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# Photoinhibition affects the non-heme iron center in photosystem II

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Effects on the PS II acceptor side caused by exposure to strong white light (180 W/m²) of PS II membrane fragments (spinach) at pH 6.5 and 0°C were analyzed by measuring low temperature EPR signals and flash-induced transient changes of the fluorescence quantum yield. The following results were obtained: (a) the extent of the light induced $g = 1.9$ EPR signal as a measure of photochemical $Fe^{2+}Q_A^-$ formation declines with progressing photoinhibition. The half-life of this effect is independent of the absence or presence of an exogenous electron acceptor during the photoinhibitory treatment; (b) in samples photoinhibited in the absence of an electron acceptor and subsequently incubated with $K_3[Fe(CN)_6]$ in the dark, the extent of the $g = 8$ EPR signal (reflecting the oxidized $Fe^{3+}$ form of the endogenous non-heme iron center) and of the flash-induced change of the fluorescence yield (as a measure of fast electron transfer from $Q_A^-$ to $Fe^{3+}$ after the first flash; [see (1992) Photosynth. Res. 31, 113–126] exhibits the same dependence on photoinhibition time as the $g = 1.9$ EPR signal; (c) in samples photoinhibited in the presence of an exogenous electron acceptor, the signals reflecting $Fe^{2+}$-formation and fast electron transfer from $Q_A^-$ to $Fe^{3+}$ decline faster than the $g = 1.9$ EPR signal. These results provide for the first time direct evidence that the endogenous non-heme iron center located between $Q_A$ and $Q_B$ is susceptible to modifications by light stress. The implications of this finding will be discussed.

Photosynthesis; Photosystem II; Photoinhibition; Non-heme iron; *Spinacea oleracea*

## 1. INTRODUCTION

The key steps of photosynthetic water cleavage into dioxygen and metabolically bound hydrogen take place in photosystem II (for a review see [1]). The overall reaction sequence comprises: (a) the transformation of light into a 'stable' radical pair $P680^+$ $Pheo\,Q_A^-$, (b) water oxidation to $O_2$ with $P680^+$ as driving force, and (c) PQ-reduction to $PQH_2$ via a two-step univalent redox reaction sequence with $Q_A^-$ as reductant. Based on striking homologies, the functional groups P680, Pheo, $Q_A$ and the $Q_B$ site for $PQH_2$ formation are assumed to be arranged within the protein matrix consisting of polypeptides D1 and D2 in a similar way as the corresponding redox components (special pair, Pheo, $Q_A$, $Q_B$) in the heterodimer of the L and M subunit in purple bacteria reaction centers (for review see [2]). These similarities are confined to (a) and (c) because of the structural and functional requirements to perform water oxidation in PS II. Apart from this basic phenomenon, there exists another remarkable difference, i.e. the susceptibility to harmful effects of strong visible light. In contrast to purple bacteria, the functional activity of the PS II complex severely declines due to processes referred to as photoinhibition (for a recent review see [3]). This process comprises a sequence of events which can be generalized in the following way: light induced modification of primary target(s) → triggering of endogenous proteolysis → degradation of the apoprotein of PS II, especially of polypeptide D1. It is now clear that the detailed mechanism of photoinhibition depends on the functional integrity of the PS II complex and on the experimental conditions. At least three types of reactions were found to be susceptible to photoinhibition: (i) formation of the stabilized radical pair $P680^+PheoQ_A^-$ [4–7], (ii) PS II donor side [8–11], and (iii) PS II acceptor side [12–15]. Likewise, the proteolytic degradation pattern of D1 was also found to be variable [16]. In a recent study the endogenous non-heme iron center located between $Q_A$ and $Q_B$ and by analogy to purple bacteria coordinated by four histidine residues of polypeptides D1 and D2 was inferred to become modified by photoinhibition. This communication provides direct evidence for changes of the properties of this iron center caused by photoinhibition prior to D1 degradation. The implications of these findings will be discussed.

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*Abbreviations:* PS II, photosystem II; $Q_A$ and $Q_B$, primary and secondary plastoquinone acceptor; MES, 4-morpholinethane sulphonic acid; DCBQ, 2,6-dichloro-*p*-benzoquinone; EPR, electron paramagnetic resonance; $F_o$, fluorescence level in the dark adapted state; $F_v$, variable fluorescence; $F_{max}$, maximum fluorescence.

## 2. MATERIALS AND METHODS

PSII-membrane fragments were prepared from spinach according to the procedures described by Winget et al. [17] and Berthold et al. [18] (with modifications by Völker et al. [19]).



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For photoinhibition, aliquots of 1.3 (or 7) ml sample suspension (20 mM MES/NaOH, pH 6.5, 10 mM NaCl, 10 mM $CaCl_2$, 100 $\mu$M Chl, 0°C) in a 2 (or 5) cm wide circular Petri dish, kept in an ice bath, were exposed to white light (500 W tungsten lamp, heat filter K3 from Schott) of an incident light intensity of 180 W/m$^2$. For control measurements, the samples were kept under the same incubation conditions in complete darkness or under dim light. The control treatment did not cause any harmful effect on the functional activity of the samples. In some experiments 2 mM $K_3[Fe(CN)_6]$ was added as indicated in the figure legends.

Transient changes of fluorescence quantum yield induced by a train of laser flashes (Nd:YAG laser, 15 mJ/pulse, FWHM: 3 ns) were measured with a home-build equipment (Gleiter [20]) as described in [15]. The time resolution of the equipment was of the order of 5 $\mu$s.

Electron paramagnetic resonance at cryogenic temperatures was carried out using a JEOL RE1X spectrometer as described in [21]. Photoreduction of $Q_A$ at 77 K was performed in a silvered dewar using strong white light (650 W source) for 10 min. For measurement of the signal due to $Fe^{3+}$, control and photoinhibited samples were incubated in the dark in the presence of 10 mM $K_3[Fe(CN)_6]$ for 60 min on ice at 0°C. EPR conditions were microwave power 10 mW, temperature 4.7 K, field modulation 1.25 mT, modulation frequency 100 kHz.

## 3. RESULTS

Fig. 1 shows typical traces of transient fluorescence yield changes induced by a sequence of four saturating laser flashes in dark adapted PS II membrane fragments. The kinetically unresolved rise mainly reflects the formation of the state P680 Pheo $Q_A^-$ and the relaxation indicates the $Q_A^-$ reoxidation (for a detailed discussion see [15,22]). A comparison of traces A and B reveals that in control samples the extent and relaxation kinetics of the signal induced by the first flash strongly depend on the presence of $K_3[Fe(CN)_6]$ during the dark incubation before measurements, while the signals induced by the subsequent flashes remain almost invariant. The marked decrease of the detected maximum and the faster relaxation in the dark is a consequence of the very fast electron transfer from photoreduced $Q_A^-$ to the oxidized $Fe^{3+}$ form of the endogenous iron center located between $Q_A$ and $Q_B$ [15] and references therein). As the time between the flashes is short compared with the oxidation kinetics of the endogenous high spin $Fe^{2+}$ by $K_3[Fe(CN)_6]$ [23], the signals induced by the subsequent flashes remain practically unaffected by preincubation with $K_3[Fe(CN)_6]$. Accordingly, the normalized amplitude ratio $[F_{var,2}(100\ \mu s) - F_{var,1}(100\ \mu s)]/F_{var,2}(100\ \mu s)$ can be used as a measure of the amount of $Fe^{3+}$ formed by dark incubation with $K_3[Fe(CN)_6]$. A comparison of the signals measured in control and photoinhibited samples, respectively, reveals two striking phenomena: (i) the variable fluorescence of the signals induced by each flash but the first one markedly decreases in the photoinhibited samples, and (ii) the ratio $[F_{var,2}(100\ \mu s) - F_{var,1}(100\ \mu s)]/F_{var,2}(100\ \mu s)$ is significantly reduced due to photoinhibition. The former effect is well established and will not be discussed here.

The latter effect, however, indicates that also the non-heme iron center is susceptible to modifications by photoinhibition. A suppression of the very fast $Q_A^-$ reoxidation could be explained by two alternative models: (a) elimination of the $K_3[Fe(CN)_6]$ induced $Fe^{3+}$ formation (as an indispensable prerequisite of the very fast $Q_A^-$ reoxidation) either by a shift of the oxidation potential to more positive values or a largely increased shielding of the endogenous non-heme iron center, or (b) a drastic retardation of the electron transfer from $Q_A^-$ to $Fe^{3+}$ due to increase of the effective distance between the redox centers and/or changes of the reorganisation energy. In order to analyze these alternatives, EPR measurements were performed which permit both direct detection of $Fe^{2+}$ formation by dark incubation with $K_3[Fe(CN)_6]$ and light induced $Q_A^-Fe^{2+}$ generation. To exclude possible interference by D1 degradation, photoinhibition was performed at 0°C and at pH 6.5 [24,25]. The EPR signals obtained are depicted in Fig. 2. Traces A and B show the $Q_A^-Fe^{2+}$ EPR signals [21,26,27] of control and photoinhibited samples, respectively, illuminated with actinic light (10 min) at 77 K. A comparison of the signal amplitudes readily reveals a harmful effect of photoinhibition. This result is in perfect agreement with recent findings [28,29]. The traces C and D were meas-

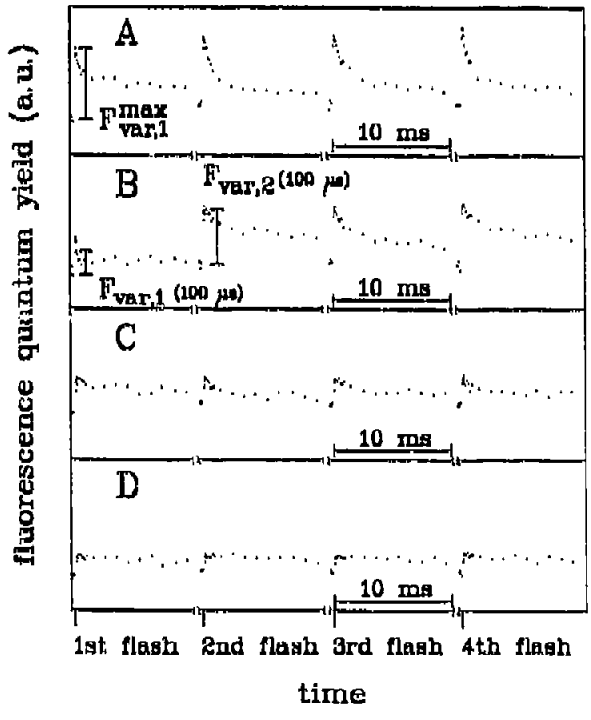


Fig. 1. Transient changes of fluorescence quantum yield induced by a train of four laser flashes in PS II membrane fragments: (A) control; (B) as (A) but sample incubated in the dark for 5 min with 2 mM $K_3[Fe(CN)_6]$; (C) samples photoinhibited (60 min) and subsequently dark incubated (5 min) in the absence of $K_3[Fe(CN)_6]$; (D) sample photoinhibited (60 min) in the presence of 2 mM $K_3[Fe(CN)_6]$ before dark adaptation for 5 min in the presence of 2 mM $K_3[Fe(CN)_6]$ and subsequent measurement. The following symbols were used to describe the fluorescence parameter used in this study: $F^{max}_{var,1}$, maximum extent of variable fluorescence induced by the first flash and $F_{var,n}(100\ \mu s)$, extent of variable fluorescence 100 $\mu$s after excitation with the $n^{th}$ flash ($n$ = 1,2).

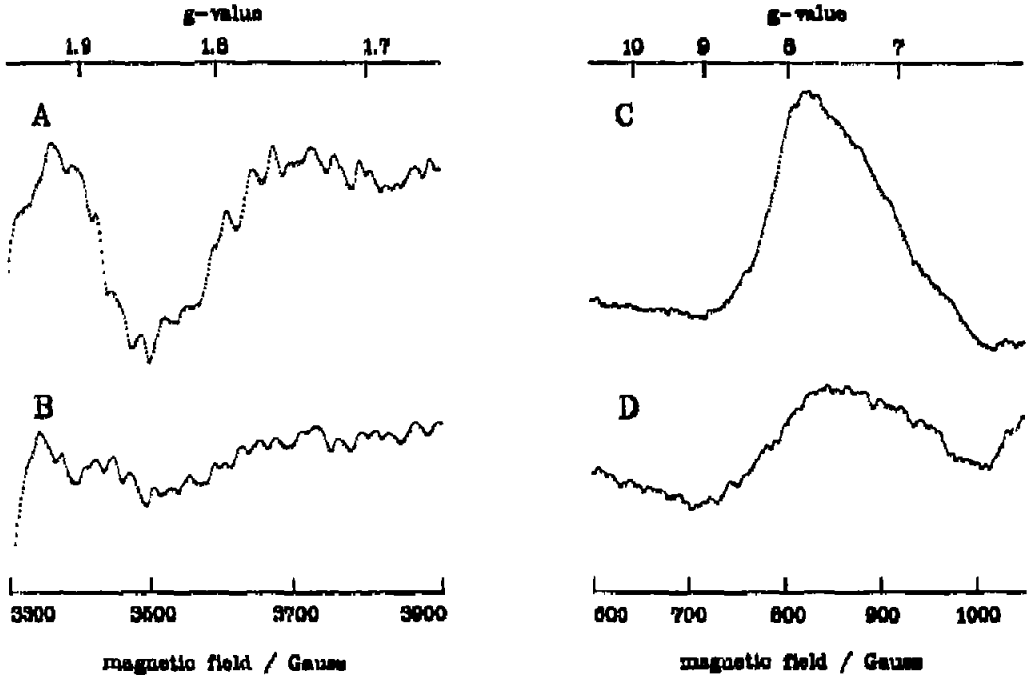


Fig. 2. Low temperature EPR signals induced by actinic illumination (10 min) at 77 K (traces (A) and (B), signal from $Q_A^-Fe^{2+}$) or by dark incubation of the sample (60 min) with 10 mM $K_3[Fe(CN)_6]$ (traces (C) and (D), signal from non-heme iron $Fe^{3+}$). Signals (A) and (C) were obtained in the control, (B) and (D) samples photoinhibited for 60 min in the absence of 2 mM $K_3[Fe(CN)_6]$, respectively.

ured in samples preincubated in the dark with $K_3[Fe(CN)_6]$. The EPR spectrum C exhibits a pronounced signal at $g = 8$ which reflects the formation of $Fe^{3+}$ by dark incubation with $K_3[Fe(CN)_6]$ [30,31]. A marked decrease of this signal is observed in trace D,

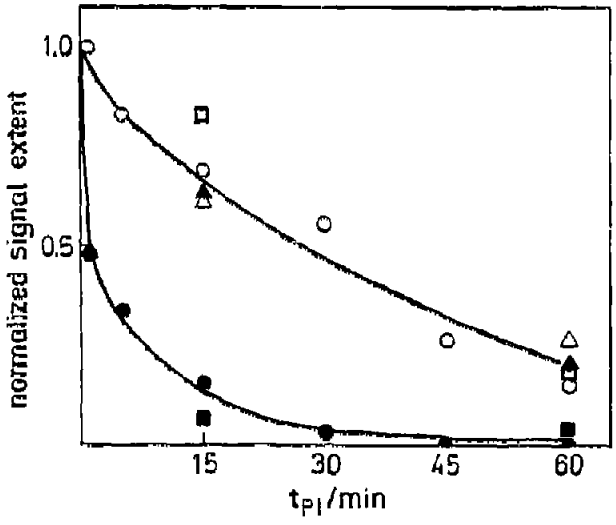


Fig. 3. Ratio of variable fluorescence $(F_{var,2}(100\ \mu s) - F_{var,1}(100\ \mu s))/F_{var,2}(100\ \mu s)$ measured 5 min after dark incubation with 2 mM $K_3[Fe(CN)_6]$ (○), amplitude of $g = 1.9$ signal induced by actinic illumination (10 min) at 77 K (△) and amplitude of $g = 8.0$ signal induced by 60 min incubation in the dark with 10 mM $K_3[Fe(CN)_6]$ (□) as a function of exposure time of photoinhibitory light. Photoinhibition was carried out in the absence (open symbols) or presence (filled symbols) of 2 mM $K_3[Fe(CN)_6]$. Data were normalized to the values of the untreated samples. For the $g = 8$ signal, values were corrected for the amplitude in fully photoinhibited samples (i.e. after 60 min photoinhibition in the presence of $K_3[Fe(CN)_6]$).

i.e. photoinhibition causes a diminution of the $K_3[Fe(CN)_6]$ induced $Fe^{3+}$ population. This finding provides direct evidence for a modification of the oxidizability of the endogenous non-heme $Fe^{2+}$ (to $Fe^{3+}$) by $K_3[Fe(CN)_6]$ in the photoinhibited samples. A modification of the iron center could also affect the magnetic coupling between $Fe^{2+}$ and $Q_A^-$ thus affecting the $g = 1.9$ signal. In order to analyze a possible relation between the disappearance of $Q_A^-Fe^{2+}$ and of $Fe^{3+}$, the extent of the corresponding EPR signals was measured as a function of photoinhibition time in the absence and presence of $K_3[Fe(CN)_6]$ in the suspension during exposure to deleterious light intensities. In the case of the $g = 8$ signal induced by dark incubation with 10 mM $K_3[Fe(CN)_6]$ a residual signal (about 30% of the control sample) remains even after severe photoinhibition (60 min). At present, the origin of this $Fe^{3+}$ center is not unambiguously clarified. For a comparison of the time course of photoinhibition this residual signal has been subtracted. The results obtained are summarized in Fig. 3. Three striking effects can be extracted from these data: (1) samples photoinhibited in absence of $K_3[Fe(CN)_6]$ exhibit practically the same susceptibility of $Q_A^-Fe^{2+}$ and $Fe^{3+}$ formation to deleterious illumination; (2) if photoinhibition is performed in the presence of $K_3[Fe(CN)_6]$, the capability of $K_3[Fe(CN)_6]$ to oxidize the endogenous $Fe^{2+}$ in the dark to $Fe^{3+}$ disappears at much shorter times than the $g = 1.9$ signal which reflects the light induced $Fe^{2+}Q_A^-$ formation; (3) the ability of $K_3[Fe(CN)_6]$ to cause the very fast $Q_A^-$ reoxidation after the first flash, as reflected by the ratio

$[F_{var.2}(100\ \mu s) - [F_{var.1}(100\ \mu s)]/F_{var.2}(100\ \mu s)$, declines with progressing photoinhibition in parallel with the loss of $K_3[Fe(CN)_6]$ induced $Fe^{3+}$ formation, regardless of the absence or presence of this oxidant during exposure to deleterious light. This finding shows that the photoinhibitory elimination of the very fast $Q_A^-$ reoxidation after the first flash is due to a redox potential shift of the iron center preventing $Fe^{3+}$ formation by $K_3[Fe(CN)_6]$ rather than a blockage of the electron transfer reaction from $Q_A^-$ to $Fe^{3+}$. The idea of a modified microenvironment around the non-heme iron due to photoinhibition is also supported by the alteration in the lineshape of the Pheo-/$Fe^{2+}Q_A^-$ split signal (indicating weaker interaction after photoinhibition) especially if exposure to light stress is performed in the presence of $K_3[Fe(CN)_6]$ (data not shown).

## 4. DISCUSSION

The results of this study unambiguously show that the properties of the endogenous high spin $Fe^{2+}$ are modified by photoinhibition under conditions (0°C, pH 6.5) where proteolytic degradation of polypeptide D1 can be neglected [24,25]. The loss of the $K_3[Fe(CN)_6]$ inducable $g = 8$ EPR signal in photoinhibited samples can be explained by structural changes which either render the iron center inaccessible to the exogenous oxidant or cause a shift of its oxidation potential to more positive. Although the former possibility cannot be totally excluded, a redox potential shift appears to be much more likely because only minor structural modifications are sufficient to cause drastic changes of the redox properties in heme iron proteins (e.g. [32] and references therein). The microenvironment of the endogenous $Fe^{2+}$ in reaction centers of anoxygenic purple bacteria differ from that of PS II. One striking difference between both types is the binding of bicarbonate in PS II. Accordingly, it might be attractive to speculate that the modification of the endogenous $Fe^{2+}$ by photoinhibition also affects the properties of bicarbonate binding. However, as the $g$-value of the EPR-signal due to $Fe^{2+}Q_A^-$ in the presence of $HCO_3^-$ at $g = 1.9$ remains invariant to photoinhibition (the bicarbonate free form is characterized by a $g$-value of 1.8; see [33]) a modification of $HCO_3^-$ binding is unlikely to be related to the redox potential shift. Therefore, other effects are responsible for the change of the redox properties due to photoinhibition. Our data do not permit to present a model for the structural changes that elicit this effect. Regardless of the detailed mechanism, the present results clearly show that photoinhibition induces structural changes in the microenvironment of the non-heme iron center, as reflected by the loss of its $K_3[Fe(CN)_6]$ induced oxidation to $Fe^{3+}$. It remains to be clarified whether these modifications provide a trigger signal for the subsequent proteolytic degradation of the D1 protein at room temperature.

It is interesting to note that in samples photoinhibited in the presence of $K_3[Fe(CN)_6]$ the oxidizability of the non-heme iron center is much more sensitive to deleterious light than the formation of the $g = 1.9$ EPR signal indicative of $Fe^{2+}Q_A^-$. This finding shows that changes in the microenvironment which prevent the formation of $Fe^{3+}$ by dark incubation with $K_3[Fe(CN)_6]$ do not drastically affect the magnetic interaction between $Fe^{2+}$ and $Q_A^-$. On the other hand, when the samples are exposed to strong light in the absence of an exogenous electron acceptor, the photoinduced $g = 1.9$ signal and the $g = 8$ signal due to dark incubation with $K_3[Fe(CN)_6]$ exhibit the same dependence on the exposure time to photoinhibition. If one neglects the highly unlikely possibility of an $Fe^{2+}$ loss (this would certainly require the presence of a strong chelator), this effect can be explained by two alternatives: (i) photoinhibition induces structural changes that lead to a redox potential shift of the non-heme iron center together with a blockage of $Q_A^-$ formation; or (ii) the modifications in the neighborhood of the endogenous iron center simultaneously lead to a drastic change of the magnetic interaction between $Fe^{2+}$ and $Q_A^-$ which causes disappearance of the $g = 1.9$ EPR signal without affecting the capacity to form $Q_A^-$. Although the former explanation (i) seems to be more likely in the light of the results obtained in samples photoinhibited in the presence of $K_3[Fe(CN)_6]$ (vide supra), the latter possibility (ii) cannot be totally excluded. This might render the question whether a loss of the $g = 1.9$ EPR signal due to photoinhibition can be really used under all circumstances as an unambiguous proof for a blockage of $Q_A^-$ formation or a double reduction of $Q_A$. Further experiments are required to clarify this very important point.

In summary, the present study shows that the properties of the high spin iron center provide a sensitive probe to monitor subtle structural changes at the acceptor side that are caused by photoinhibition prior to proteolytic degradation of polypeptide D1.

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## REFERENCES

[1] Renger, G. and Wydrzynski, T. (1991) Biol. Metals 4, 73–80.
[2] Michel, H. and Deisenhofer, J. (1988) Biochemistry 27, 1–7.
[3] Barber, J. and Andersson, B. (1992) Trends Biol. Sci. 17, 61–66.
[4] Krause, G.H., Köster, S. and Wong, S.C. (1985) Planta (Berl.) 165, 430–438.
[5] Arntz, B. and Trebst, A. (1986) FEBS Lett. 194, 43–49.
[6] Allakhverdiev, S.I., Setlikova, E., Klimov, V.V. and Setlik, I. (1987) FEBS Lett. 226, 186–190.
[7] Vass, I., Mohanty, N. and Demeter, S. (1988) Z. Naturforsch. 43c, 99–103.
[8] Callahan, F.E., Becker, D.W. and Cheniae, G.M. (1986) Plant Physiol. 82, 261–269.

[9] Zhao, J. and Brandt, J.J. (1988) Arch. Biochem. Biophys. 264, 657–664.
[10] Blubaugh, D.J., Atamian, M., Babcock, G.T., Golbeck, J.H. and Cheniae, G.M. (1991) Biochemistry 30, 7586–7597.
[11] Eckert, H.-J., Geiken, B., Bernarding, J., Napiwotzki, A., Eichler, H.J. and Renger, G. (1991) Phot. Res. 27, 97–108.
[12] Kyle, D.J., Ohad, I. and Arntzen, C.J. (1984) Proc. Natl. Acad. Sci. USA 81, 4070–4074.
[13] Kirilovski, D.J., Aljani, G., Picaud, M. and Etienne, A.A. (1989) Plant Mol. Biol. 13, 355–363.
[14] Ohad, I., Adir, N., Koike, H., Kyle, D. and Inoue, Y. (1990) J. Biol. Chem. 265, 1972–1974.
[15] Haag, E., Gleiter, H.M. and Renger, G. (1992) Photosynth. Res. 31, 113–126.
[16] De Las Rivas, J., Andersson, B. and Barber, J. (1992) FEBS Lett. 301, 246–252.
[17] Winget, G.D., Izawa, S. and Good, N.E. (1965) Biochem. Biophys. Res. Commun. 21, 438–441.
[18] Berthold, D.A., Babcock, G.T. and Yocum, C.A. (1981) FEBS Lett. 134, 231–234.
[19] Völker, M., Ono, T., Inoue, Y. and Renger, G. (1985) Biochim. Biophys. Acta 806, 25–34.
[20] Gleiter, H. (1988) Diploma thesis (in German), Technical University Berlin.
[21] Hallahan, B.J., Ruffle, S.V., Bowden, S.J. and Nugent, J.H.A. (1991) Biochim. Biophys. Acta 1059, 181–188.
[22] Renger, G., Hanssum, B., Gleiter, H., Koike, H. and Inoue, Y. (1988) Biochim. Biophys. Acta 936, 435–446.
[23] Renger, G., Wacker, U. and Völker, M. (1987) Photosynth. Res. 13, 167–189.
[24] Kuhn, M. (1989) Ph.D. Thesis (in German), University of Konstanz, Germany.
[25] Aro, E.M., Hundell, T., Carlsberg, I. and Andersson, B. (1990) Biochim. Biophys. Acta 1019, 269–275.
[26] Nugent, J.H.A., Diner, B.A. and Evans, M.C.W. (1981) FEBS Lett. 124, 241–244.
[27] Rutherford, A.W. and Zimmermann, J.L. (1984) Biochim. Biophys. Acta 767, 168–175.
[28] Styring, S., Virgin, I., Ehrenberg, A. and Andersson, B. (1990) Biochim. Biophys. Acta 1015, 269–278.
[29] Vass, I., Styring, S., Hundal, T., Koivuniemi, A., Aro, E.-M. and Andersson, B. (1992) Proc. Natl. Acad. Sci. USA 89, 1408–1412.
[30] Petrouleas, V. and Diner, B.A. (1986) Biochim. Biophys. Acta 849, 264–275.
[31] Aasa, R., Andreasson, L.E., Styring, S. and Vänngard, T. (1989) FEBS Lett. 243, 156–160.
[32] Babcock, G.T., Widger, W.R., Cramer, W.A., Oertling, W.A. and Metz, J.G. (1985) Biochemistry 24, 3638–3645.
[33] Bowden, S.J., Hallahan, B.J., Ruffle, S.V., Evans, M.C.W. and Nugent, J.H.A. (1991) Biochim. Biophys. Acta 1060, 89–96.