Protein Expression and Purification 78 (2011) 156–166

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

Cloning, expression and purification of the luminal domain of spinach photosystem 1 subunit PsaF functional in binding to plastocyanin and with a disulfide bridge required for folding

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ARTICLE INFO

Article history:
Received 10 January 2011
and in revised form 18 February 2011
Available online 25 February 2011

Keywords:
NMR
Photosynthesis
Plastocyanin
Protein–protein interaction
PsaF
Thioredoxin

ABSTRACT

The photosystem 1 subunit PsaF is involved in the docking of the electron-donor proteins plastocyanin and cytochrome c_6 in eukaryotic photosynthetic organisms. Here we report the expression, purification and basic characterization of the luminal domain of spinach PsaF, encompassing amino-acid residues 1–79. The recombinant protein was expressed in *Escherichia coli* BL21 (DE3) using a pET32 Xa/LIC thioredoxin fusion system. The thioredoxin fusion protein contained a His_6 tag and was removed and separated from PsaF through proteolytic digestion by factor Xa followed by immobilized metal affinity chromatography. Further purification with size-exclusion chromatography resulted in a final yield of approximately 6 mg PsaF from one liter growth medium. The correct identity after the factor Xa treatment of PsaF was verified by FT-ICR mass spectrometry which also showed that the purified protein contains an intact disulfide bridge between Cys residues 6 and 38. Secondary structure and folding was further explored using far-UV CD spectroscopy indicating a α-helical content in agreement with the 3.3 Å-resolution crystal structure of photosystem I Ref. [5] and a helix-coil transition temperature of 29 °C. Thermofluorescence studies showed that the disulfide bridge is necessary to keep the overall fold of the protein and that hydrophobic regions become exposed at 50–65 °C depending on the ionic strength. The described expression and purification procedure can be used for isotopic labeling of the protein and ^{15}N-HSQC NMR studies indicated a slow or intermediate exchange between different conformations of the prepared protein and that it belongs to the molten-globule structural family. Finally, by using a carboxyl- and amine-reactive zero-length crosslinker, we have shown that the recombinant protein binds to plastocyanin by a specific, native-like, electrostatic interaction, hence, confirming its functionality.

Introduction

Photosystem I (PSI)[1] is a membrane-bound protein super-complex responsible for translocating electrons across the thylakoid membrane in plants, algae and cyanobacteria – the oxygen-evolving photosynthetic organisms. Electrons are transferred from the electron-carrier proteins plastocyanin (Pc, a copper protein) or cytochrome (cyt) c_6 (a mono-heme protein) in the lumen to ferredoxin in the stroma via a series of redox cofactors in PSI. This process is made favorable through absorption of sunlight by antenna pigments and subsequent energy transfer to the special chlorophyll pair P700 in PSI, where charge separation takes place.

PSI consists of a core complex and a peripheral antenna, see [1,2] for recent reviews. In plants, these two functional units are assembled from at least 19 protein subunits. The PSI core complex contains 15 subunits named PsaA to PsaL and PsaN to PsaP, of which many are homologous to their cyanobacterial counterparts. The subunit explored in the present work, PsaF, is a nuclear-encoded 17.3 kDa protein that extends through the thylakoid membrane. It consists of both a hydrophobic transmembrane helical region (amino-acid residues 84–94, see Fig. 1a) and two hydrophilic regions exposed to either side of the membrane [3–9]. The N-terminal region of PsaF, located in the thylakoid lumen, contains a lysine-rich helix-loop-helix motif [3] that has been demonstrated to interact with Pc in plants and with Pc or cyt c_6 in algae in a very specific manner (see below).

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[1] *Abbreviations used*: Cyt, cytochrome; DTT, DL-dithiothreitol; EDAC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, hydrochloride; GEE, Glycine ethyl ester; IMAC, immobilized metal affinity chromatography; IPTG, isopropyl-β-D-1-thiogalactopyranoside; Pc, plastocyanin; PMSF, phenylmethylsulphonylfluoride; PSI, photosystem I; SEC, size-exclusion chromatography; TF, thermofluorescence; Trx, thioredoxin.

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doi:10.1016/j.pep.2011.02.007

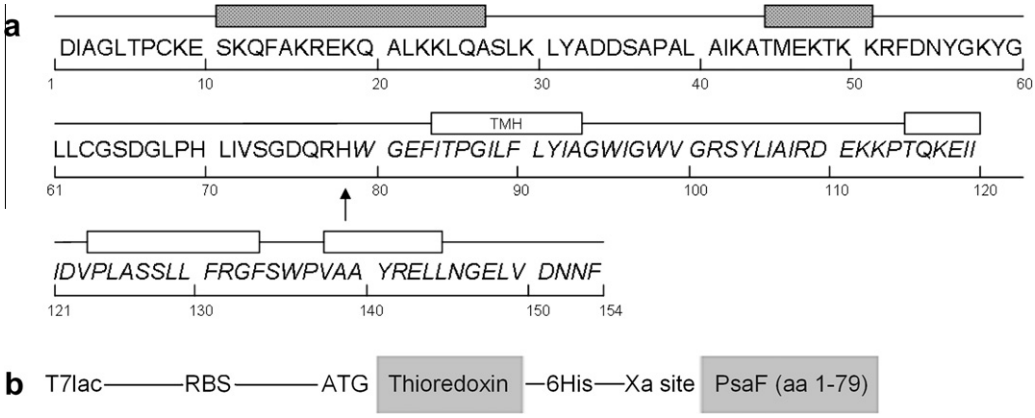

Fig. 1. (a) Amino-acid sequence and secondary structure of the entire PsaF subunit including the lumen-exposed domain estimated to stretch from Asp 1 to His 79 (indicated with an arrow). The trans-membrane helix (TMH) going from Ile 84 to Ala 94 and the remainder of the sequence are written in italics. Secondary-structure elements are indicated as defined by the authors in the PDB file 3LW5. Alpha helices are shown as rectangles, diamond-filled rectangles indicate helices exposed to the thylakoid lumen, helices otherwise not exposed to the lumen are shown as empty rectangles. (b) Block diagram illustrating the functional elements of the pET32-Xa/LIC expression vector.

The interactions between PSI and its electron donors have been elucidated with site-directed mutagenesis combined with flash-induced time-resolved absorption spectroscopy. These studies have revealed three important areas on Pc: the hydrophobic, northern end, where the copper is situated, and two acidic patches (residues 42–45 and 59–61, surrounding Tyr83) on the eastern side [10–18]. The hydrophobic area binds to the PSI subunits PsaA and PsaB and mediates the electron transfer to P700. In the case of algae and higher plants, the acidic patches form salt bridges with positively charged residues on the lumen-exposed domain of subunit PsaF.

Early work showed that PsaF (then called subunit III) was required for electron transfer from Pc to P700 [19,20]. Subsequently, it was demonstrated that Pc cross-linked to PSI is capable of fast electron transfer to P700 and the cross-linking partner was identified as PsaF [7,9]. Site-directed mutagenesis of Pc revealed that one of the acidic patches (residues 42–45) is more important in the interaction than the other (residues 59–61) [11–13,15,18]. Analysis of the cross-linked Pc–PsaF complex showed that the interaction involves positive charges located among residues 9–23 in the luminal domain of PsaF [12]. Indeed, neutralizing the lysine residues 16 and 23 by site-directed mutagenesis of an algal PsaF significantly weakens the interaction [21].

In cyanobacteria and algae, cyt c_6 fulfills the role of Pc as electron donor to PSI. But in some cyanobacteria and in most algae, Pc is expressed instead of cyt c_6 if copper is available [22]. Plants have a cyt c_{6A}, similar to cyt c_6, but with a reduction potential which is too low for electron transfer between cyt *f* and PSI [23]. Thus, Pc has gradually taken over the role of cyt c_6 in the evolution of oxygen-evolving organisms. The evolution of PsaF and its extended lysine-rich region appear to be correlated with the progression of Pc, leading to a much faster electron transfer in the eukaryotes compared to the cyanobacteria. Generally, the latter lack a fast phase in the kinetics, characteristic for plants and algae [24]. The development of acidic patches on Pc or cyt c_6 together with complementary basic residues in the N-terminal domain of PsaF seems to be crucial for fast electron transfer. As a matter of fact, the lysine-rich region in PsaF is located in a sequence of 27 amino acids which folds into a helix-loop-helix motif and is highly conserved in eukaryotes [3,5,12]. This stretch replaces a shorter sequence of nine amino acids with only a few basic residues in cyanobacteria. The helices are much shorter in cyanobacteria and PsaF does not seem to be involved in the docking of Pc or cyt c_6 in these organisms [12,25–27]. However, replacing the shorter N-terminal portion of a cyanobacterial PsaF with a longer from algal PsaF results in faster electron transfer from algal Pc and cyt c_6 to the modified cyanobacterial PSI [28].

In order to further elucidate the mechanism of electron donation to PSI, we decided to overexpress PsaF for structural characterization and interaction studies with Pc and cyt c_6. NMR spectroscopy provides an important tool in such studies [29], however, restrictions are set on the size of the system. In order to circumvent the low NMR signal/noise associated with long correlation times of large protein systems, we chose to isolate only the soluble lumen-exposed N-terminal domain of PsaF from a plant source. After trying several constructs, we found that a DNA fragment encoding for amino-acid residues 1–79 of spinach PsaF, cloned into a pET thioredoxin (Trx) expression vector and overexpressed in *Escherichia*, resulted in a high yield of protein after purification. We report here a characterization of the secondary structure and stability of the protein using circular dichroism (CD) and thermofluorescence (TF) spectroscopy. The α-helical content is consistent with the crystal structure resolved at 3.3 Å [5]. We pay particular attention to Cys 8 and 63 which are in close proximity but in the dithiol form in the crystal structure of pea PSI [5] while they form a disulfide bridge in the structure of a cyanobacterial PSI [26]. Using mass spectrometry we find that the disulfide bridge is present in our PsaF protein and the TF data show that it is necessary for the stability of the protein. In addition we have performed cross-linking studies with the PsaF protein and spinach Pc (wild-type as well as chemically and genetically modified) which show a native-like behavior, but only when the disulfide bridge is intact. Finally, we show that the reported expression protocol is suitable for the production of isotopically labeled protein using minimal media as the source. This will be valuable for future heteronuclear NMR studies.

Materials and methods

Materials

Oligonucleotides and Platinum® *Pfx* DNA polymerase were purchased from Invitrogen. pET 32 Xa/LIC vector kit and BL21 (DE3) competent cells were obtained from Merck Novagen. Gel extraction kit, miniprep plasmid purification kit, factor Xa and Ni–NTA superflow cartridge were purchased from Qiagen. S-100 sephacryl column was obtained from Pharmacia. Vivaspin concentration