{\cdot}Fe^{2+}$ [18], the relaxation of which complex is dominated by the high-spin iron [19], while Petrouleas measured $P_{1/2} \approx 5$ mW at 4.6 K for the $g = 8$ signal arising from Fe^{3+} (personal communication). Taking into account the spectrometer variability inherent in such measurements we conclude that the $P_{1/2}$ values and thus the relaxation behaviour of Fe^{2+} and Fe^{3+} are rather similar. Thus, cycling the Fe-ion through the 2+ and 3+ oxidation states will little influence the relaxation of $D^{\cdot}$ and consequently, the dependence of this relaxation on flash number must be due to changes in the redox state of the Mn-cluster [3]. Earlier we have calculated the distance between the Mn-cluster and $D^{\cdot}$ (30-40 Å) from the temperature dependence of $\bar{\tau}$ in the S_0 state [3]. The underlying assumption is that dipolar enhancement induced by the Mn-cluster dominates over that induced by Fe^{2+}/Fe^{3+}, an upper limit for the latter given by the relaxation of $D^{\cdot}$ in the S_1-state, which is about equal to that of $D^{\cdot}$ in Mn-depleted material [3]. This assumption seems to be justified since the S_0-state has an average relaxation (at temperatures between 5 K and 20 K) of a factor 2–3 faster than that of the S_1-state [3]. In addition the temperature dependence of the relaxation in the S_1-state is much less pronounced than that in the S_0-state [3], while the temperature dependence in the S_1, S_2 and S_3-states (containing predominantly Fe^{2+}, 40% Fe^{3+} and Fe^{2+}, respectively) is very similar.

We conclude that a likely explanation for the absence of Fe^{2+}/Fe^{3+} induced oscillations in the relaxation of $D^{\cdot}$

is that both high spin Fe-ions have similar spin-lattice relaxation times. In addition it is likely that Fe is responsible for the enhanced relaxation of D˙ in Mn-depleted PS II preparations compared to Tyr⁺ in vitro (in agreement with earlier suggestions [5,6,7,20]), while the temperature dependence of the relaxation of D˙ in the S_0-state is dominated by the enhancement due to the Mn-cluster. This validates our earlier estimate of the distance between D˙ and the Mn-cluster.

Acknowledgements: The authors wish to thank Dr V. Petrouleas for providing unpublished $P_{1/2}$-data on the g=8 signal and Dr P. Gast for critically reading the manuscript. This work was supported by the Netherlands Foundation of Chemical Research (SON) with financial aid from the Netherlands Organization for Scientific Research (NWO). S.S. was supported by the Swedish Natural Science Research Council, the Erna and Victor Hasselblad Foundation and the Knut and Alice Wallenberg Foundation. A.W.R. was supported by the CNRS (URA 1290).

REFERENCES

[1] Babcock, G.T. (1987) in: Photosynthesis (Amesz, J. ed.), New Comprehensive Biochemistry, Vol. 15, pp. 125–158, Elsevier, Amsterdam.
[2] Miller, A.-F. and Brudvig, G.W. (1991) Biochim. Biophys. Acta 1056, 1–18.
[3] Evelo, R.G., Styring, S., Rutherford, A.W. and Hoff, A.J., Biochim. Biophys. Acta (1989) 973, 428-442.
[4] Styring, S.A. and Rutherford, A.W. (1988) Biochemistry 27, 4915-4923.
[5] Beck, W.F., Innes, J.B. and Brudvig, G.W. (1990) in: Current Research in Photosynthesis (Baltscheffsky, M. ed.), Vol. I, I.3.817, Kluwer, Dordrecht, The Netherlands.
[6] Evelo, R.G. (1991) Doctoral Dissertation, Leiden University, The Netherlands.
[7] Evelo, R.G. and Hoff, A.J. (1991) J. Magn. Res. (in press).
[8] Zimmermann, J.-L. and Rutherford, A.W. (1986) Biochim. Biophys. Acta 851, 416–423.
[9] Renger, G. (1972) Biochim. Biophys. Acta 256, 428–439.
[10] Berthold, D.A., Babcock, G.T. and Yocum, C.F. (1981) FEBS Lett. 134, 231–234.
[11] Ford, R.C. and Evans, M.C.W. (1983) FEBS Lett. 160, 159–164.
[12] Beck, W.F., Innes, J.B., Lynch, J.B. and Brudvig, G.W. (1991) J. Magn. Reson. 91, 12–29.
[13] Petrouleas, V. and Diner, B.A. (1986) Biochim. Biophys. Acta 849, 264–275.
[14] de Groot, A, Plijter, J.J., Evelo, R, Babcock, G.T. and Hoff, A.J. (1986) Biochim. Biophys. Acta 848, 8–15.
[15] Aasa, R., Andréasson, L.-E., Styring, S. and Vänngård, T. (1989) FEBS Lett. 243, 156–160.
[16] Petrouleas, V. and Diner, B.A. (1987) Biochim. Biophys. Acta 893, 126–137.
[17] Michel, H. and Deisenhofer, J. (1988) Biochemistry 27, 1-7.
[18] Evans, M.C.W., Diner, B.A. and Nugent, J.H.A. (1982) Biochim. Biophys. Acta 682, 97–105.
[19] Calvo, R., Butler, W.B., Isaacson, R.A., Okamura, M.Y., Fredkin, D.R. and Feher, G. (1982) Biophys. J. 37, Abstr. M-PM-E7.
[20] Hirsch, D.J., Beck, W.F., Innes, J.B. and Brudvig, G.W. (1991) Biophys. J. 59, Abstr. Tu-AM-A3.