Available online at www.sciencedirect.com
ScienceDirect
Biochimica et Biophysica Acta 1767 (2007) 624–632
Biochimica et Biophysica Acta
BBA
www.elsevier.com/locate/bbabio

Protective effect of bicarbonate against extraction of the extrinsic proteins of the water-oxidizing complex from Photosystem II membrane fragments

Olga V. Pobeguts *, Tatiana N. Smolova, Olga M. Zastrizhnaya, Vyacheslav V. Klimov

Institute of Basic Biological Problems, Russian Academy of Sciences, Pushchino, Moscow Region 142290, Russia

Received 15 October 2006; received in revised form 18 December 2006; accepted 20 December 2006
Available online 4 January 2007

Abstract

A protective effect of bicarbonate (BC) against extraction of the extrinsic proteins, predominantly the Mn-stabilizing protein (PsbO protein), during treatment of Photosystem II (PS II) membrane fragment from pea with 2 M urea, and at low pH (using incubation in 0.2 M glycine–HCl buffer, pH 3.5 or 0.5 M citrate buffer, pH 4.0–4.5) was detected. It was shown that the extraction of the proteins with Mw 24 kDa (PsbP protein) and 18 kDa (PsbQ protein) by the use of highly concentrated solutions of NaCl does not depend on the presence of BC in the medium. An optimal concentration of BC at which it produces the maximum protecting effect was shown to be between 1 mM and 10 mM. The addition of formate did not influence the protein extraction but it reduced the stabilizing effect of BC. Independence of the stabilizing effect on the presence of the functionally active Mn within the water-oxidizing complex indicates that the protecting effect of BC is not related to its interaction with Mn ions. The fact that there is a preferable sensitivity of the PsbO protein to the absence of BC in the medium during all the treatments makes it possible to suggest that either BC interacts directly with the PsbO protein or it binds to some other sites within PS II and this binding facilitates the preservation of the native structure of this protein.

Keywords: Photosystem II; Extrinsic proteins; Manganese-stabilizing protein (PsbO protein); 24 kDa protein (PsbP protein); 18 kDa protein (PsbQ protein); Bicarbonate

1. Introduction

Bicarbonate (BC) is known to be necessary for the maximal activity of Photosystem II (PS II) (for a recent review, see Ref. [1]. Initially, its site of action was thought to be in the oxygen-evolving system [2,3]. However, later strong evidence for the action of BC on the acceptor side of PS II, providing efficient re-oxidation of the first plastoquinone electron acceptor, Q_A, has been presented [4], and the idea was supported by a number of data [1]. The non-heme Fe between Q_A and the secondary plastoquinone electron acceptor, Q_B, has been shown to play an essential role in BC binding [5,6]. Strong evidence for BC requirement within the water-oxidizing complex (WOC) of PS II has been presented [7–12]. The following explanations for the involvement of BC in the events within the WOC are considered: (1) BC serves as an electron donor (alternative to water or as a way of involvement of water molecules in the oxidative reactions) to the Mn-containing WOC; (2) BC facilitates re-assembly of the WOC from apo-WOC and Mn (II); (3) BC is an integral component of the WOC essential for its function and stability; it may be considered as a direct ligand to the Mn-cluster or as a base with an appropriate pK; (4) BC stabilizes the WOC indirectly, through its binding to other components of PS II, in particular, with the protein components of PS II (the integral proteins of PS II or extrinsic proteins with Mw 33 kDa (PsbO protein), 24 kDa (PsbP protein) and 18 kDa (PsbQ protein) that are included in the WOC and associated with the lumenal surface of thylakoid membrane) that is important for structural organization of PS II complex. It is quite possible that amino acid residues of the protein components of PS II core complex may serve as ligands to BC.

Abbreviations: DCBQ, 2,5-dichloro-1,4-benzoquinone; Chl, chlorophyll; PS II, photosystem II; MES, 4-(N-morpholino)ethanesulfonic acid; SDS, sodium dodecyl sulfate; Tris, 2-Amino-2-(hydroxymethyl)-1,3-propanediol; WOC, water-oxidizing complex; BC, bicarbonate

* Corresponding author. Tel.: +1 496 773 1966; fax: +1 496 733 0532.
E-mail address: nikitishena@mail.ru (O.V. Pobeguts).

0005-2728/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbabio.2006.12.010

The first isolation of the extrinsic proteins from PS II membrane fragments was performed by 0.8 M Tris–HCl buffer treatment at pH 8.4 [13]. At present time there are a number of methods to isolate these extrinsic proteins from WOC that are in addition to the Tris-treatment described above as follows: treatments with 0.25–1 M NaCl [14], 1 M $CaCl_2$ [15], 3–5 min heat-treatment at 50 °C [16], treatment at acidic pH [17], treatment with 2.6 M urea in the presence of NaCl [18]. As was shown earlier, the extraction of the PsbO protein is accompanied by the release of Mn from the WOC [19]. However, as a result of urea and 1 M $CaCl_2$ treatment the release of Mn does not occur [15,17]. All the extrinsic proteins are essential for stabilization and functioning of the WOC. The PsbP and PsbQ proteins cooperate to optimize the levels of Ca^{2+} and Cl^-, which are essential cofactors for the water splitting reaction [20]. Extraction of the PsbO protein from PS II particles strongly diminishes the oxygen evolution rate and affects the turnover and the stability of the higher redox states of the WOC and Mn binding [18,21,22]. Therefore the extrinsic PsbO protein is often referred to as Mn stabilizing protein. The 3.5 Å resolution X-ray analysis of a cyanobacterial PS II [6] produced an eight-strand β-barrel structure for the PsbO protein that is located in the thylakoids lumen with the C- and N-termini directed towards the luminal side. It has been shown that the PsbO protein is an elongated protein composed of two major domains [23]. An apparent contradiction between an extraordinary stability and flexibility presented in the same time in the PsbO protein can be explained by the existence of one rigid "solid" part of the PsbO protein (domain I) and one flexible "liquid" part (domain II). One of the most interesting properties of the PsbO protein is the existence of hysteresis [24,25]. This PsbO protein property can be explained by the existence of two steady states of the protein that differ in their proton accepting ability. The PsbO protein comprises several carboxylic groups with unusually high pK_a values of about 5.7 that is a unique feature of this protein [24,25]. These last properties suggest that the PsbO protein might participate in proton transfer from the WOC into the lumen through a hydrogen bond network [23,25].

Despite the fact that numerous data demonstrating the participation of BC in PS II functioning have been reported, its binding sites and its role in the photosynthetic oxidation of water mechanisms remain unclear. In this paper we present evidence for a protecting effect of BC against extraction of the extrinsic proteins of the WOC from PS II membrane fragments in the course of treatments by the high concentration of urea (0.2–3 M), NaCl (0.25–1.5 M) and at acidic pH using glycine–HCl buffer (pH 3.5) and citrate buffer (pH 4.0–4.5).

2. Materials and methods

2.1. Isolation of membrane fragments of PS II

The PS II membrane fragments ("BBY-particles") were isolated from pea leaves (*Pisum sativum*) as described earlier [26] with some modifications [27]. The preparations were centrifuged for 15 min at 20,000×*g* and resuspended in 0.1 M Mes–NaOH buffer (pH 5.5 or pH 6.5) with 200 mM NaCl, 0.3 M sucrose and depleted of CO_2/HCO_3^- (buffer A).

2.2. Membrane fragments treatment at acidic pH, with urea and NaCl

Three ways for dark extraction of the extrinsic proteins at 4 °C were selected for investigation: treatment of membrane fragments with highly concentrated urea, NaCl solutions and treatment at acidic pH (pH < 5.0). In the first case 30 μl of sample containing 3 mg of chl·ml^{-1} were placed in a glass tube (0.5 ml) with screw cap and were diluted 10-fold by the buffer A to a concentration of 300 μg chl·ml^{-1}. Then an amount of BC was applied on the tube wall. After that in the case of urea treatment, 6 M solution of urea (the final concentration was from 0.2 M to 3 M) was added to the solution in the tube and the resulting mix was shaken for 15 min in an ice-cold bath at 4 °C. After the incubation the samples were centrifuged at 20,000×*g* for 10 min and supernatants and pellets were analyzed. The proteins in the supernatant were precipitated using a 20% trichloroacetic acid solution and cooled acetone. Pellets were then suspended in the electrophoresis buffer. In the second treatment, right after the addition of BC we added a high molar solution of NaCl to the samples so that its final concentration was equal to 0.25 M, 0.5 M, 0.75 M, 1 M, 1.5 M. After that the samples were incubated during 15 min in an ice-cold bath at 4 °C. Then the samples were centrifuged at 20,000×*g* for 10 min. The proteins in the supernatants were precipitated as described earlier. The third way of protein extraction we used, a treatment at acidic pH, was conducted as follows: an amount of 30 μl of the analyzed sample containing 3 mg chl·ml^{-1} was placed in a 0.5 ml glass tube with a screw cap. Then an amount of BC was dripped on the tube wall. After that a 0.2 M glycine–HCl buffer (pH 3.5) or a 0.5 M citrate buffer (pH 4.0, pH 4.5) were added. The samples were diluted 10 fold so that the final concentration was 300 μg chl·ml^{-1}. After 20-min incubation at 4 °C the samples were centrifuged at 20,000×*g* for 15 min. The proteins remaining in supernatants after membrane fragments incubation were precipitated as described above.

2.3. Electrophoretic analysis

After the treatment the polypeptides in initial preparations, supernatants and pellets of treated membrane fragments were analyzed in a PAAG electrophoresis under denaturing conditions (12–18% polyacrylamide, 0.1% (w/v) SDS) [28]. The upper electrode Tris–glycine buffer (150 mM, pH 8.3) contained 0.1% (w/v) SDS. The gels were stained with 0.25% (w/v) Coomassie G-250 in order to view the protein distribution. After staining, the gels were photographed by digital camera Power Shot A420 (Canon) and processed by the means of Total Lab program (v. 2.01). Molecular markers (Bio-Rad Laboratories, Hercules, CA) were used for the protein molecular weight estimation.

2.4. Quantitation of photosynthetic activity

Photosynthetic oxygen evolution was measured with a Clark-type oxygen electrode for the first 30–60 s of illumination. The measurements were carried out at 25 °C in the presence of 0.1 mM 2,5-dichloro-1,4-benzoquinone (DCBQ) and 0.5 mM $K_3[Fe(CN)_6]$ used as exogenous electron acceptors. The measurements medium was 0.1 M Mes–NaOH solution (pH 6.5) containing 0.3 M sucrose, 10 mM NaCl and 40 mM $CaCl_2$. Red actinic light at an intensity of 2000 μmol photons·m^{-2}·s^{-1} passed through a heat-absorbing filter (3% solution of $CuSO_4$) and a red optical filter (KS-11, Russia).

2.5. Measurement of the Mn-content in membrane fragments of PS II

The content of Mn in preparations was determined by the means of atomic-absorption on the Perkin-Elmer 503 spectrophotometer (USA) using graphitic atomizator HGA-74 [29].

3. Results

3.1. Treatment of PS II membrane fragments with urea

Fig. 1 shows the release of 51% of the Psb0 protein, 73% of PsbP protein and 95% of PsbQ protein after a 15-min incubation

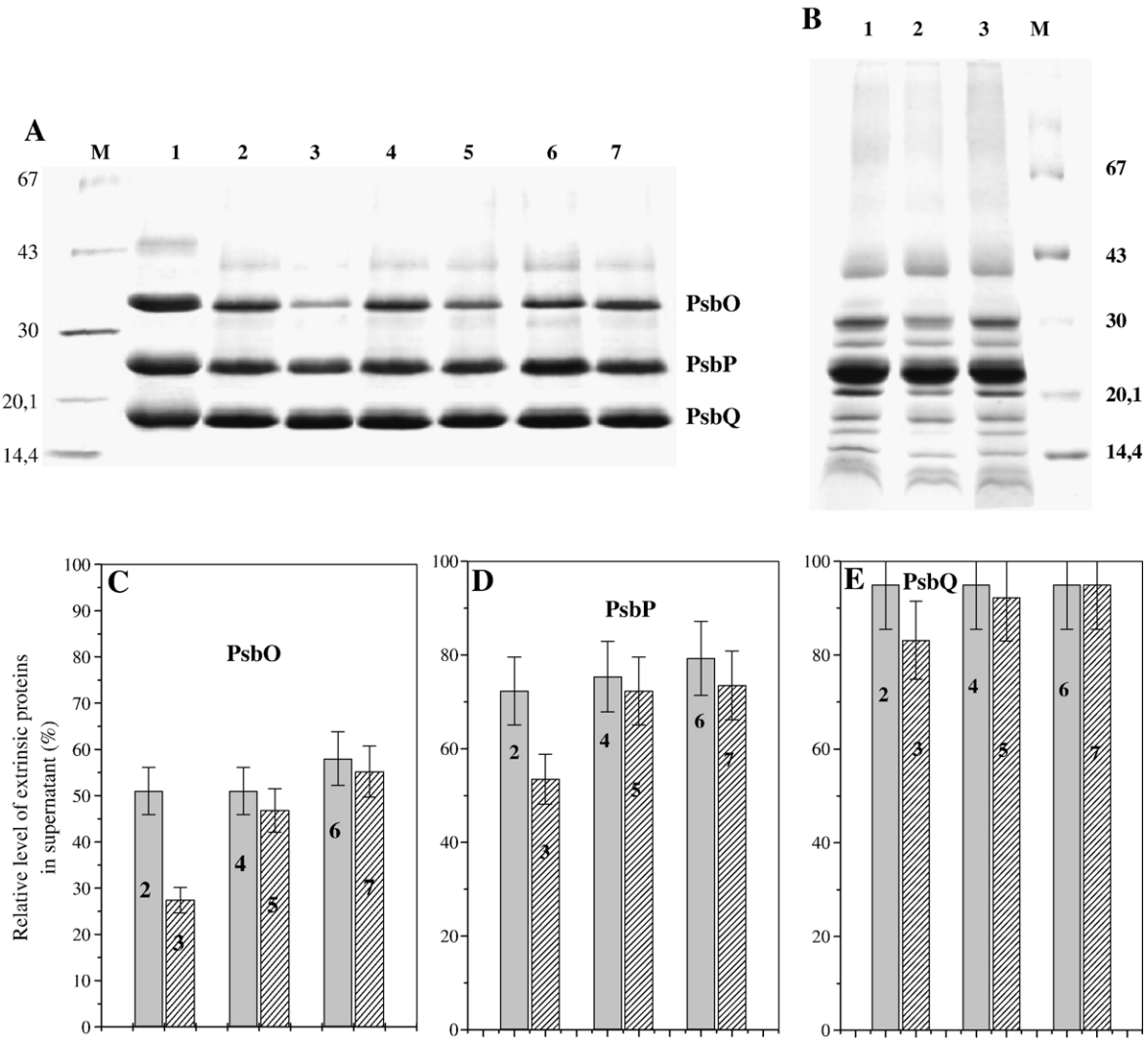

Fig. 1. Effect of added bicarbonate and formate on extraction of extrinsic PsbO, PsbP and PsbQ proteins from PS II membrane fragments upon treatment with 2 M urea. (A) Electrophoregrammes of the supernatants obtained after a 10-min centrifugation at 12000×*g* of PS II membrane fragments treated with urea (15 min at 4 °C at chlorophyll concentration of 0.3 mg ml^{-1}) in the medium contained 100 mM MES–NaOH (pH 5.5), 0.2 M NaCl and 0.3 M sucrose and depleted of HCO_3^-/CO_2 in the absence of other additions (2) and after the addition (during the incubation) of 5 mM $NaHCO_3$ (3); 1 mM formate (4); 1 mM formate and 5 mM $NaHCO_3$ (5); 2 mM formate (6); 2 mM formate and 5 mM $NaHCO_3$ (7). Lane 1 is electrophoregramme of the supernatant obtained after a 10-min centrifugation at 12,000×*g* of PS II membrane fragments treated with 0.8 M Tris–HCl buffer at pH 8.0 (30 min at 4 °C at chlorophyll concentration of 0.3 mg ml^{-1}). (B) Electrophoregrammes of the pellets obtained in the experiments described in A in the absence (2) and presence (3) of 5 mM $NaHCO_3$; (1), the initial (untreated) PS II membrane fragments. M, standards containing albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin (20.1 kDa) and α-lactalbumin (14.4 kDa). (C, D and E) Quantitative analysis of the data shown in A, the numbers of the diagram columns in C, D and E correspond to the numbers of the electrophoretic lanes in A. 100% corresponds to the initial content of the protein in the PS II preparation. The data are an average of three experiments.

of membrane fragments in the presence of 2 M urea in the medium A depleted of CO_2/HCO_3^-. The quantity of extracted proteins decreases if 5 mM $NaHCO_3$ is added prior to the treatment so that it becomes equal to 27%, 54% and 84% for the Psb0, PsbP and PsbQ proteins, respectively. So, BC protects the extrinsic proteins against extraction and the stabilizing effect of BC is especially pronounced for the Psb0 protein: its extraction efficiency is nearly two times lower in the presence of $NaHCO_3$. In contrast to BC, formate added at concentration of 1 mM or 2 mM does not prevent (and even stimulates) extraction of the Psb0 protein from the membrane (Fig. 1A, C). (In order to obtain comparable concentrations of formate and BC in the medium, formate was added at lower concentrations than BC taking into account the difference in pKs: pK 6.4 for bicarbonate and pK 4.3 for formate). When BC is added during incubation of the sample containing formate, a very low (3–5%) retardation of the extraction of the extrinsic proteins induced. Thus, the addition of BC in contrast to formate efficiently stabilizes the binding of the PsbO, PsbP and PsbQ proteins to the membrane during the treatment of PS II preparations with 2 M urea in the medium depleted of CO_2 /HCO_3^-, and the PsbO protein is a protein component the most sensitive to the presence of BC in the medium.

Fig. 2 shows that during the 15-min incubation of PS II membranes in medium A (at pH 5.5) depleted of CO_2/HCO_3^- in the presence of 2 M urea, the optimal concentration of added

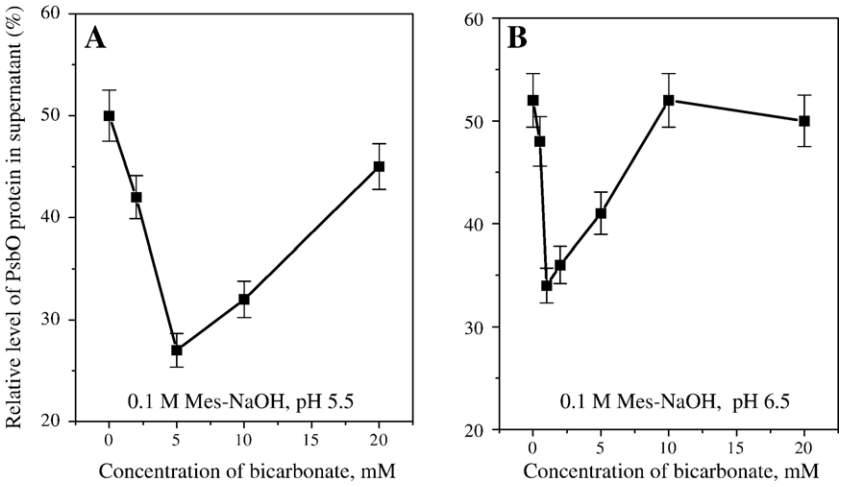

Fig. 2. Dependence of the PsbO protein extraction from PS II membrane fragments with 2 M urea in the medium contained 100 mM Mes–NaOH, 0.2 M NaCl and 0.3 M sucrose and depleted of HCO_3^-/CO_2 (as described in Fig. 1) on concentration of added $NaHCO_3$ at pH 5.5 (A) and pH 6.5 (B). The data are an average of three experiments. A pH shift upon the addition of 10 mM and 20 mM BC at pH 5.5 and pH 6.5 was less than 0.1 units.

BC (inducing the maximal stabilizing effect) is 5 mM (Fig. 2A), while in the same medium at pH 6.5 it is equal to 1 mM (Fig. 2B). Evaluation of the equilibrium concentration of added BC at these two pH shows that the optimal concentration of anion HCO_3^- at pH 5.5 and pH 6.5 is 0.62 mM and 0.6 mM, respectively. Upon further increase of BC concentration the protecting effect becomes lower so that at concentration of added BC equal to 10 mM (at pH 6.5) and 20 mM (at pH 6.5 and pH 5.5) the stabilizing effect is practically absent.

3.2. Treatment of membrane fragments with high molar NaCl

In order to compare the effects of BC on the extrinsic proteins of PS II, the high NaCl concentrations treatment of membrane fragments was used, what results in a predominant extraction of the PsbP and PsbQ proteins Da [13,14,30]. The concentration of NaCl varied from 0.25 M to 1.5 M. Fig. 3 shows that a 15-min treatment of membrane fragments in 0.1 M Mes–NaOH buffer (pH 5.5) depleted of CO_2/HCO_3^- in the presence of 1 M NaCl results in a predominant extraction of the PsbP protein (52%) and the PsbQ protein (41%) along with an extraction of the PsbO protein (11%), in agreement with the literature data [30]. The addition of 5 mM $NaHCO_3$ to the incubation medium does not prevent the removal of the PsbP and PsbQ proteins, while there is some retardation in the extraction of the MSP so that only 6% is found in supernatant. Similar results were obtained as a result of the treatment with 0.25 M and 0.5 M NaCl. Thus, the removal of PsbP and PsbQ proteins by the treatment of PSII membrane fragments with highly concentrated NaCl solutions is not sensitive to BC in the medium.

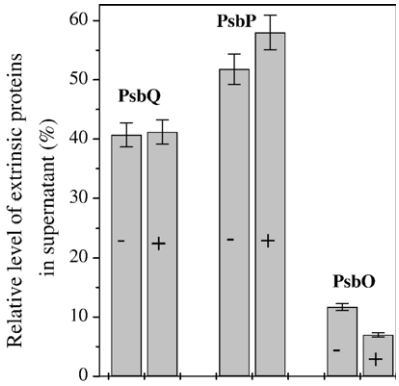

Fig. 3. Diagram columns correspond of the supernatants obtained after a 10-min centrifugation at 12,000×*g* of PS II membrane fragments treated with 1 M NaCl (15 min at 4 °C at chlorophyll concentration of 0.3 mg ml^{-1}) in the medium containing 100 mM MES–NaOH (pH 5.5), 0.3 M sucrose and depleted of HCO_3^-/CO_2 in the absence of other additions (−) and after the addition (during the incubation) of 5 mM $NaHCO_3$ (+). 100% corresponds to the initial content of the protein in the PS II preparation. The data are an average of three experiments.

3.3. Treatment of PS II membrane fragments at low pH

Fig. 4A shows the effect of added $NaHCO_3$ on the extraction of the extrinsic proteins during a 20-min treatment of membrane fragments in 0.2 M glycine–HCl buffer (pH 3.5). Without an addition of BC, 56% of the PsbO protein, 24% of the PsbP protein and 5–6% of the PsbQ protein are revealed in supernatant, while after the addition of 10 mM $NaHCO_3$ to the incubation medium the protein extraction is equal to the portions of 5%, 2% and 0%, respectively. A considerable protecting effect against the extraction of the PsbO protein is observed already at 2 mM $NaHCO_3$ (Fig. 4B, curve 1) and nearly 95% of the protein is not removed to the medium in the presence of 10 mM $NaHCO_3$ (i.e. practically all the protein remains bound to the membrane). This conclusion is supported by the data on the PsbO protein content in the membrane: in the presence of added 10 mM $NaHCO_3$ a protein portion of almost 95% remains bound to the membrane (Fig. 4B, curve 2). On the other hand, the stabilizing effect of BC is some what lowered upon the increase of BC concentration from 10 mM to 20 mM. Replacement of anion HCO_3^- by an equivalent quantity of NaCl

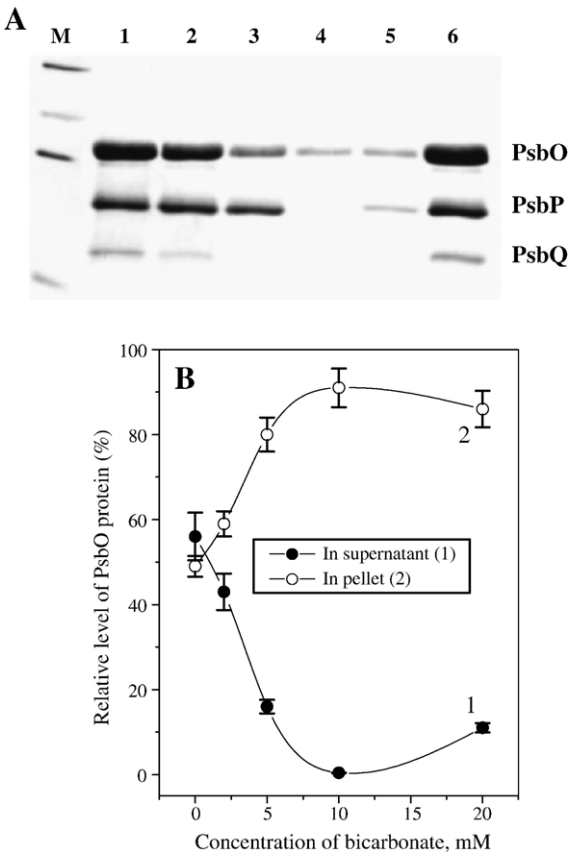

Fig. 4. Effect of bicarbonate on extraction of the extrinsic PsbO, PsbP and PsbQ proteins from PS II membrane fragments at pH 3.5. (A) Electrophoregrammes of the supernatants after a 10-min centrifugation at 12,000×*g* of PS II membrane fragments (0.3 mg Chl ml^{-1}) incubated for 20 min at 4 °C in the medium containing 0.2 M glycine–HCl-buffer, pH 3.5 without other additions (lane 1) and after the addition of 2 mM $NaHCO_3$ (lane 2), 5 mM $NaHCO_3$ (lane 3), 10 mM $NaHCO_3$ (lane 4), 20 mM $NaHCO_3$ (lane 5) and 10 mM NaCl (lane 6). M, standards containing albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin (20.1 kDa) and α-lactalbumin (14.4 kDa). (B) Dependence of the content of the PsbO protein in the supernatant (curve 1) and pellet (curve 2) on concentration of BC added to the medium during the treatment of PS II membranes described in A. The data are an average of three experiments. The initial content of the PsbO protein in the PS II preparation is taken as 100%.

did not affect the protein extraction (Fig. 4A). In a number of separate experiments we have checked that the PS II sample by itself does not shift the pH of the medium (the shift was lower than 0.05 unit), while the addition of 5 mM, 10 mM and 20 mM of $NaHCO_3$ shifts pH of the incubation medium by 0.05, 0.15 and 0.3 units, respectively. The observed decrease of the protecting effect of BC upon the increase in BC concentration from 10 mM to 20 mM (accompanied by the highest shift of pH) indicates that the stabilizing effect upon the addition of BC at concentration from 2 mM to 10 mM is not related to the pH shift.

For a more correct quantitative evaluation of the content of the extrinsic proteins remaining bound to the membrane after the treatment at low pH, the membrane fragments were subjected to a secondary treatment with 0.8 M Tris–HCl (pH 8.0), that is often used to remove the extrinsic proteins [13]. The results of the electrophoretic analysis of the supernatants and pellets after the primary (at low pH) and secondary (by Tris) treatments are shown in Fig. 5. Comparison of the lanes 2, 4 and 6 (as well as the data shown on Fig. 4B) shows that in the presence of BC the quantity of the PsbO protein remaining bound to the membrane after the first treatment and extracted during the secondary treatment (with Tris) increases considerably. However, for the two other proteins (PsbP and PsbQ proteins) the results are less certain. At first sight, the protecting effect of BC against extraction of these proteins is also observed (Fig. 4A). On the other hand, the results shown in Fig. 5 disprove this conclusion: in the electrophoretic lanes corresponding to the pellets obtained after the treatment of the membranes at pH 3.5 (Fig. 5, lanes 3 and 4) as well as in the lanes of corresponding supernatants obtained after the treatment of the membranes by Tris (Fig. 5, lanes 5 and 6) the bands corresponding to the PsbP and PsbQ proteins are practically not seen (in contrast to the PsbO protein). So, after the treatment of the membranes at acidic pH these two proteins do not remain bound to the membrane regardless of the presence or absence of BC. They are extracted and evidently form high molecular aggregates which are not dissolved neither by Tris during the treatment nor by SDS and mercaptoethanol during the electrophoresis. So, in the experiments on treatment at acidic pH, only in the case of the PsbO protein it is clear that its binding to the membrane is considerably stabilized upon the addition of BC.

The dependence of the extraction of the extrinsic proteins upon the treatment of the membrane fragments by 0.5 M citrate buffer (pH 4.5) on concentration of added BC is shown in Fig. 6. (It was checked that in these experiments the shift of pH upon the addition of $NaHCO_3$ or PS II samples is less than 0.05 units). After a 20-min incubation, 36% of the PsbO protein, 21% of the PsbP protein and 15% of the PsbQ protein are in the

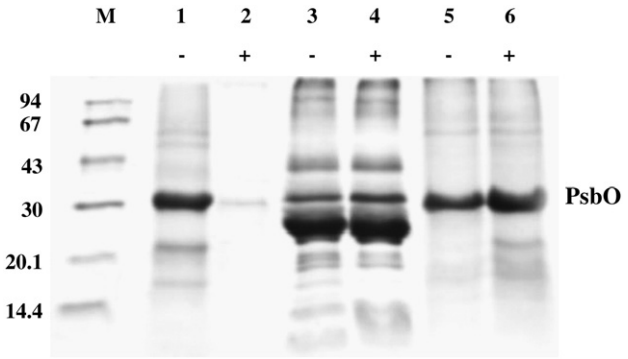

Fig. 5. Electrophoregrammes of the supernatants (lane 1, 2) and pellets (lane 3, 4) after a 10-min centrifugation at 12,000×*g* of PS II membrane fragments incubated for 20 min at 4 °C in the medium containing 0.2 M glycine–HCl buffer, pH 3.5 without other additions (−) and after the addition of 10 mM $NaHCO_3$ (+). Lanes 5 and 6 are the supernatants after subsequent incubation of the glycine-treated membrane fragments (pellets 3 and 4, respectively) for 30 min at 4 °C in the medium containing 0.8 M Tris–HCl (pH 8.0) and a 10-min centrifugation at 12,000×*g*. M, standards containing albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin (20.1 kDa) and α-lactalbumin (14.4 kDa).

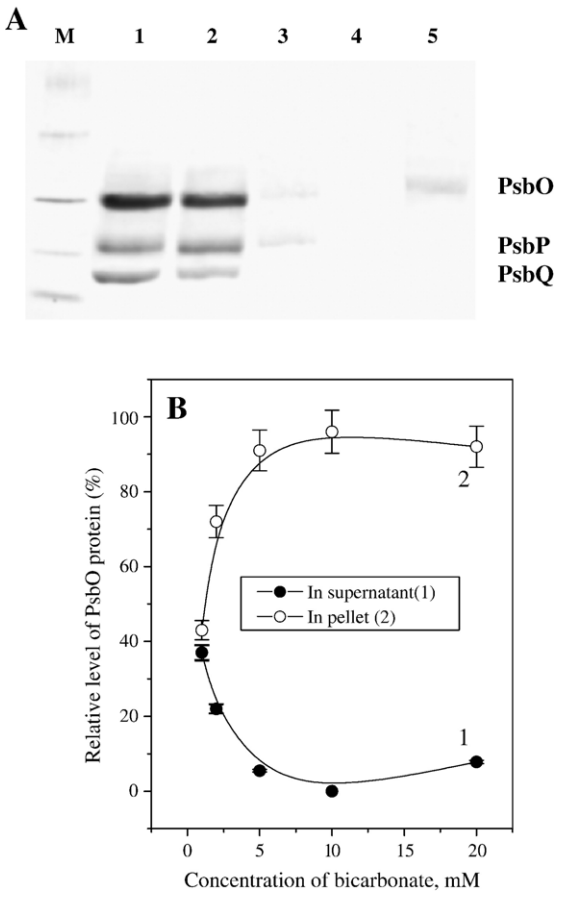

Fig. 6. Effect of bicarbonate on extraction of the extrinsic PsbO, PsbP and PsbQ proteins from PS II membrane fragments at pH 4.5. (A) Electrophoregrammes of the supernatants after a 10-min centrifugation at 12,000×*g* of PS II membrane fragments (0.3 mg Chl·ml^{-1}) incubated for 20 min at 4 °C in the medium containing 0.5 M citrate-buffer, pH 4.5 without other additions (lane 1) and after the addition of 2 mM $NaHCO_3$ (lane 2), 5 mM $NaHCO_3$ (lane 3), 10 mM $NaHCO_3$ (lane 4), 20 mM $NaHCO_3$ (lane 5). M, standards containing albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin (20.1 kDa) and α-lactalbumin (14.4 kDa). (B) Dependence of the content of the PsbO protein in supernatant (curve 1) and pellet (curve 2) on concentration of BC added to the medium during the treatment of PS II membranes described in A. The data are an average of three experiments. The initial content of the PsbO protein in the PS II preparation is taken as 100%.

supernatant, while the addition of 10 mM $NaHCO_3$ during the incubation results in a practically complete absence of the proteins in the supernatant. The protecting effect is already detected at 2 mM $NaHCO_3$ and it achieves its maximal level at 10 mM $NaHCO_3$. Upon the increasing of BC concentration the decrease in the PsbO protein amount in supernatant (curve 1) is accompanied by its increased content in the membrane (curve 2). Secondary treatment of membranes with 0.8 M Tris–HCl buffer (pH 8.0) showed (as is in the case of the treatment in glycine buffer) that the PsbP and PsbQ proteins do not remain bound to the membrane after an incubation in 0.5 M citrate (pH 4.5) and they are evidently extracted and form high molecular aggregates (data not shown). A similar extrinsic proteins extraction level dependence on the BC concentration in the medium was shown in citrate buffer at pH 4.0 (data not shown).

3.4. Effect of the treatments on the Mn content and the O_2-evolving activity in PS II membrane fragments

The Mn content in the PS II membranes was equal 0.95±0.05 μg·mg^{-1} Chl (corresponding to 4±0.1 Mn per PS II reaction center if we accept that the ratio Chl:PS II reaction center equals 250 [31]), the content did not change upon the treatments with 2 M urea or with 0.2 M glycine–HCl buffer (pH 3.5) (0.98±0.05 μg·mg^{-1} Chl or 0.97±0.05 μg·mg^{-1} Chl, respectively), independent of the presence of BC in the medium. In contrast, the treatment with 0.5 M citrate buffer (pH 4.5) resulted in a considerable decrease of Mn content in the PS II membranes: it was equal to 0.29±0.05 μg·mg^{-1} Chl, independent of the presence of BC in the medium that corresponds to 1.25±0.25 Mn per PS II reaction center. So, nearly 3 of 4 Mn atoms are removed after the latter treatment.

To determine the O_2-evolving activity, the membrane fragments treated with 2 M urea at low pH were pelleted by centrifugation at 20,000×*g* for 15 min and, in order to measure their activity, were resuspended in medium (see Materials and methods). After the treatment of the PS II membrane fragments with 2 M urea in the absence and presence of 5 mM $NaHCO_3$ the remaining activity was nearly 14% and 58%, respectively (the initial activity was 420 μmol O_2·mg^{-1} Chl·h^{-1}). A treatment with citrate buffer (pH 4.5) resulted in a considerable loss of the O_2-evolving activity: after incubation in the absence or presence of 10 mM $NaHCO_3$ it was equal to 12% and 18% of the initial activity, respectively. In contrast, upon the treatment with 0.2 M glycine–HCl buffer (pH 3.5) in the absence and the presence of 10 mM $NaHCO_3$ the residues of 85% and 97% of the initial activity were detected.

4. Discussion

The results presented above clearly demonstrate that BC added to the incubation medium considerably stabilizes the binding of the extrinsic PsbO, PsbP and PsbQ proteins of the WOC to the membrane during the treatment of PS II preparations with 2 M urea, while in the case of treatment at low pH the protective effect of BC is revealed only against extraction of the PsbO protein.

In the course of the treatment of PS II membrane fragments with urea, the binding of the three proteins depends in a different way on the presence of BC. The PsbO protein is the most sensitive extrinsic protein to the absence of BC in the medium (in the presence of 5 mM $NaHCO_3$ the PsbO protein extraction is 2 times lower than in the medium depleted of BC (Fig. 1). It is not excluded that the extraction of the PsbO protein can affect the binding of the two other proteins. There are different views on the interaction of the extrinsic proteins with each other and with the PS II membrane. From the experiments with bifunctional cross-linking agents it was concluded that the proteins are associated with each other. The PsbP protein has two binding sites: one for the PsbO protein and another for an integral component of PS II [31,32] while the PsbQ protein is bound to the PsbP protein [31]. On the other hand, there is no specific interaction between these proteins in solution, though

when the PsbO protein is bound to the membrane it facilitates the binding of the PsbP protein, and both of them, in turn, facilitate the binding of the PsbQ protein either by a direct providing of a binding site or by inducing structural changes in other PS II components [33]. Enami and co-workers suggested that there is an interaction between the PsbO protein and the pigment–protein complex CP47, as well as between the PsbP and PsbQ proteins [34]. Evidently the three proteins interact with each other and it is not excluded that the protecting effect of BC against the urea-induced extraction of the PsbP and PsbQ proteins can be mediated by the BC effect on binding of the PsbO protein.

In the case of treatment at acidic pH, it is only the protective effect of BC against extraction of the PsbO protein that is clearly demonstrated. The addition of 10 mM $NaHCO_3$ during the incubation both in 0.2 M glycine–HCl buffer (pH 3.5) and in 0.5 M citrate buffer (pH 4.5) practically completely prevents the extraction of the PsbO protein from the membrane while the binding of the PsbP and PsbQ proteins in this case is not stabilized by BC. The difference in pI (for the PsbO PsbP and PsbQ proteins it were 5.2, 6.5 and 9.2, respectively) and in the binding constants for these proteins [35] as well as the existence of the unique β-barrel-like structure in the PsbO protein [6,23], evidently determines the observed difference in behavior of the proteins in response to the low pH treatment and the addition of BC.

It is interesting that while the addition of BC increases in a stepwise manner its concentration in solution, the BC growing protective effect against protein extraction changes into lowering of this effect. Thus for each treatment type there is an optimal concentration of $NaHCO_3$ that provides an effective protection. During the treatment with urea the optimal concentrations of the added BC at pH 6.5 and pH 5.5 are 1 mM and 5 mM, respectively. The equilibrium concentration in both cases equals nearly 0.6 mM (that corresponds to 400 HCO_3^- per one reaction centre of PS II). At acidic pH the optimal concentration for the added $NaHCO_3$ at both pH 4.5 and pH 3.5 comprises a value around 10 mM that corresponds to the equilibrium anion HCO_3^- concentration of 150 μmol and 15 μmol (or nearly 100 and 10 HCO_3^- per one PS II reaction centre), respectively. These data provide a suggestion that in PSII there is a certain amount of specific HCO_3^- binding sites that show high-affinity and that are important for the stabilizing effect. A surplus increase in BC concentration may lead to its non-specific interaction with PS II that interferes with the stabilizing effect. A similar effect is probably caused by formate anion (which does not protect the proteins against extraction and even interferes with the stabilizing effect of BC (Fig. 1A and B). There is an earlier data for the existence of at least two different binding sites with different affinity for BC in thylakoids [12,36].

As a few literature sources state, the treatment of thylakoids at acidic pH (pH < 5.0) results in both the release of Mn [37] and the inactivation of electron transport on the donor side of PS II [38]. Ono and Inoue have shown that a 5-min treatment of PS II membrane fragments with citrate buffer at pH 3.0 leads to the removal of one Ca^{2+} per PS II without changing the extrinsic proteins content [40]. In contrast to these data in our experiments the removal of the extrinsic proteins from the membrane is observed upon the treatment of PS II membrane at pH 3.5 and pH 4.5. The discrepancy between the results obtained earlier [39,40] and those obtained in our work could be due to the difference in the content of the incubation medium, incubation time and the concentration of PS II membranes.

The preferable sensitivity of the PsbO protein to BC in the medium during all the treatments used in this work suggests that there is either a direct BC interaction with the PsbO protein or there is a BC binding to some other site(s) within PS II that indirectly facilitates the preservation of the native structure of this protein. Evidently, the possible binding of anion HCO_3^- to Mn within the WOC [7–12] is not important for the revealing of the protecting effect of BC against extraction of the PsbO protein since the stabilizing effect of BC on binding of this protein to the membrane is clearly seen in both cases: when all Mn atoms remain bound to the membrane (treatment with 2 M urea or with 0.2 M glycine–HCl buffer, pH 3.5) and when Mn is removed from the membrane (treatment with 0.5 citrate buffer, pH 4.5). The participation of the non-heme Fe^{2+} (acting between Q_A and Q_B) as a ligand for BC [4–6] in providing the stabilizing effect described here is doubtful since Fe atoms are located on the acceptor side of PS II while the extrinsic proteins are attached to the luminal surface of the membrane. Besides, it has been shown that the non-heme Fe^{2+} is removed during citrate buffer treatment (pH 3.0) [41], while the protective effect of BC against the PsbO protein extraction is observed in the pH range from pH 3.5 to pH 4.5. However, we cannot exclude completely the possibility of indirect participation of BC binding to Fe^{2+} in the effects described here.

The protective effect of bicarbonate against extraction of the PsbO protein can appear due to the direct interaction of anion HCO_3^- with positively charged amino acid residues of either the PsbO protein itself or integral PS II proteins. The BC protein binding has been demonstrated earlier [42,43]. BC was shown to bind to mitochondrial superoxidedismutase [42] that facilitates the diffusion of H_2O_2 to the active center at physiological pH so that the peroxide activity is observed only in the presence of BC. It is known that HCO_3^- binds to arginine residue rather than to metals (Cu, Zn). Maeda and co-workers have shown that a membrane protein-transporter (CmpA) from *Synechococcus* sp. binds HCO_3^- with a high affinity and transfers it through the membrane [43]. The suggestion that BC interacts with the PsbO protein directly is in agreement with the data on carbonic anhydrase activity of the PsbO protein reported recently [44].

The sites in the PsbO protein responsible for its binding to the integral PS II proteins are not well studied though there are data suggesting the existence of two domains on the N-termini of the MSP that are important for its WOC interaction [45]. Both of these domains are required for binding and both ensure the maximal recovery of the O_2-evolving activity. Besides, the 148V-246Q part of the protein contains a few positively and a few negatively charged amino acid residues required for both functioning and binding of PsbO protein to the integral part of PS II [46]. Frankel and co-workers have

shown that modifications in carboxylic groups in domains (157D–168D and 212E–247Q) of C-termini of the PsbO protein lower its capability to bind to PS II [47]. On the other hand, chemical modification of positively charged residues also alters the PsbO protein binding [48]. Thus, both positively and negatively charged amino acid residues can participate in the interaction of the PsbO protein with the membrane. One can suggest that a specific interaction of BC with the positively charged amino acid residues of proteins can change the binding of the PsbO protein to the membrane (though participation of CO_2 in the effect also cannot be excluded).

Thus, we show that the addition of BC to incubation medium considerably stabilizes the binding of the extrinsic PsbO, Psbp and PsbQ proteins of PS II to the membrane in the course of the 2 M urea treatment. In the case of acidic pH treatment (pH<4.5) the protecting effect of BC is revealed exclusively for the PsbO protein. The mechanism of the stabilizing effect of BC is not yet clear. The binding to Mn appears not to be important for the BC stabilizing effect. The predominant protection of the PsbO protein against the extraction from the membrane during treatment with 2 M urea and at acidic pH as well as the independence of the NaCl-induced extraction of the PsbP and PsbQ proteins on the presence of BC provide the suggestion that the stabilizing effect of BC is based either on its direct specific binding to the PsbO protein or to PS II integral components. This interaction can stabilize a functional conformation and binding of the PsbO protein to the WOC.

Acknowledgements

This work was supported by the grants from the Russian Foundation of Basic Research (05-04-49671 and 06-04-81024), MCB RAS and CRDF-FACI.

References

[1] J.J.S. van Rensen, V.V. Klimov, Bicarbonate interactions, in: T. Wydrzynski, K. Satoh (Eds.), Photosystem II: The Light-driven Water: Plastoquinone Oxidoreductase, Springer, The Netherlands, 2005, pp. 329–345.
[2] A. Stemler, Govindjee, Bicarbonate ion as a critical factor in photosynthetic oxygen evolution, Plant Physiol. 52 (1973) 119–123.
[3] A. Stemler, Inhibition of Photosystem II by formate: possible evidence for a direct role of bicarbonate in photosynthetic oxygen evolution, Biochim. Biophys. Acta 593 (1980) 103–112.
[4] T. Wydrzynski, Govindjee, A new site of bicarbonate effect in Photosystem II of photosynthesis: evidence from chlorophyll fluorescence transients in spinach chloroplasts, Biochim. Biophys. Acta 387 (2) (1975) 403–408.
[5] B.A. Diner, V. Pertrouleas, Formation by NO of nitrosyl adducts of redox components of the Photosystem II reaction center: II. Evidence that bicarbonate/CO2 bind to the acceptor side non-heme iron, Biochim. Biophys. Acta 1015 (1990) 141–149.
[6] K.N. Ferreira, T.M. Iverson, K. Maghlaoui, J. Barber, S. Iwata, Architecture of the photosynthetic oxygen-evolving center, Science 303 (2004) 1981–1997.
[7] V.V. Klimov, S.I. Allakhverdiev, Y.M. Feysiev, S.V. Baranov, Bicarbonate requirement for the donor side of photosystem II, FEBS Lett. 336 (1995) 251–255.
[8] V.V. Klimov, R.J. Hulsebosch, S.I. Allakhverdiev, H. Wincencjusz, H. van Gorkom, A.J. Hoff, Bicarbonate may be required for ligation of manganese in the oxygen evolving complex of photosystem II, Biochemistry 36 (1997) 16277–16281.
[9] S.I. Allakhverdiev, I. Yruela, R. Picorel, V.V. Klimov, Bicarbonate is an essential constituent of the water-oxidizing complex of photosystem II, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 5050–5054.
[10] Yu.N. Kozlov, S.K. Zharmukhamedov, K.G. Tikhonov, J. Dasgupta, A.A. Kazakova, G.C. Dismukes, V.V. Klimov, Oxidation potentials and electron donation to photosystem II of manganese complexes containing bicarbonate and carboxylate ligands, Phys. Chem. Chem. Phys. 6 (2004) 9405–9411.
[11] V.V. Klimov, S.V. Baranov, S.I. Allakhverdiev, Bicarbonate protects the donor side of photosystem II against photoinhibition and thermoinactivation, FEBS Lett. 418 (1997) 243–246.
[12] S. Baranov, A. Tyryshkin, D. Katz, G. Dismukes, G. Ananyev, V.V. Klimov, Bicarbonate is a native cofactor for assembly of the manganese cluster of the photosynthetic WOC. Kinetics of reconstitution of O2 evolution by photoactivation, Biochemistry 43 (2004) 2070–2079.
[13] Y. Yamamoto, M. Doi, N. Tamura, M. Nishimura, Release of polypeptides from highly active O_2 evolving Photosystem-2 preparation by Tris treatment, FEBS Lett. 133 (1981) 265–268.
[14] H.-E. Åkerlund, B. Andersson, Reconstitution of photosynthetic water splitting in inside-out thylakoid vesicles and identification of a participating polypeptide, Biochim. Biophys. Acta 681 (1982) 1–10.
[15] T. Ono, Y. Inoue, Mn-preserving extraction of 33-, 24- and 16-kDa proteins from O_2-evolving PS II particles by divalent salt-washing, FEBS Lett. 165 (1983) 255–260.
[16] Y. Yamamoto, M. Nishimura, Association of two manganese atoms with the PS II reaction center in a highly active O_2-evolving Photosystem II preparation, Biochim. Biophys. Acta 724 (1983) 294–297.
[17] T. Kuwabara, N. Murata, Inactivation of photosynthetic oxygen evolution and concomitant release of three polypeptides in the Photosystem II particles of spinach chloroplasts, Plant Cell Physiol. 23 (1982) 533–539.
[18] M. Miyao, N. Murata, Role of the 33 kDa polypeptide in preserving Mn in the photosynthetic oxygen-evolving system and its replacement by chloride ions, FEBS Lett. 170 (1984) 350–354.
[19] Y. Yamamoto, Molecular organization of oxygen-evolution system in chloroplast, Bot. Mag. (Tokyo) 102 (1989) 565–582.
[20] A. Seidler, The extrinsic polypeptides of PS II, Biochim. Biophys. Acta 1277 (1996) 35–60.
[21] T. Ono, Y. Inoue, Reconstitution of photosynthetic oxygen evolving activity by rebinding of 33 kDa protein to $CaCl_2$-extracted PS II particles, FEBS Lett. 166 (1984) 381–384.
[22] I. Vass, T. Ono, Y. Inoue, Stability and oscillation properties of thermoluminescent charge pairs in the O_2-evolving system depleted of Cl^- or the 33 kDa protein, Biochim. Biophys. Acta 892 (1994) 224–235.
[23] J. De Las Rivas, M. Balsera, J. Barber, Evolution of oxygenic photosynthesis: genome-wide analysis of the OEC extrinsic proteins, Trends Plant Sci. 9 (2004) 18–25.
[24] T. Shutova, M. Khristin, V. Opanasenko, G. Ananyev, V. Klimov, Proton-acceptor properties of the water-soluble 33 kDa protein from spinach PS II, Biol. Membr. (Moscow) 9 (1992) 836–844.
[25] T. Shutova, K.-D. Irrgang, V. Shubin, V. Klimov, G. Renger, Analysis of pH-induced structural changes of the isolated extrinsic 33 kDa protein of PS, Biochemistry 36 (1997) 6350–6358.
[26] D.A. Berthold, G.T. Babcock, C.F. Yocum, A highly resolved, oxygen-evolving photosystem II preparations from spinach thylakoids membranes, EPR and electron-transport properties, FEBS Lett. 134 (1981) 231–234.
[27] M.S. Khristin, O.V. Nikitishena, T.N. Smolova, O.M. Zastrizhnaya, Extraction of functionally active Photosystem II pigment–protein complexes from pea thylakoids and their purification on Sepharose DEAE 6B, Biol. Membr. (Moscow) 14 (2) (1997) 133–142.
[28] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[29] N.I. Geletuk, B.N. Zolotareva, Using of the method of nonfair atomic-absorption spectroscopy for analysis in samples of different components of biosphere, Preprint, Pushchino (1980) 1–24.
[30] T. Suzuki, O. Tada, M. Makimura, A. Tohri, H. Ohta, Y. Yamamoto, I. Enami, Isolation and characterization of oxygen-evolving Photosystem II

complexes retaining the PsbO, p and Q proteins from *Euglena gracilis*, Plant Cell Physiol. 45 (9) (2004) 1168–1175.
[31] M. Miyao, N. Murata, Partial disintegration and reconstitution of the photosynthetic oxygen evolution system, Biochim. Biophys. Acta 75 (1983) 87–93.
[32] C. Larsson, C. Jansson, U. Ljungberg, H.-E. Åkerlund, B. Andersson, C. Cybesma (Eds.), Advances in Photosynthesis Research, 1, Martinus Nijhoff/Dr. W. Junk Publishers, The Hague, 1984, pp. 363–366.
[33] M. Miyao, N. Murata, The mode of binding of three extrinsic proteins of 33 kDa, 23 kDa and 18 kDa in the photosystem II complex of spinach, Biochim. Biophys. Acta 977 (1989) 315–321.
[34] I. Enami, M. Kitamura, T. Tomo, Y. Isokawa, H. Ohta, S. Katoh, Is the primary cause of thermal inactivation of oxygen evolution in spinach PS II membranes release of the extrinsic 33 kDa protein or of Mn? Biochim. Biophys. Acta 1186 (1994) 52–58.
[35] Y. Yamamoto, S. Shimada, M. Nishimura, Purification and molecular properties of 3 polypeptides released from a highly active O_2-evolving Photosystem II preparation by Tris-treatment, FEBS Lett. 151 (1983) 49–53.
[36] A. Stemler, The binding of bicarbonate ions to washed chloroplast grana, Biochim. Biophys. Acta 460 (1977) 511–522.
[37] M.P.J. Pulles, H.J. van Gorkom, G.A.M. Verschoor, Primary reactions of Photosystem II at low pH. Light-induced changes of absorption and electron spin resonance in spinach chloroplasts, Biochim. Biophys. Acta 440 (1976) 98–106.
[38] C. Spetea, E. Hideg, I. Vass, Low pH accelerates light-induced damage of Photosystem II by enhancing the probability of the donor-side mechanism of photoinhibition, Biochim. Biophys. Acta 1318 (1997) 275–283.
[39] T. Ono, Y. Inoue, Discrete extraction of the Ca atom functional for O_2 evolution in higher plant Photosystem II by a simple low pH treatment, FEBS Lett. 227 (2) (1988) 147–152.
[40] K.A.V. Meulen, A. Hobson, C.F. Yocum, Calcium depletion modifies the structure of the Photosystem II O_2-evolving complex, Biochemistry 41 (2002) 958–966.
[41] B.K. Semin, M. Seibert, A carboxylic residue at the high-affinity, Mn-binding site participates in the binding of iron cations that block the site, Biochim. Biophys. Acta 1757 (3) (2006) 189–197.
[42] S. Sankarapandi, J.L. Zweier, Bicarbonate is required for the peroxidase function of Cu,Zn-superoxide dismutase at physiological pH, J. Biol. Chem. 27 (3) (1999) 1226–1232.
[43] S.-I. Maeda, G. Dean Price, M.R. Badger, C. Enomoto, T. Omata, Bicarbonate binding activity of the CmpA protein of the cyanobacterium Synechococcus sp. Strain PCC 7942 involved in active transport of bicarbonate, J. Biol. Chem. 275 (27) (2000) 20551–20555.
[44] Y.-K. Lu, A. Stemler, Extrinsic Photosystem II carbonic anhydrase in maize mesophyll chloroplasts, Plant Phys. 128 (2002) 643–649.
[45] H. Popelkova, M.M. Im, C.F. Yocum, N-terminal truncations of manganese stabilizing protein identify two amino acid sequences requires for binding of the eukaryotic protein to PS II and reveal the absence of one binding-related sequence in cyanobacterial, Biochemistry 41 (2002) 10038–10045.
[46] A. Motoki, M. Usui, T. Shimazu, M. Hirano, S. Katoh, A domain of the Mn-stabilizing protein from *Synechococcus eleongatus* involved in functional binding to Photosystem II, J. Biol. Chem. 227 (2002) 14747–14756.
[47] L.K. Frankel, J.A. Cruz, T.M. Bricker, Carboxylate groups on the manganese-stabilizing protein are required for its efficient binding to Photosystem II, Biochemistry 38 (43) (1999) 14271–14278.
[48] T. Miura, J.R. Shen, S. Takahashi, M. Kamo, E. Nakamura, H. Ohta, A. Kamei, Y. Inoue, N. Domae, K. Takio, K. Nakazato, Y. Inoue, I. Enami, Identification of domains on the extrinsic 33 kDa protein possibly involved in electrostatic interaction with Photosystem II complex by means of chemical modification, J. Biol. Chem. 272 (1997) 3788–3798.