High-specific binding of Fe(II) at the Mn-binding site in Mn-depleted PSII membranes from spinach

B.K. Semin[a], I.I. Ivanov[a], A.B. Rubin[a], F. Parak[b,*]

[a]*Biophysics Department, Biological Faculty, Moscow State University, Moscow, 119899, Russian Federation*
[b]*Fakultät für Physik, E17, Technische Universität München 85748 Garching, Germany*

Received 26 July 1995; revised version received 13 October 1995

Abstract The interaction of Fe(II) and Fe(III) with the 'high-affinity Mn-binding site' in Mn-depleted photosystem II (PSII) was investigated using diphenilcarbazide (DPC)/2, 6-dichlorophenol-indophenol (DCIP) inhibition assay. Fe(III) was ineffective in the inhibition of DPC–DCIP reaction while Fe(II) decreased the rate of DCIP photoreduction supported by DPC in the same concentration range as Mn(II). The effectivity of the interaction of Fe(II) with the high affinity Mn-binding site depends on different anions in the same manner as for Mn(II) and coincides with hierarchy observed for the stimulation of O_2 evolution. The Fe(II) binding is accompanied by its oxidation. By using reductants it was shown that the high affinity site contains a redox-active component and the reduction of this component totally prevents the binding of Fe(II).

Key words: Photosynthesis; Photosystem II; Oxygen evolving complex; Manganese; Iron

1. Introduction

The photosynthetic oxygen evolution in higher plants occurs in the water-splitting complex at the lumen side of the reaction center of PSII. A manganese cluster consisting of four manganese ions plays the central role in the accumulation of four positive charges in a cycle S_0–S_4 required for water oxidation process (see [1] for a review).

Although several models have been proposed [1] for the structure of the Mn complex, the residues binding the Mn atoms remain uncertain. The Mn atoms can be removed easily from PSII by different treatments including exposure to a reductant such as NH_2OH [2] or to a high concentration of Tris free base [3]. The active water splitting enzyme can be reconstituted by incubation of Mn-depleted PSII membranes in the presence of Mn [4]. The restoration of the oxygen evolution activity in Mn-depleted PSII called photoactivation was used for the investigation of the ligands of Mn [5].

Another test to probe the Mn-binding site in Mn-depleted PSII membranes was developed by Hsu et al. [6]. In samples containing Mn-depleted PSII, DPC and DCIP, DCIP can be reduced by photo-induced electron donation of DPC. Hsu et al [6] found that micromolar concentrations of Mn(II) can inhibit this electron donation. The inhibition can be measured by the electron transport efficiency, κ, giving the photoreduction rate of DCIP after some treatment of the sample (e.g. with Mn(II)-ions) with respect to the Mn-depleted PSII. The inhibition is determined by the binding of the Mn cation to the high-affinity Mn-binding site. This interaction prevents the donation by DPC to one of the two intrinsic acceptors through which DPC can donate electrons to PSII [7,8].

Cation-binding sites can be studied by a selective substitution with other metal ions. In the case of PSII a number of trivalent lanthanides have been used as suitable probes for Ca^{2+}-binding site in the oxygen-evolving complex [9]. It has also been shown that Co^{2+} and Zn^{2+} can interact with the high-affinity Mn-site [6]. But a larger concentration (>20 μM) is necessary than for Mn(II) [6]. In this respect Fe cations may be more suitable probes for the Mn sites owing to the possibility of redox transitions utilized in many enzymes. The ionic radii and the coordination number are also important factors for the substitution. For manganese and iron cations the coordination number is six. The ionic radii are very close: 0.80A/0.66 Å and 0.74A/0.64 Å for Mn(II)/Mn(III) and Fe(II)/Fe(III), respectively. Another motivation is the fact that enzymes exist which carry out the same function but utilize either Fe or Mn. One example is the superoxide dismutases (SOD) in which the coordination of the different metal cations is very similar [10].

In this paper we study the interaction of Fe(II) and Fe(III) with the high affinity Mn-site in Mn-depleted PSII membranes. A very weak interaction for Fe(III) was found whereas Fe(II) was effective in the same degree as Mn(II). Ligation of Fe(II) is accompanied by its oxidation and in the process of the iron cation binding a redox active component participates.

2. Materials and methods

Photosystem II-enriched membranes (BBY-type) were prepared from spinach according [11]. Mn depletion of PSII membranes (PSII-Mn) was accomplished by a Tris treatment according Hsu et al. [6] incubating PSII membranes (0.5 mg of chl/ml) for 15 min in 0.8 Tris buffer with pH 8.5 under room light and temperature (20°C). The membranes were collected by centrifugation, washed once and resuspended in buffer A (0.4 M sucrose, 50 mM MES/NaOH, pH 6.5, 15 mM NaCl).

Photoreduction of DCIP was monitored spectroscopically by the change of the absorbance at 600 nm. The rate of DCIP (ionized form) photoreduction in Mn-depleted PSII membranes with DPC as do-nor of electrons was around 200 (μmol of DCIP) (mg of chl)$^{-1}\cdot$h^{-1} using extinction coefficient for deprotonated form $\varepsilon = 21.8$ mM$^{-1}\cdot$cm^{-1}[12].

$MnCl_2$ and $FeSO_4$ were added from stocks prepared before the experiment in bidistillated water. Practically no oxidation of Fe(II) in the stock solution (pH 5.6) was observed during 5 h incubation at room temperature. The decreasing was less than 2%. A formation of ferrous hydroxide at used concentrations and pH (stock solution: pH 5.6, 0.55 mM; buffer A: pH 6.5, < 10 μM) does not take place [13]. The stock solution of Fe(III) (1 mM) was prepared as soluble and stable complex of ferric iron with sucrose described by Charley et al. [14]. The addition of the manganese or the iron cations to the PSII membranes was done at room light (intensity about 15 μmol quanta/m^2/s). The time between the addition of the cations and the start of the measurement of the

*Corresponding author.

SSDI 0014-5793(95)01215-X

DCIP photoreduction was between 1 and 1.5 min. Other incubation times are indicated in the legends.

3. Results

The photoreduction of DCIP in Mn-depleted PSII membranes supported by DPC is inhibited by micromolar concentrations of $MnCl_2$ [6,8,15]. Fig. 1 shows the typical $MnCl_2$ inhibition profile measured by the decrease of κ. At concentration higher than 2 μM the Mn (II) prevents the path of electron donation of DPC determined by the Mn-binding site. This is reflected in the decrease of electron-transport efficiency κ to about 0.4. Addition of 5–10 μM Fe Cl_3 to the DPC–DCIP system decreases κ only to about 0.97 (Fig. 1). Increasing the concentrations up to 80 μM did not further increase the inhibition. The weak effect of Fe(III) can not be explained by the very pure solubility of iron(III) chloride at pH > 2 because in this experiment a stable solution of a Fe(III)–sucrose complex has been used [14]. The possibility of the interaction of this Fe(III)-complex with PSII membranes is demonstrated by very intensive unspecific binding of Fe(III) with PSII membranes components [16].

Opposite to Fe(III), we observed a very strong influence of Fe(II) on the DPC photoreduction in Mn-depleted PSII membranes (Fig. 1). The treatment with $FeSO_4$ yields j values close to those of $MnCl_2$. This indicates the high affinity of the Mn-binding site to Fe(II) which is comparable with the affinity to Mn(II).

Hsu et al. [6] found that the affinity of Mn(II) to this site is influenced by the presence of different anions with a hierarchy

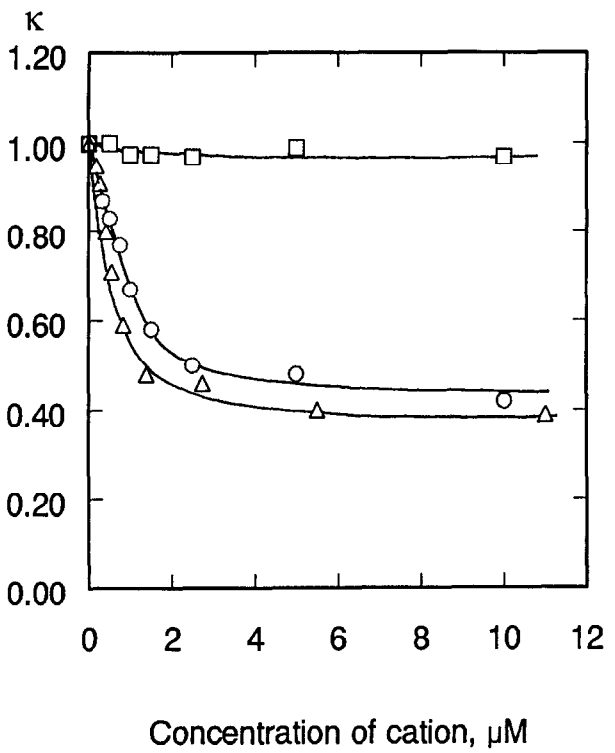

Fig. 1. The effect of Mn(II), Fe(II) and Fe(III) on the electron transport efficiency, κ, in Mn-depleted PSII membranes. The experiments were performed in room-light in buffer A containing 12 μg Chl/ml PSII membranes, 40 μM DCIP, 200 μM DPC and $MnCl_2$ (○), $FeSO_4$ (△) and $FeCl_3$ (□) in different concentrations.

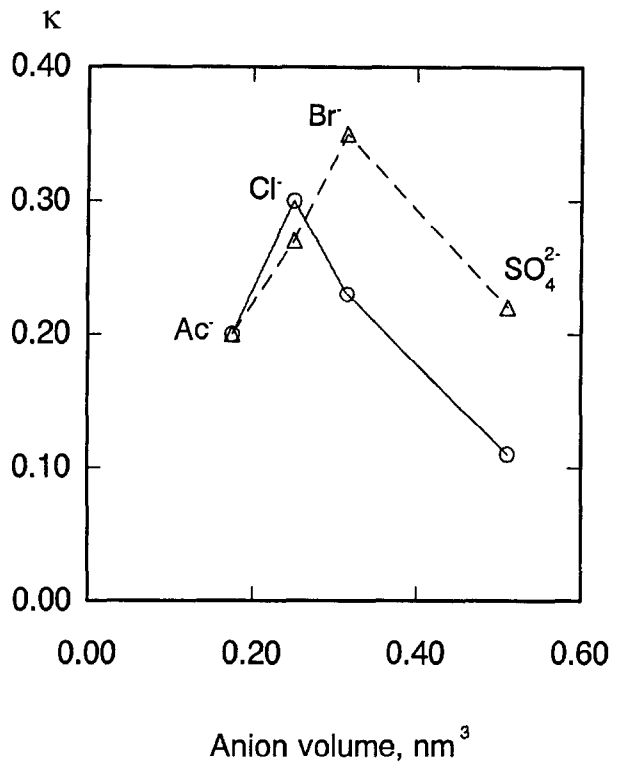

Fig. 2. Inhibition of electron transport by 1 μM $MnCl_2$ (○) and 0.55 μM $FeSO_4$ (△) in PSII-Mn membranes in the presence of various anions vs. the ion volume. The anions were added as sodium salts at a concentration of 50 mM. The experiments were performed in a medium containing 15 mM MES adjusted to pH 6.1 by Tris. The anion volumes were taken from [6].

similar to that observed for stimulation of O_2 evolution [6]. In the view of the fact that a high similarity exists between the affinity of the Mn-binding site to Mn(II) and Fe(II) we compared the effect of some anions, Cl^-, Br^-, CH_3COO^- and SO_4^{2-}, on the interaction of Fe(II) and Mn(II) with this site. The tested sodium salts prevented the binding of Mn(II) in the following order of effectiveness $SO_4^{2-} > CH_3COO^- > Br^- > Cl^-$. This order is consistent with the result received by Hsu et al. [6] excluding the reverse position for Br^- and Cl^-. The binding of Fe(II) was also inhibited by these anions actually in the same way: $SO_4^{2-} > CH_3COO^- > Cl^- > Br^-$. In Fig. 2 we have shown the inhibition of DCIP photoreducion by Mn(II) and Fe(II) in the presence of different anions as a function of the anion volumes. The plots for Fe(II) and Mn(II) are very similar and resemble the plot of reactivation of O_2-evolution by various anions in Cl^--depleted thylakoids [17]. The results indicate that several anions have practically the same influence on the binding of Mn(II) and Fe(II) to the high-affinity Mn-binding site in Mn-depleted PSII membranes. We also found that the optimal pH for the interaction of the metal cations with the high-affinity Mn site is from pH 6.0 to 6.5 for both Fe(II) and Mn(II).

The inhibition of the DPC–DCIP electron transport by Mn(II) was found to be reversible and its reactivation was observed upon decreasing the concentration of Mn(II) [6]. The removing of Mn(II) by centrifugation after some minutes of incubation of PSII-Mn membranes with Mn(II) under room light confirmed this observation (κ = 0.4 in the Mn-depleted

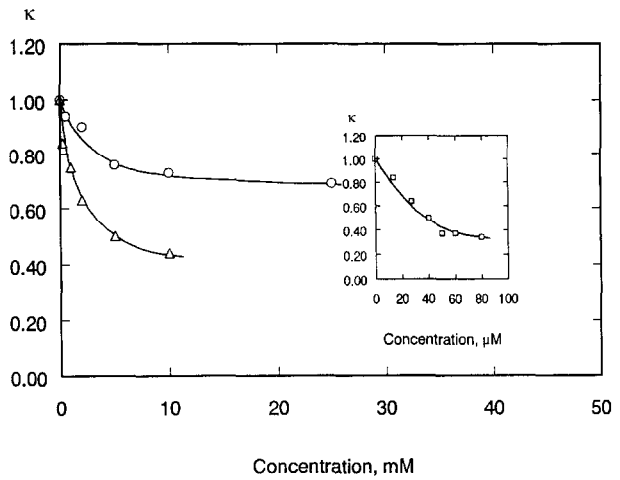

Fig. 3. Inhibition the DPC-supported DCIP photoreduction in Mn-depleted PSII membranes by reductants. Reductants: NaI (○), potassium ferrocyanide (△). (Inset) Sodium ascorbate (□). Assays were performed as described in Fig. 1, excluding the experiment with sodium ascorbate. In this case DCIP was added in excess compared to sodium ascorbate and the measurements were started 3–5 min after the addition of DCIP.

PSII membranes with 5 μM Mn(II) and κ = 1.05 after removing of Mn(II) by centrifugation). Such a reversibility was not found for Fe(II) (κ = 0.38 and 0.43 after 1 and 6 min incubation with $FeSO_4$ respectively and κ = 0.57 and 0.47, respectively, after removing of Fe(II) by centrifugation). Fe(II) bound to the high-affinity Mn-site is resistant to the action of different chelators (compare Table 1). The ineffectiveness of chelators in the removing of iron cation after its interaction with the Mn site indicates that during binding of the iron it may be oxidized. This suggestion was confirmed in an experiment in which dipyridyl was applied together with sodium dithionite (Table 1). The addition of this reductant with dipyridyl to Mn-depleted PSII-membranes after incubation with 2.75 μM $FeSO_4$ reconstituted the DPC–DCIP activity. These results indicate that the binding of Fe(II) to the Mn-binding site is accompanied by an oxidation of the cation. The oxidation can result from an autoxidation upon ligation or by an interaction with an oxidizing component at the binding site. In the experiment with the anions we observed the inhibition of the DPC–DCIP electron transport in Mn-depleted PSII membranes by NaI which is a reductant [18]. Taking into account these facts we investigated the effects of some reductants like NaI (E_0 = 0.53 V), potassium ferrocyanide (E_0 = 0.43 V) and ascorbate (E_0 = 0.08 V) in the DPC/DCIP assay conditions. The results are shown in Fig. 3.

Table 1
Effects of chelators on the electron transport efficiency, κ, in Mn-depleted, Fe(II)-substituted PSII membranes

Chelator	κ	
	without $FeSO_4$	with $FeSO_4$
	1	0.35
Ethylenediamide tetraacetic acid	0.88	0.49
o,o-Dipyridyl	0.87	0.46
o,o-Dipyridyl + Sodium dithionite*		0.93
Nitrilotriacetic acid	0.97	0.54
Chelex X-100	1.02	0.46
Tris (carboxymethyl)-ethylenediamine	0.81	0.59

Assays were performed in buffer A as described in Fig. 1. The chelators were added after 3 min incubation of Mn-depleted PSII membranes with 2.75 μM $FeSO_4$ in room-light. The incubation time with chelator was 1 min. Concentration of chelators = 1 mM; content of Chelex X-100 = about 30% (v/v) relative to PSII membranes suspension.
* After 6 min incubation of Mn-depleted PSII membranes with 2.75 μM $FeSO_4$ sodium dithionite and then dipyridyl were added followed by centrifugation after 1 min. The pellet was washed twice by buffer A and suspended in assay medium contained 60 μM DCIP.

We found that these reductants reduced κ in Mn-depleted membranes. The character of inhibition resembles to that of Mn(II). Depending on the reductant the κ-value is decreased only by 30–70% rather than 100%. The order of efficiency of the tested reductants (concentration for 50% inhibition) is sodium ascorbate>potassium ferrocyanide>NaI which coincides with the order of their redox potentials. The inhibition profile of the reducing reagents implies that reductants prevent the donation of electrons by DPC through the site which is dependent on the high affinity Mn site. This means that the high affinity Mn site contains a redox active component and the reduction of this component makes impossible the interaction of the high affinity site with DPC. One might expect that the interaction of Mn(II) or Fe(II)) with the Mn-binding site depends on this component, too.

The effect of the reductant was found to be reversible: a reactivation of κ up to 100% takes place after removing the reductant by centrifugation (compare Table 2). Using this result the influence of the redox component on the binding of Fe(II) was checked. The results shown in Table 2 demonstrate that the addition of sodium ascorbate after the addition of Fe(II) did not influence the interaction of the iron cation with the Mn-binding site. But an addition of the reductant before the addition of Fe(II) totally prevents the cation binding. This result shows the participation of the redox-active component in the ligation of Fe(II), its reduction prevents the binding of cation.

4. Discussion

The suggestion that the high-affinity Mn-binding site in Mn-depleted PSII-membranes is the native site in the water splitting system was experimentally established in the original work of Hsu et al. [6] and confirmed in the following investigations [8,15]. According to our results the high-affinity Mn-binding site in Mn-depleted PSII-membranes has a comparable affinity for Fe(II) and Mn(II). Moreover, the affinity of Fe(II) to this site was influenced by different anions in a similar way as observed for the Mn(II) binding and for the stimulation of oxygen evolution [17]. The optimum of pH for Fe(II) binding coincides with the optimum for Mn(II) binding. These facts suggest that the Fe(II) interacts with the high-affinity Mn-binding site and that sterical arrangement of the Mn ligands in the high-affinity site is suitable for the ligation of Fe(II). This is not unique in biological important molecules. Their exist a number of enzymes where the catalytic center can contain iron or manganese although the function is the same. To such proteins belong the family of SODs utilizing either Fe or Mn to catalyse the dismutation of O_2^-. In these enzymes the coordination of ligands at the Fe site of FeSOD closely corresponds to the Mn coordination found in MnSOD [10]. Morover, in the

Table 2
Influence of sodium ascorbate on the binding of Fe(II) to the high-affinity Mn-binding site in Mn-depleted PSII membranes

Sample	κ	
	Before centrifugation	After centrifugation
(PSII-Mn)	1	0.80
(PSII-Mn) + Fe(II)	0.46	0.54
(PSII-Mn) + sodium ascorbate	0.30	0.93
(PSII-Mn) + Fe(II) [3 min incubation] + sodium ascorbate [3 min incubation]		0.53
(PSII-Mn) + sodium ascorbate [3 min incubation] + Fe(II) [3 min incubation]		0.80

The assays were performed as described in Fig. 1 and Table 1. Concentrations: ascorbic acid = 50 μM; $FeSO_4$ = 2.75 μM. After centrifugation the pellet was washed twice in buffer A and resuspended in the assay medium.

SOD of *Propionibacterium shermanii* the Fe can be replaced by Mn without changing the activity [19].

An important step in the Mn ligation is its oxidation induced by light. In our experiments we also observed the oxidation of Fe(II) during the interaction with the high-affinity Mn-binding site. In this respect the binding of Fe(II) resembles to Mn(II). In contrast to Mn(II) the oxidation of Fe(II) is either a high quantum yield process and weak (room) light short illumination is enough for the oxidation. However, an autooxidation of Fe(II) taking place upon coordination cannot be excluded. Such a process occurs for example also during the ligation of Mn(II) to superoxide dismutase [20].

The present study reveals that reductants inhibit DPC supported DCIP photoreduction and its efficiency grows with the decreasing of its redox potential. What is the mechanism of this inhibition? It is known that I^- ion, ferrocyanide and ascorbate can be donors to Mn-depleted PSII although they are weaker donors than DPC [18]. The oxidation products of NaI and ferrocyanide can oxidize the reduced $DCIPH_2$ and this way mimic the inhibition of DCIP photoreduction. However, we imply that such side reactions do not interfere with the assay because: (i) ferrocyanide is a weaker donor than I^- [18] but more effective in the inhibition of electron donation by DPC; (ii) the redox potential of the couple ascorbate/dehydroascorbate (0.08 V) is less than redox potential of $DCIPH_2$/ DCIP (0.22 V), nevertheless sodium ascorbate inhibits the reduction of DCIP supported by DPC; (iii) DPC electron donation is decreased only around 50% like in the case of Mn(II). These facts show that reductants prevent the donation of electrons by DPC acting on the Mn-binding site. This implies the presence of a redox-component at this site. A reduction of this component totally prevents the binding of Fe(II). This means that it acts either as a redox-active ligand for Fe(II) ligation or it has strong influence on this process.

There are two hints suggesting the existence of a redox-active component at the donor side of PSII membranes. First, after the depletion of Ca^{2+} from the oxygen evolving complex an EPR signal with $g \sim 2$ can be detected in the S_3 state of the Mn cluster. On the basis of the absorption spectrum in the ultraviolet [21] and the FTIR difference spectroscopy [22] this radical species was proposed to be a histidine residue. Second, a photooxidizable His residue on the donor side of the PSII was inferred from the observation that the thermoluminescence A_T-band in Mn-depleted samples is reversibly quenched by a treatment with a histidine modifier diethylpyrocarbonate [23]. This residue is also proposed to mediate the oxidation of Mn(II) during photoactivation and provides a redox-active ligand for Mn.

Inhibitory effects of various reducing agents on the photoactivation induced by flash or continuous light have been shown [24,25]. It is belived that these reagents reduce the Mn(III) formed by the photooxidation in the photoligation process. Our results suggest that the reductant (at least in the case of Fe(II)) can interact not only with metal cation but also with the redox component of the binding site.

In summary, the present study shows that the binding of Fe(II) to the high-affinity Mn-binding site in Mn-depleted PSII membranes is high specific and provides a good complementary tool to investigate the assembly of water splittig complex.

Acknowledgements: This work was supported by the Deutsche Forschungsgemeinschaft and the Fond der Chemischer Industrie.

References

[1] Debus, R.J. (1992) Biochim. Biophys. Acta 1102, 269–352.
[2] Cheniae, G.M. and Martin, I.F. (1971) Plant Physiol. 47, 568–575.
[3] Cheniae, G.M. and Martin, I.F. (1978) Biochim. Biophys. Acta 502, 321–344.
[4] Tamura, N. and Cheniae, G.M. (1987) Biochim. Biophys. Acta 890, 179–194.
[5] Blubaugh, D.J. and Cheniae, G.M. (1992) Photosynth. Res. 34, 147–152.
[6] Hsu, B.D., Lee, J.-Y. and Pan, R.-L. (1987) Biochim. Biophys. Acta 890, 89–96.
[7] Blubaugh, D.J. and Cheniae, G.M. (1990) Biochemistry 29, 5109–5118.
[8] Preston, C. and Seibert, M. (1991) Biochemistry 30, 9615–9633.
[9] Bakou, A., Buser, C., Dandulakis, G., Brudvig, G. and Ghanotakis, D.F. (1992) Biochim. Biophys. Acta 1099, 131–136.
[10] Lah, M.S., Dixon, M.M., Pattrige, K.A., Stallings, W.C., Fee, J.A. and Ludwig, M.L. (1995) Biochemistry 34, 1646–1660.
[11] Ghanotakis, D.F. and Babcock, G.T. (1983) FEBS Lett. 153, 231–234.
[12] Armstrong, J.M. (1964) Biochim. Biophys. Acta 86, 194–197.
[13] Kragten J. (1978) in: Atlas of Metal–Ligand Equilibria in Aqueous Solution (Chalmers, R.A. and Masson, M. eds.) p. 285, Ellis Horwood Ltd., New York/London/Sydney/Toronto.
[14] Charley, P.J., Sarkar, B., Stitt, C.F. and Saltman, P. (1963) Biochim. Biophys. Acta 69, 313–321.
[15] Preston, C. and Seibert, M. (1991) Biochemistry 30, 9625–9633.
[16] Semin, B.K., Alexandrov, A. Ju., Novakova, A.A., Ivanov, I.I., Rubin, A.B., Parak, F. (1995) Biol. Membrani (Russ.) in press.
[17] Critchley, C., Baianu, I.C., Govindjee, Gutowski, H.S. (1982) Biochim. Biophys. Acta 682, 436–445.
[18] Izawa, S. and Ort, D.R. (1974) Biochim. Biophys. Acta 327, 127–143.
[19] Meier, B., Barra, D., Bossa, F., Calabrese, L. and Rotilio, G. (1982) J. Biol. Chem. 257, 13977–13980.
[20] Brock, C.J. and Walker, J.E. (1980) in: Chemical and Biochemical Aspects of Superoxide Dismutase (Bannister, J.V. and Hill, H.A.O. eds.) p. 237, Elsevier, Amsterdam.
[21] Boussac, A., Zimmermann, J.L., Rutherford, A.W. and Lavergne, J. (1990) Naturc 347, 303–306.
[22] Bertomieu, C. and Boussac, A. (1995) Biochemistry 34, 1541–1548.
[23] Ono, T.-A. and Inoue, Y. (1991) FEBS Lett. 278, 183–186.
[24] Ono, T.-A. and Inoue, Y. (1987) Plant Cell Physiol. 28, 1293–1299.
[25] Tamura, N. and Cheniae, G.M. (1987) in: Progress in Photosynthesis Res. (Biggins, J. ed.) pp. 621–624, Martinus Nijhoff.