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Identification of the plastocyanin binding subunit of photosystem I

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Plastocyanin is specifically cross-linked by incubation with *N*-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) to a subunit of photosystem I in stroma lamellae and in isolated photosystem I complex. SDS-PAGE shows the disappearance of a 18.5 kDa subunit and the appearance of a new 31.5 kDa protein which was recognized by anti-plastocyanin antibodies. The isolated subunit was identified by its N-terminal amino acid sequence as the mature peptide coded by the nuclear gene *psa*F [Steppuhn et al. (1988) FEBS Lett. 237, 218–224]. $P700^+$ was reduced by cross-linked plastocyanin with the same halftime of 13 μs as found in the native complex. This is evidence that cross-linking conserved the orientation of the complex and that the 18.5 kDa subunit provides the conformation of photosystem I necessary for the extremely rapid electron transfer from plastocyanin to $P700^+$.

Photosynthesis; Photosystem I; Subunit III; Peptide sequencing; Plastocyanin; (Spinach)

1. INTRODUCTION

Photosystem I (PS I) is a multipeptide complex localized in non-appressed thylakoid membrane regions. A PS I complex being competent in mediating the electron transfer from plastocyanin to ferredoxin is assembled from more than 7 subunits [1,2]. The two large chlorophyll (chl) *a* containing subunits Ia and Ib are plastid encoded and carry the core of the reaction center with the primary electron donor P700 and the electron acceptors phylloquinone and Fe-S_X (reviewed in [2]). Four of the nuclear encoded subunits have been sequenced very recently [3–5]. The largest of these subunits, subunit II, has been shown by cross-linking studies to ensure the docking of ferredoxin for the electron transfer from Fe-S_A and Fe-S_B contained in the small subunit VII [6]. At the lumenal side of PS I subunit III appeared to be necessary for the oxidation of plastocyanin [1]. It does not carry a prosthetic group and has been proposed to provide efficient binding of plastocyanin to PS I [7]. But its attribution to a particular band in SDS-PAGE is ambiguous because subunits III and IV alter their relative position in different gel systems [8]. Its participation in the electron transfer from plastocyanin to P700 in purified PS I has been questioned (reviewed in [9]). In a different approach P700 has been made accessible to external plastocyanin at minimal disturbance of the PS I complex [10]. In this study the subunit has been shown to be positively charged which enables the fast electron transfer to the oxidizing site of the PS I reaction center of extremely negative surface charge density. Plastocyanin bound to PS I in situ reduces $P700^+$ with a halftime of 12–14 μs [11,12] which has also been observed in vitro [13]. In a recent study Wynn and Malkin [14] have cross-linked plastocyanin to a PS I subunit of 19 kDa and demonstrated that the carboxyl groups of plastocyanin are necessary for this reaction. However, the function of this subunit and the assignment to one of the PS I subunits remains to be established.

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2. MATERIALS AND METHODS

2.1. *Isolation and cross-linking of photosystem I and plastocyanin*

Stroma lamellae enriched in PS I (Y-100) were isolated from spinach chloroplasts by Yeda press fractionation [15] with modifications [10]. Isolation of PS I particles followed the procedure given for PS I-200 [14]. Plastocyanin was isolated from spinach leaves [10] and had an absorbance ratio A_{278nm}/A_{597nm} of 1.2–1.4. Stroma lamellae in the presence of 0.1% (w/v) Triton X-100 [10] or PS I particles were incubated at 0.2 mg chl/ml with 5 mM *N*-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) for 15 min in ambient light at room temperature in the presence of 1 mM sodium ascorbate, 0.1 mM diaminodurene, 0.2 mM methyl viologen, 30 mM 3-(*N*-morpholino)propanesulfonic acid (Mops) buffer, pH 6.5, and 50 μM plastocyanin. The reaction was terminated by adjusting a concentration of 0.2 mM ammonium acetate in the sample. After dilution to a 20-fold volume with 30 mM Mops buffer, pH 6.5, the sample was centrifuged for 15 min at 18000 $\times$ *g*. This step was repeated once to remove residual EDC and plastocyanin.

2.2. *Electrophoresis and immunoblotting*

SDS-PAGE was carried out at 4°C on a gradient from 10.2 to 15.4% T at 2.6% C using the Laemmli buffer system [16], except that 2 mM EDTA was present and the samples were incubated for 15 min at 65°C in the presence of 0.2 M dithiothreitol. Electroblotting to nitrocellulose, immuno-gold staining and characterization of the monospecific antibodies raised against plastocyanin has been described [12]. Preparative SDS-PAGE was as described in [17] except that a 2-mm gel was loaded over the full width with isolated PS I containing 350 μg chl. After staining with Coomassie brilliant blue G-250 (Serva) the band at 18.5 kDa was excised, extracted overnight with 75% formic acid and the supernatant dried in a vacuum centrifuge (Speed Vac). The residue was dissolved in sample buffer and rerun on a gel with high resolution at low molecular masses [18]. All chemicals were of analytical grade except for acrylamide and SDS used for the latter gel which were of sequencing grade (Biorad). Markers were the low molecular mass standards from Biorad (14.4–97.4 kDa) and the peptide molecular mass standards from Pharmacia (2.6–17.2 kDa) using the correction in [18] for the CNBr fragments of myoglobin from sperm whale.

2.3. *Peptide sequencing*

The latter gel was used for electroblotting [12] to an Immobilon membrane (Millipore) in 20 mM CAPS buffer, pH 10, and 20% methanol for 4.5 h at 0.2 A and 4°C. The membrane was stained with 0.001% (w/v) Coomassie brilliant blue G-250 in methanol/acetic acid (40:4, v/v). The dark blue band was excised and the stain extracted with 90% methanol. Several bands were collected and transferred to a gas-phase protein sequencer (Applied Biosystems, model 477A).

2.4. *Spectroscopy*

Absorbance changes of P700 were induced by repetitive (0.4 Hz) xenon flashes of 2 μs (FWHM) duration and monitored with light of 703 nm at an intensity of 42 μW/cm^2 using an apparatus described in [10] at an electrical bandwidth ranging from d.c. to 1 MHz and a 2-μs dwell time of the transient recorder. The suspension of stroma thylakoid membranes or of PS I particles in the 1 $\times$ 1 cm cuvette contained 0.1 mM diaminodurene, 1 mM sodium ascorbate, 1 mM $MgSO_4$, 0.2 mM methyl viologen as electron acceptor, and 30 mM Mops buffer, pH 6.5.

3. RESULTS

3.1. *Cross-linking of plastocyanin and PS I*

At low concentrations of 0.1% (w/v) Triton X-100 has been used to make the oxidizing site of PS I accessible to externally added plastocyanin in stroma lamella without disturbing the subunit composition of PS I [10]. Incubation with EDC under these conditions in the presence of plastocyanin shows on SDS-PAGE the specific disappearance of a band at 18.5 kDa and a new one at 31.5 kDa (fig.1, lanes C and D). After EDC incubation isolated plastocyanin shows a band at

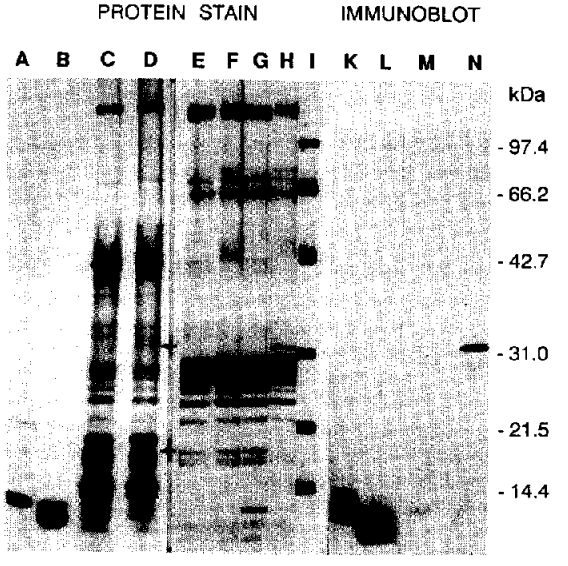

Fig.1. SDS-PAGE analysis of stroma lamellae and PS I-particles cross-linked with plastocyanin. Lanes A–I, protein stain; lanes K–N, immunoblot probed with anti-plastocyanin antibody and stained with protein A–gold. Stroma lamellae and the PS I-particles contained 2.5 μg chl per lane. Lane A, K, 10 μg plastocyanin; lane B, L, 10 μg plastocyanin treated with EDC; lane C, stroma lamellae (Y-100) treated with plastocyanin; lane D, stroma lamellae treated with EDC + plastocyanin; lane E, PS I-particles (PS I-200); lane F, PS I-particles treated with EDC; lane G, M, PS I-particles treated with plastocyanin. The band near 12 kDa shows traces of residual plastocyanin; lane H, N, PS I-particles treated with EDC + plastocyanin; lane I, markers (phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, lysozyme).

slightly lower but none at higher molecular mass than native plastocyanin (fig.1, lanes A and B). An assignment of the cross-linked subunit to a known polypeptide is difficult due to the high number of peptides in stroma lamellae.

Therefore isolated PS I was incubated with EDC and plastocyanin and analyzed by SDS-PAGE (fig.1, lanes E–H). The 2 large subunits Ia and Ib were found at molecular masses of 69 and 64 kDa, the subunits of the light harvesting complex LHCI at 24–29 kDa, and the smaller subunits of PS I at 21.4, 18.5, 17.4, 16.9, 11.2, 10.1 and 9.1 kDa. After cross-linking of plastocyanin the band at 18.5 kDa disappeared completely and a new band was observed at 31.5 kDa as with stroma lamellae. Electroblotting of the gel and immuno-gold staining with antibodies to plastocyanin showed that the new band at 31.5 kDa contained cross-linked plastocyanin (fig.1, lane N).

3.2. *Identification of subunit III*

The subunits of isolated PS I were separated by preparative SDS-PAGE and the band at 18.5 kDa excised. The protein was eluted from the gel and purified on a gel system of high resolution at low molecular masses [18]. Using standard proteins and fragments of myoglobin as molecular weight markers we estimate 20.2 and 17.7 kDa, respectively, as the apparent molecular mass of this subunit (fig.2). This band was collected by electroblotting to a Teflon membrane. The N-terminal sequence was determined in a gas-phase sequencer. Positions with uncertain or no identification of the amino acids are represented by X. By comparison with the protein sequences currently available and the translated genes of PS I subunits, subunit III is unambiguously identified with the mature protein of the nuclear encoded sequence of the *psa*F gene derived from the P6SocPI4-7 DNA clone [5]:

Deduced sequence –				
spinach *psa*F gene	1	5	10	15
product	D I	A G L T P	C K E S K	Q F A
Isolated subunit III of				
spinach PS I	X I	A G L X P	X K E X K	Q F

A first amino acid analysis of the protein was consistent with the composition of the mature pro-

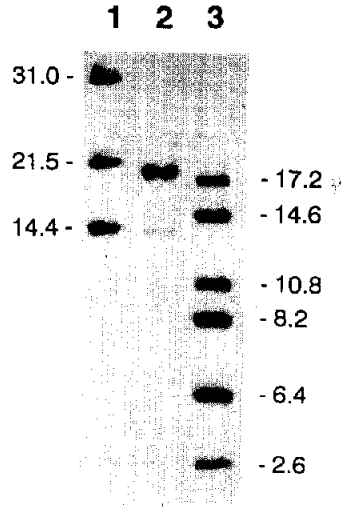

Fig.2. SDS-PAGE of subunit III at high resolution. The band at 18.5 kDa was excised from a preparative gel, eluted and concentrated (see section 2) before application to lane 2. Lanes: 1, low molecular mass standards; 3, peptide molecular mass standards [18].

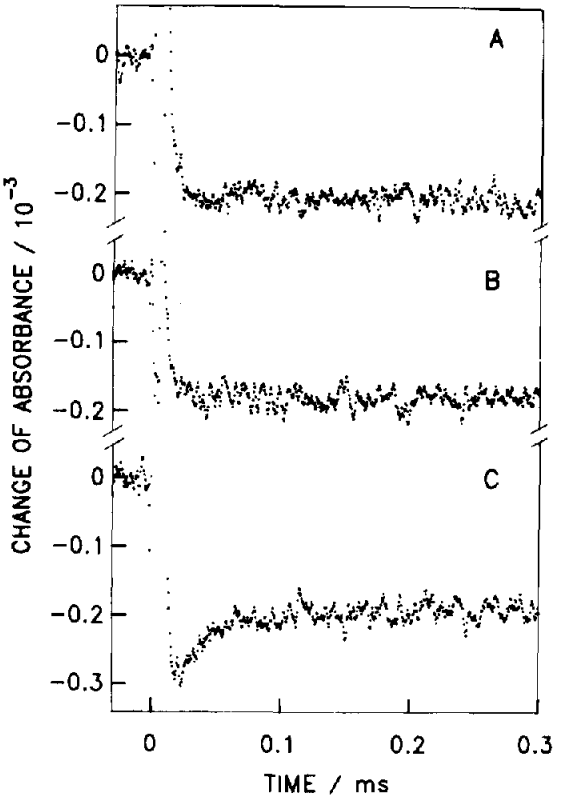

Fig.3. Absorbance changes of P700 in PS I particles (PS I-200) at 703 nm induced by a flash of saturating intensity. A, PS I treated with plastocyanin; B, PS I treated with EDC; C, PS I treated with EDC + plastocyanin. For each trace 100 signals were averaged.

tein derived from the translated sequence (not shown).

3.3. *Reaction kinetics*

Although plastocyanin is selectively cross-linked to one subunit the linked amino acid residues may not interact in the functional complex. Therefore we studied the flash induced kinetics of P700. The sample with cross-linked plastocyanin shows fast reduction kinetics with a halftime of 13 μs in contrast to the control samples (fig.3). We estimate that the observed amplitude of the fast component represents 55% of the total amplitude. It may be slightly greater if a shorter flash is used for excitation.

4. DISCUSSION

The term subunit III has been coined by Nelson for the subunit involved in the interaction between PS I and plastocyanin [1]. The uncertainty in its attribution to a band in SDS-PAGE is settled now by cross-linking of the subunit at 18.5 kDa to plastocyanin in [14] and this study. It has been found as the third largest subunit of PS I in this study and in [6,14,19] giving 18.5, 18, 19, 18.5 kDa, respectively. The new band of the cross-linked protein at 31.5 kDa has been shown with the help of antibodies to contain this subunit [14] in addition to plastocyanin (cf. fig.1N). The N-terminal amino acid sequence is that predicted from the *psa*F gene for the mature protein of 154 amino acids with a molecular mass of 17.3 kDa [5]. This is in good agreement with the values of 18.5 and 17.7 kDa estimated for subunit III by SDS-PAGE in figs 1 and 2, respectively. The subunit attributed to the *psa*F gene [5] will be renamed subunit III (Herrmann, R., personal communication). There are other properties of the predicted sequence consistent with previous observations. (i) Recent in organello import studies using the precursor peptides made from 5 cDNA clones of PS I subunits [4,5] by in vitro transcription and translation showed that the *psa*F gene product is the only inaccessible subunit (Steppuhn and Herrmann, manuscript in preparation) in agreement with the location of plastocyanin [20] and the function of subunit III. (ii) The two hydrophobic domains [5] may be involved in the binding to the large subunits of PS I as suggested by the high Triton X-100 concentration needed to release subunit III [1,10]. (iii) The excess of 7 positively charged residues is consistent with studies of the surface charge density at the oxidizing site of PS I which provided first evidence that subunit III is positively charged [10]. The positive charges may not only compensate the high negative surface charge density at the electron transfer site to P700 but also provide a suitable protein environment for rapid binding [7] while the bulk of the repulsing negatively charged lipid membrane may facilitate the diffusion of plastocyanin in the thylakoid lumen [12].

The time of the electron transfer from cross-linked plastocyanin to $P700^+$ of 13 μs (fig.3C) is the same as that of the complex in vivo [11,12]. This is evidence that cross-linking with EDC conserved the orientation of the complex and that subunit III provides the conformation of PS I necessary for the extremely rapid electron transfer from plastocyanin to $P700^+$. It is tempting to conclude that one of the carboxyl groups of residues 44–46 and 59–61 being conserved in all plastocyanins [21] may interact with a lysine residue of subunit III in the complex. The identification of the cross-linked amino acid residues will help to understand more structural details.

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