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Orientation and nearest neighbor analysis of *psb*I gene product in the photosystem II reaction center complex using bifunctional cross-linkers

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Two distinct cross-linked products containing *psb*I gene product were generated in the photosystem II reaction center complex from spinach by treatment with bifunctional reagents directed to amino groups. The first product, which was generated by a 3,3′-dithiobis(succinimidyl propionate) treatment, is deduced to be formed between the ε-amino group of Lys^3 of the *psb*I gene product, and a side-chain amino group present on the stromal extension of the D2 protein. The CNBr cleavage analysis of the cross-linked product predicted that the amino group of the D2 protein engaged in the cross-linking is either one of the three lysine residues on the N-terminal fragment (from N-terminus to Met^{19}) or the Lys^{268} on the 6th fragment (from Val^{248} to Met^{275}). The second product, which was also generated on the stromal side by a 1,6-hexamethylene diisocyanate treatment preferentially in alkaline conditions, is predicted to be formed between the ε-amino group of Lys^3 of the *psb*I gene product and the N-terminal α-amino group of the α-subunit of cytochrome b_{559}.

Chemical cross-linking; Cytochrome b_{559}; D2 protein; Photosystem II; *psb*I gene product; Reaction center; Spinach

1. INTRODUCTION

The isolated reaction center (RC) complex of photosystem II (PSII) consists of five protein subunits, i.e. D1 and D2 proteins, α- and β-subunits of cytochrome b_{559} and *psb*I gene product (*psb*I protein) [1]. The D1 and D2 proteins are significantly homologous in their primary structure to the L and M subunits of purple bacterial RC, whose structure has recently been determined by an X-ray crystallographic analysis [2–4]. Based on the similarity in the amino acid sequence mentioned above, as well as in the electron transport chain on the reducing side for both photosystems, it is assumed that the functional role of D1 and D2 subunits in the PSII RC complex is to provide a site for primary photochemistry, in a similar manner to its purple bacterial counterparts [5,6]. On the other hand, the functional role of two other transmembrane components, tightly associated with the D1 and D2 subunits in PSII RC complex, i.e. cytochrome b_{559} and *psb*I protein, has not been elucidated at present, although there are some speculations and proposals concerning the role of the former component [7,8].

The present study was intended to analyze the orientation and nearest neighbors of the *psb*I protein, in the PSII RC complex, in order to provide basic information for the forthcoming structural analysis of the RC. For this purpose, two bifunctional reagents directed to amino groups, 3,3′-dithiobis(succinimidyl propionate) (DSP) and 1,6-hexamethylene diisocyanate (HMDI), in combination with immunological detection using specific antibodies raised against each component, were employed to the isolated PSII RC complex from spinach. The results clearly demonstrated that the N-terminal portion of the *psb*I protein is present on the stromal side in close vicinity to the part of D2 protein and to the N-terminal end of α-subunit of cytochrome b_{559}.

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2. MATERIALS AND METHODS

The PSII RC complex was prepared from spinach grana thylakoids as described by Nanba and Satoh [1]. For cross-linking, DSP or HMDI was added to the PSII RC complex (0.4 mg Chl · ml^{-1}), dissolved in a 40 mM MES–NaOH (pH 6.5) buffer containing 0.2% digitonin. The cross-linker was freshly prepared by dissolving in dimethylsulfoxide prior to each experiment. The concentration of dimethylsulfoxide in the reaction mixture was less than 4%. The cross-linking reaction was carried out at room temperature for 10 min and then terminated by adding glycine in 100-fold molar excess over the cross-linker. For analysis of cross-linked products, the sample was treated with 3% LDS in case of DSP treatment, or with 3% LDS and 60 mM dithiothreitol in case of HMDI, for 30 min at room temperature, and then separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli [9] with modifications described by Ikeuchi and Inoue [10]; the analyzing gels contained 16–22% acrylamide and 7.5 M urea. After electrophoresis, the gels were stained with CBB R-250 (Nakarai, Osaka). For immunoblotting, proteins resolved by SDS-PAGE were transferred to a nitrocellulose membrane and detected by enzyme-linked immunoblot analysis [11] or by staining with Ponceau S (Aldrich, Milwaukee). The specific antibodies raised against each subunit of PSII RC complex, expect for the β-subunit of cytochrome b_{559}, i.e. D1 protein, D2 protein, α-subunit of cytochrome b_{559} and *psb*I protein, respectively, were provided by Ikeuchi [12].

For CNBr-cleavage, cross-linked products were electroeluted and then concentrated to 100 μl by lyophilization. The CNBr cleavage was conducted at 10 mg · ml^{-1} CNBr in 70% formic acid for 3 h at 20°C. After the cleavage, peptide fragments were lyophilized and then dissolved in a solution containing 40 mM MES–NaOH, (pH 6.5), 3% LDS and 0.4 M sucrose. After separation by SDS-PAGE, peptide fragments were electrophoretically transferred onto nitrocellulose membranes and then processed for detection by an antibody mediated enzymatic reaction [11].

3. RESULTS AND DISCUSSION

3.1. *DSP-treatment*

Fig. 1A shows the polypeptide profile analyzed by SDS-PAGE of the PSII RC complex, treated with different concentrations of DSP. Each polypeptide band, except for the β-subunit of cytochrome b_{559}, in the control sample, was identified as shown on the left side of the figure, using specific antibodies raised against each component (lane 1). Two prominent phenomena as affected by DSP treatment can be observed on the chromatogram; decrease in the intensity of the *psb*I protein band of about 5 kDa ((I) in Fig. 1A) and upper shift in the apparent molecular mass of D2 protein band (together with similar band shift in the molecular mass of D1/D2 band). The concentration dependence on DSP of these two phenomena observed on SDS-PAGE was nearly the same, suggesting that the disappearance of the *psb*I protein correlates with a molecular mass shift in the D2 protein band, i.e. shift in the molecular size of the D2 protein is due to association of the *psb*I protein.

Immunoblotting analysis, using specific antibodies raised against the *psb*I protein, was conducted in the experiment shown in Fig. 1B in order to trace the *psb*I protein which disappeared by the treatment on the chromatogram at the position of about 5 kDa. The result of analysis clearly demonstrated that the modified D2 band, formed by the treatment with DSP (thick line in Fig. 1A), contains the *psb*I protein, in agreement with the above prediction that the D2 protein and the *psb*I protein cross-linked by the DSP treatment. This conclusion was also supported by an experiment using specific antibodies raised against the D2 protein (data not shown).

In order to specify the site of cross-linking between these two proteins, the cross-linked product (modified D2 band) obtained by the DSP-treatment at 0.3%, was cleaved by CNBr as described in section 2 and then the cleavage products containing the *psb*I protein were detected after SDS-PAGE, by specific antibodies raised against *psb*I protein, in the experiment shown in Fig. 2. The deduced sequence, of D2 protein of spinach, contains 8 cleavage sites for CNBr, i.e. carboxyl-side of methionine, and thus the treatment is expected to produce 9 fragments of different size, as shown in Table I. While, in the experiment shown in Fig. 2, only one immuno-reactive fragment for the *psb*I protein of the molecular mass of 5–7 kDa, a value slightly higher than that of *psb*I protein band of untreated sample (lane 2), was obtained from the modified D2 band. Based on estimated size of the cross-linked product and the expected molecular size of the CNBr fragments which contain free amino groups, it can be deduced that, out of 9 fragments, only the 1st and 6th components in

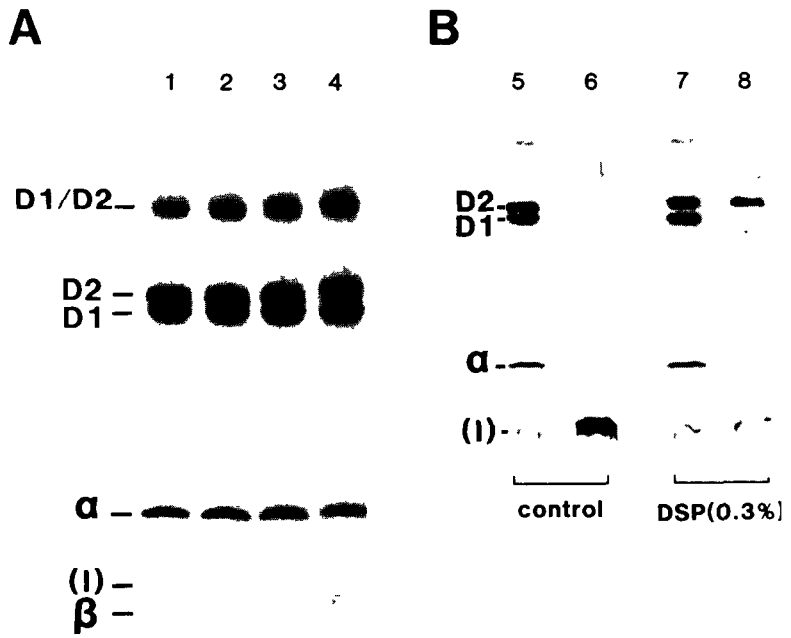

Fig. 1. DSP-treatment of the PSII RC complex. (A) SDS-polyacrylamide gel electrophoresis of the PSII RC complex treated with various concentrations of DSP. Concentration of DSP: 0% (lane 1), 0.005% (lane 2), 0.02% (lane 3), 0.3% (lane 4). (B) Immunoblot analysis of the cross-linked products. PSII RC complex was incubated in the absence (lanes 5 and 6) or presence (lanes 7 and 8) of 0.3% DSP. Polypeptides were stained with Ponceau S (lanes 5 and 7) or detected by antibodies raised against *psb*I gene product (lanes 6 and 8).

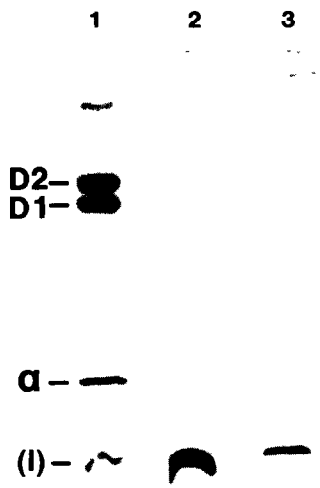

Fig. 2. Immunological detection of CNBr fragments using specific antibodies raised against *psb*I gene product. PSII RC complex was treated with 0.3% DSP (lane 1, stained with Ponceau S). The modified D2 band generated by the treatment was cleaved with CNBr as described in section 2 and then separated by SDS-PAGE and detected by antibodies raised against *psb*I gene product (lane 3). Polypeptides of untreated PSII RC were detected by antibodies raised against *psb*I gene product in lane 2.

Table I are candidates for partner of the *psb*I protein in cross-linking. Thus, it is concluded that the N-terminal portion of the *psb*I protein, which contains an ε-amino group at the 3rd residue, (Lys^3) and a formylated N-terminal α-amino group [12], cross-linked at the lysine residue with the ε-amino group on the stromal extension of D2 protein, since the N-terminal α-amino group of D2 protein is also reported to be blocked by acetylation [13]. The CNBr cleavage analysis of the cross-linked product further concluded that the fragment of D2 protein, engaged in the cross-linking, corresponds either to the N-terminal fragment which contains three lysine residues or the 6th fragment which contains only one lysine residue at the 268th position (Lys^{268}), both of which are predicted to be present on the stromal side of membranes in its native state.

Table I

The CNBr fragments of D2 protein of spinach and their predicted location in thylakoid membrane

No.[a]	No. of amino acid	No. of free amino group	Predicted location[b]
1	18	3[c]	Stroma
2	108	0	Transmembrane
3	72	0	Transmembrane
4	1	0	Membrane
5	47	0	Membrane and stroma
6	26	1	Mostly in stroma
7	9	0	Membrane
8	48	1	Mostly in lumen
9	23	0	Lumen

[a] The numbers start from the N-terminal fragment.
[b] Based on Trebst (1987).
[c] The N-terminal α-amino group is blocked [13].

Recently, a cross-linking experiment using DSP was conducted for PSII RC by Moskalenoko et al. [14]. However, the cross-linked products obtained in their study were entirely different from those reported in this paper, possibly because of a difference in experimental conditions; they used SDS for terminating cross-linking reactions, while we used glycine.

3.2. *HMDI-treatment*

Further analysis was conducted by using another homobifunctional cross-linking reagent, HMDI, which cross-links amino groups at a distance of about 11 Å [15]. At pH 6.5, HMDI treatment of PSII RC complex resulted in the formation of a distinct CBB stainable band of about 15 kDa, in a concentration dependent fashion, in the range of 0.01–0.08%. Formation of the 15 kDa band accompanied with loss of both the *psb*I protein and the α-subunit of cytochrome b_{559}, suggesting the possibility that this component is a cross-linked product formed between these two lower molecular weight components of the PSII RC complex. Another prominent phenomenon caused by the treatment is that both the D1 and D2 proteins dramatically decrease in intensity accompanied with an increase of the D1/D2 band. However, the phenomenon was not analyzed in detail in this study.

Fig. 3A also shows a pH dependence of the formation of the cross-linked product by HMDI-treatment. It is well documented that HMDI interacts with amino groups, but the specificity is low at higher concentrations, or at low pH in the reaction mixture, where the thiol, imidazol, aromatic hydroxyl and carboxyl groups of proteins are also reactive [16]. However, in the experiment shown in Fig. 3, the efficiency of cross-linking to produce the 15 kDa component increased as pH increased, confirming that cross-linking is taking place between the amino groups, under the condition used in this experiment.

In order to specify the components engaged in the cross-linking, to produce the 15 kDa component, antibodies raised against the *psb*I protein and those against the α-subunit of cytochrome b_{559} were employed in the experiment shown in Fig. 3B, where HMDI treatment was conducted at 0.038% at pH 6.5. The result clearly demonstrated that the 15 kDa band is a cross-linked product between the *psb*I protein and the α-subunit of cytochrome b_{559}, consistent with the estimated molecular size of the cross-linked product.

Since the α-subunit of cytochrome b_{559} has only one reactive, non-blocked, amino group at the N-terminal end [17], the α-amino group must be the site of cross-linking. On the other band, the N-terminal α-amino group in the *psb*I protein of spinach is reported to be blocked as mentioned above [12], and the only one pos-

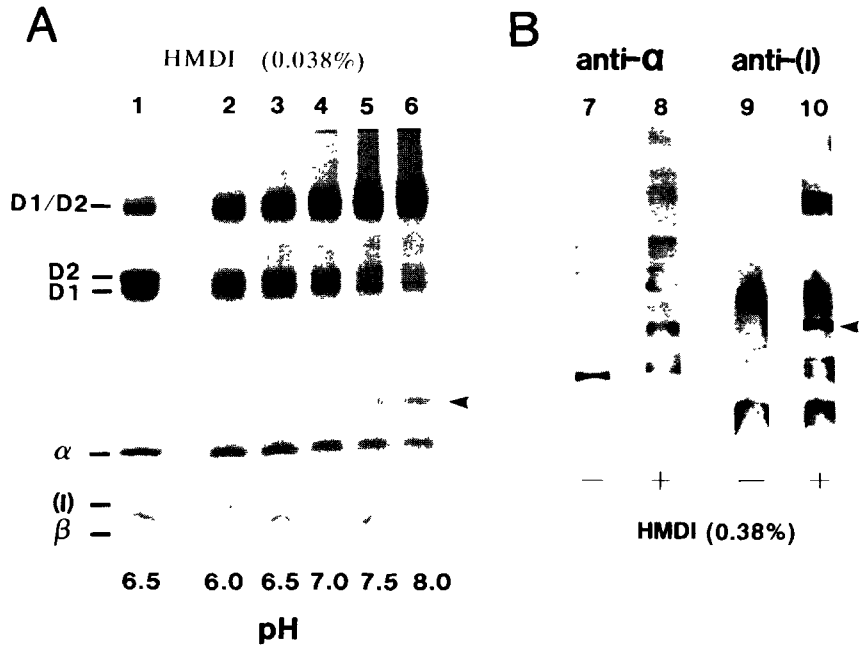

Fig. 3. HMDI-treatment of the PSII RC complex. (A) SDS-polyacrylamide gel electrophoresis of PSII RC complex incubated in the absence (lane 1) or in the presence (lanes 2–6) of 0.03% HMDI at different pH. pH values of the reaction mixture for HMDI-treatment are indicated on the bottom. Arrowheads indicate positions of the 15 kDa cross-linked product. (B) Immunoblot analysis of the cross-linked product generated by HMDI-treatment of PSII RC complex. PSII RC complex was incubated in the absence (lanes 7 and 9) or presence (lanes 8 and 10) of 0.038% HMDI. After SDS-PAGE, proteins were detected by antibodies raised against α-subunit of cytochrome b_{559} (lane 8) or against *psb*I gene product (lane 10)

sible choice for the *psb*I protein is the ε-amino group of lysine residue at the 3rd position (Lys^3) which is also engaged in the cross-linking with D2 protein when DSP is added. The conclusion reached from this analysis is that the ε-amino group of Lys^3 on the N-terminal portion of the *psb*I gene product in the PSII RC complex is in close vicinity (within 11 Å) with the N-terminal end of the α-subunit of cytochrome b_{559}.

In summary, two distinct cross-linked products, which contain the *psb*I protein, were generated in the PSII RC complex by treatment with chemical cross-linkers. The first product, which was formed by the DSP treatment, is thought to be a cross-linked product between the ε-amino group of the 3rd amino acid (Lys^3) of *psb*I protein and one of the ε-amino groups present within a distance of less than 12 Å on the stromal extension of the D2 protein. The amino group of the D2 protein engaged in cross-linking, is situated either on the N-terminal CNBr fragment (from N-terminus to Met^{19}) or on the segment which corresponds to the sequence between the 3rd and the 4th helices (from Val^{248} to Met^{275}), based on the CNBr cleavage experiment, and the predicted amino acid sequence. The 2nd product, which is produced by the HMDI treatment, preferentially in alkaline condition, is thought to be formed between the ε-amino group of lysine residue at the 3rd position (Lys^3) of the *psb*I protein and the N-terminal α-amino group of the α-subunit of cytochrome b_{559}. Further analysis is evidently needed to clarify the structure and function of the *psb*I gene product in PSII RC.

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