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Specific release of a 9-kDa extrinsic polypeptide of photosystem I from spinach chloroplasts by salt washing

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The newly reported 9-kDa polypeptide in photosystem I [(1991) FEBS Lett. 280, 332–334] is an extrinsic component located on the lumenal side of the thylakoid membrane. This subunit can be solubilized with high salt buffer and does not bind any cofactors. The photosystem I electron transfer chain remains intact and functional in the absence of this component as characterized by the photoreduction of $NADP^+$.

Photosystem I; Extrinsic subunit; $NADP^+$ reduction; Spinach

1. INTRODUCTION

Photosystem I (PSI) is a multi-subunit membrane complex that catalyzes the electron transfer from reduced plastocyanin to ferredoxin, which in conjunction with FNR, reduces $NADP^+$. Apart from the light-harvesting portion, LHCI, the PSI complex isolated from higher plants consists of at least 13 subunits with molecular weight ranging from 80 kDa to 4 kDa [1,2]. Except for the recently described 9-kDa subunit, all the subunits have their complete amino acid sequences, deduced from their DNA sequence data, and the subunits are commonly designated after corresponding genes as PSI-A through PSI-L [1,3]. The two largest subunits, PSI-A and PSI-B, bind chlorophyll and several of the primary electron carriers. PSI-C binds the two iron–sulfur centers, F_A and F_B, which serve as secondary electron acceptors [4]. The PSI-D and PSI-E are located on the stromal side of the membrane and are involved in binding ferredoxin as well as providing a shield to the PSI-C subunit [5–7]. PSI-F is located on the lumenal side of the membrane and is involved in binding of plastocyanin to PSI [8]. The function of the rest of the components in PSI remains to be clarified.

In this work, we report a simple procedure to remove specifically the 9-kDa subunit of PSI (this subunit is actually 12 kDa in the gel system we used), while leaving the rest of the complex intact. This procedure facilitated the study of structural and functional roles of this 9-kDa polypeptide. As discussed, this component is located on the lumenal side of the thylakoid membrane, and its removal does not cause a significant change in the PSI electron transfer process from plastocyanin to $NADP^+$ in vitro.

Abbreviations: DCIP, 2,6-dichlorophenol indophenol; FNR, ferredoxin-$NADP^+$ oxidoreductase; PSI, photosystem I.

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2. MATERIALS AND METHODS

2.1. *Isolation of photosystem I complex*

PSI-200 complex was prepared from green-house spinach leaves (*Spinacia oleracea*) as previously described [9]. Excessive amounts of sucrose were removed by filtration with a YM-100 membrane. PSI-enriched stromal lamellae were prepared as in [10].

2.2. *SDS-PAGE and blotting for sequencing*

Polypeptide subunits were analyzed by SDS-PAGE with a Tris-tricine buffer system [11] with some modifications. The resolving gel consisted of a 1 cm spacer of 10% T/3% C and a linear gradient of 10% T/3% C–22% T/6% C, where T is the total of acrylamide and bis-acrylamide and C is the ratio of bis-acrylamide to acrylamide. Following electrophoresis, the gel was either stained for proteins or transferred onto PVDF membranes for N-terminal amino acid sequencing, which was done in the Department of Chemistry at the Arizona State University at Tempe.

2.3. *Determination of [Chl], [P700] and F_A/F_B*

Chlorophyll concentrations were determined in 80% acetone [12]. The content of P700 was estimated photochemically at 435–444 nm with a different extinction coefficient of 44 $mM^{-1}cm^{-1}$ [13]. EPR measurements were carried out as in [14] except that isolated PSI-200 complex was used instead of whole thylakoids.

2.4. *O_2 uptake and $NADP^+$ photoreduction assays*

O_2 uptake was measured with a Rank-type electrode in a medium consisting of 50 mM Tris Cl, pH 8.0, 0.3 M sucrose, 10 mM NaCl, 3 mM sodium ascorbate, 0.1 mM DCIP and 1 μM methyl viologen [14]. $NADP^+$ photoreduction activities were measured in an SLM-Aminco DW-2000 spectrophotometer operating in split beam mode. Saturating actinic side-illumination was provided with the 150-W tungsten lamp and filtered with a combination of Corning 4-96 and a glass filter. The photomultiplier tube was protected from excessive radiation with an interference filter having a narrow transmitting band centered at 340 nm. The reaction mixture (1 ml) contained 5 μg chlo-

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rophyll of PSI-200, 5 mM ascorbate, 20 μg plastocyanin, 20 μg FNR, 20 μg ferredoxin and 0.1 mM $NADP^+$. An extinction coefficient of 6.22 $mM^{-1} \cdot cm^{-1}$ was used for calculation of the reduction rate of $NADP^+$. Both assays were conducted at room temperature.

3. RESULTS AND DISCUSSION

High salt treatment of biological membranes is frequently used for the release of electrostatically bound components, like many peripheral polypeptides. Various amounts of 2 M NaCl or $CaCl_2$ in Tris buffer, pH 8.0, were added to the isolated PSI-200 complex to make the final concentration of chlorophyll to be about 0.25 $mg \cdot ml^{-1}$. After incubation on ice for 45 min, the PSI complex was pelleted at 100,000×g to separate the

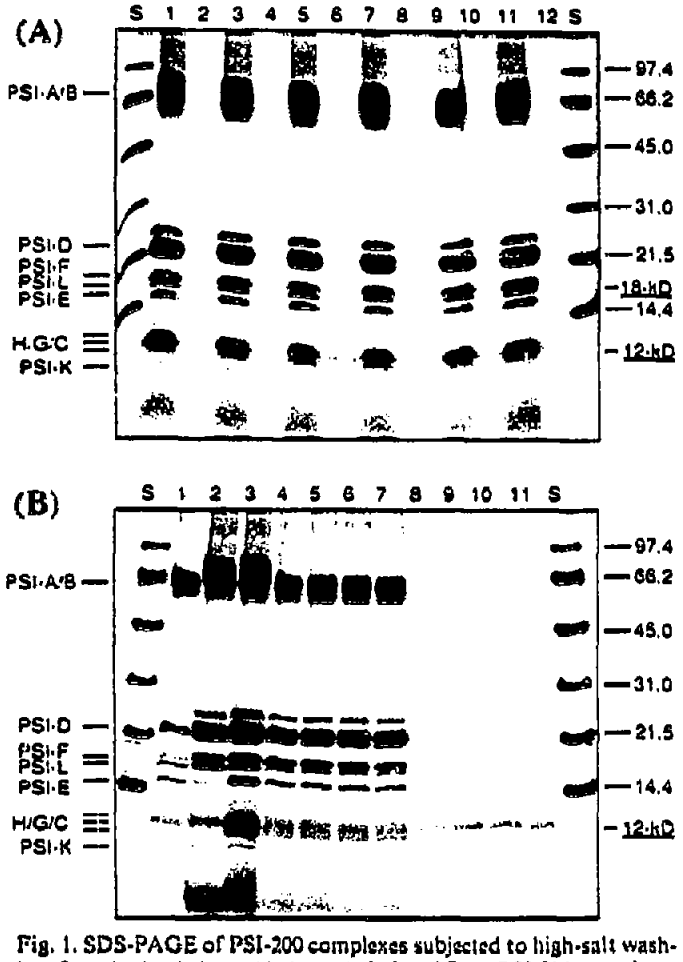

Fig. 1. SDS-PAGE of PSI-200 complexes subjected to high-salt washing. Samples loaded are: (A) lanes 1, 3, 5 and 7 are PSI-200 complexes treated with 0.1, 0.2, 0.5 and 1.0 M NaCl and lanes 2, 4, 6 and 8 are corresponding NaCl-solubilized portions. Lanes 9 and 11 are PSI-200 complexes treated with 0.05 and 0.1 M $CaCl_2$ with solubilized portions in lanes 10 and 12, respectively; (B) lane 1, PSI-100 core complex depleted of light harvesting components; lane 2, urea-treated PSI-200 complex depleted of all extrinsic polypeptides; lane 3, control PSI-200 complex; lanes 4–7, PSI-200 complexes treated with 0.4, 0.6, 0.8 and 1.0 M $CaCl_2$ and lanes 8–11 are corresponding $CaCl_2$-solubilized portions. In both (A) and (B), lanes labelled 'S' are molecular-weight markers as indicated to the right side of the gel and to the left, identifiable components are also indicated.

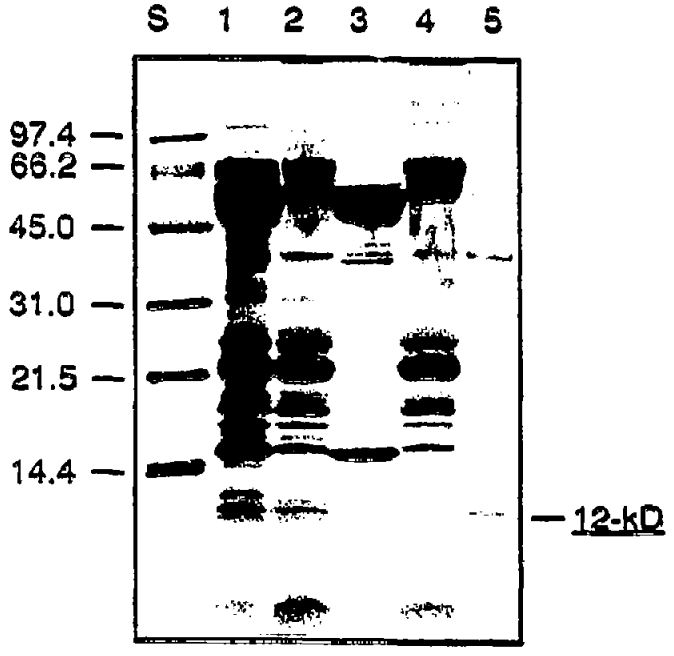

Fig. 2. SDS-PAGE of PSI-enriched stromal lamellae subjected to $CaCl_2$-washing. Lane 1 is the untreated stromal lamellae as control; lane 2 is 0.4 M $CaCl_2$-treated stromal lamellae and lane 3 is the solubilized portion. Lane 4 is the same as in lane 2 but treated again with 0.4 $CaCl_2$ together with brief sonication to disrupt the thylakoid membrane, and lane 5 is the solubilized portion.

salt-solubilized portion from the main complex and both the supernatant solution and the complex were analyzed by SDS-PAGE. As shown in Fig. 1, such a salt-washing treatment induces the specific release of a polypeptide of apparent molecular weight of 12 kDa in our gel system.

Concentrations of $CaCl_2$ and NaCl required for effective removal are 0.4 M and 1.0 M, respectively. Sometimes, especially when spinach leaves harvested in the winter were used as the starting material, an 18-kDa component was also released in addition to the above described 12-kDa subunit. Both polypeptides were subjected to N-terminal amino acid sequencing analysis and the results are:

12 kDa GVIDE YLEKS KANKE ... ...

18 kDa LSGGL PGTEN SDQAR ... ...

The 12-kDa polypeptide is identical to the 9-kDa one previously found both in spinach and pea [2] and the difference in molecular weights between our subunit and that in the previous report is due to the different gel system we used. For simplicity and consistancy, we will designate this polypeptide as 9-kDa hereafter. The 15 amino acid residues at the N-terminal of the 18-kDa is identical to that of the 18-kDa extrinsic polypeptide in the oxygen-evolving photosystem II (PSII) complex [15] but starting from the 13th residue of the mature protein.

Presumably, a short sequence of 12 amino acid residues at the N-terminal, missing from the subunit found in our PSI complex, causes the loss of specificity in binding site in PSII, as suggested previously [16].

As an analogy to the interaction of extrinsic 18-kDa component, which could be easily removed by salt washing, with the main intrinsic portion of the PSII complex [17], we concluded the 9-kDa polypeptide is also an extrinsic component and interacts with PSI core primarily via electrostatic attraction. The question remained, however, as to which side of the thylakoid membrane the 9-kDa subunit of PSI is located.

To address this question, we carried out $CaCl_2$ washing of a PSI-enriched stromal lamellae preparation, that is capable of ATP synthesis [10]. As shown by lane 3 of the gel in Fig. 2, washing the intact preparation with $CaCl_2$ releases a number of components which are mainly associated with the extrinsic portion of the coupling factor, CF_1. However, if the preparation is subjected to a brief sonication to disrupt the vesicles, then subsequent $CaCl_2$ washing causes the dissociation of an additional 12-kDa polypeptide, which was again identified as the same 9-kDa subunit by amino acid sequencing. From this, we can conclude that the 9-kDa PSI subunit is associated with the lumenal surface of the thylakoid membrane by electrostatic interaction. The lumenal side location of this 9-kDa polypeptide can also explain an earlier observation that this subunit is not available for protease digestion if the thylakoid membrane is intact [9].

This selective removal procedure should also enable us to determine the functional role of the 9-kDa polypeptide in PSI electron transfer reactions. First of all, the 9-kDa does not seem to bind any cofactors as indicated by its absorption spectrum (not shown). Its direct involvement as an electron carrier can be ruled out. Secondly, removal of this 9-kDa polypeptide by $CaCl_2$ washing does not effect the $P700^+$ photoaccumulation in the presence of ascorbate, charge recombination rate between $P700^+$ and $(F_A/F_B)^-$ at room temperature, or EPR profiles of the iron-sulfur centers at cryogenic temperature. Finally, the rates of oxygen uptake and $NADP^+$ photoreduction with purified plastocyanin added as electron donor were not altered by $CaCl_2$-treatment of the PSI-200 complex. These results are summarized in Table I.

The above results raise questions about the functional role this 9-kDa polypeptide in PSI and raise the possibility that this subunit may not be a true PSI subunit. There are, however, other possibilities. Firstly, the 9-kDa subunit may be a protease that processes transit sequences of other nuclear-encoded subunits, directing them to the lumenal side of the thylakoid membrane. Secondly, the subunit may come into function when the plant is growing in unpreferable physiological conditions. Thirdly, this subunit may function as a substitute for other polypeptides in the event of damage to the complex. For the last possibility, it is particularly interesting to note that a cyanobacterial mutant lacking the gene *psa*F can still grow photoautotrophically [18]. However, the 9-kDa has only been reported in higher plant species to date. These above points will be clarified in the future with a combination of biochemical and molecular biological tools.

Table I

Activity measurements for control and $CaCl_2$-treated PSI-200 complexes. Both oxygen-uptake and $NADP^+$ reduction activities are expressed as μmol per mg of chl per hour

Preparations	[Chl]/[P700]	F_A/F_B	O_2 uptake	$NADP^+$ reduction
Control PSI-200	220	1/1	2830 ± 180	190 ± 20
$CaCl_2$-treated PSI-200	234	1/1	3100 ± 380	230 ± 70

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