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Thioredoxin-mediated reduction of the photosystem I subunit PsaF and activation through oxidation by the interaction partner plastocyanin

Daniel Farkas, Örjan Hansson *

Department of Chemistry, University of Gothenburg, P.O. Box 462, SE-403 30 Gothenburg, Sweden

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ABSTRACT

In the photosynthetic electron-transfer chain, the photosystem I subunit PsaF is involved in the specific binding of plastocyanin. Using fluorescence electrophoresis we show here that the luminal domain of PsaF is a target for thioredoxin-mediated reduction of the Cys residues 8 and 63. Furthermore, by using NMR spectroscopy, we show that the thiolated form of PsaF has a lower affinity towards reduced plastocyanin than when the disulfide bridge is intact. Time-resolved absorbance measurements and fluorescence electrophoresis shows that oxidized plastocyanin can re-oxidize PsaF and thus restore the active form.

Structured summary of protein interactions:
PsaF and **PsaF** bind by nuclear magnetic resonance (View interaction)

1. Introduction

Photosystem I (PSI) is a protein super-complex bound to the thylakoid membrane of all oxygen-evolving photosynthetic organisms. Upon excitation of P700 (the reaction-center chlorophyll in PSI), a driving force is created that enables transfer of electrons from the thylakoid lumen to the stroma where $NADP^+$ is reduced to NADPH. In the lumen, the Cu-containing protein plastocyanin (Pc) is responsible for donating electrons to PSI. In higher plants and green algae, Pc and the membrane-spanning PSI subunit PsaF are involved in specific electrostatic interactions. Two patches (residues 42–45 and 59–61) on Pc, dominated by negatively charged residues (Asp and Glu), interact with a complementary Lys-rich region on the luminal side of PsaF [1]. In cyanobacteria, both Pc and PsaF lack the two charged regions which results in an electron donation from Pc to PSI which is a 100-fold slower [2].

The recent 3.3 Å-resolution crystal structure of a plant PSI (pdb file 3lw5) reveals that the N-terminal domain of PsaF forms a helix-turn-helix motif exposed to the luminal side of the thylakoid membrane [3]. One of the two helices includes amino acids K12, K16, R17, K19, K23 and K24 which make up the Pc binding site. At the N- and C-terminal ends of the helix-turn-helix region, two highly conserved residues C8 and C63 are found separated by 5.7 Å between the S_γ atoms, clearly in their reduced, thiolated, form in the crystal structure. This is in contrast to the crystal structure of a cyanobacterial PSI, in which the corresponding C8 and C43 are connected by a disulfide bridge (pdb file 1jb0) [4]. An oxidized state is as expected in vivo, since during the light reactions and the formation of molecular oxygen, the lumen constitutes an oxidizing environment both in plants and cyanobacteria. Another observation from the plant PSI structure is that the soluble PSI subunit PsaN, a modulator of the Pc–PSI interaction [5], contains four Cys residues which are all reduced as in the case of PsaF.

Recently we have reported on the expression and characterization of the lumen-exposed domain (residues 1–79) of PsaF from spinach, hereafter denoted PsaF′. By using MS and thermofluorescence, we have shown that a disulfide bridge is present in the isolated protein and that this is required for the protein to attain its tertiary fold. Furthermore, the ability to cross-link Pc via the amide-carboxyl selective zero-length cross-linking agent EDAC was significantly diminished when PsaF′ was in its thiolated form [6]. These findings raise the question whether the disulfide bridge is a potential target for thioredoxin (Trx)-mediated reduction and functional deactivation.

Abbreviations: DTT, DL-dithiothreitol; MIANS, 2-(4′-maleimidylanilino) naphthalene-6-sulfonic acid; Pc^{ox} and Pc^{red}, oxidized and reduced forms of plastocyanin; PsaF′, lumen-exposed domain of PsaF; PSI, photosystem I; Trx^{ox} and Trx^{red}, oxidized and reduced forms of thioredoxin

* Corresponding author. Fax: +46 31 786 3910.
E-mail address: Orjan.Hansson@chem.gu.se (Ö. Hansson).

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It has long been known that reducing equivalents, generated by the light reactions, are shuttled by means of PSI and ferredoxin to Trx in the stroma, and lead to a Trx-dependent reduction and activation of enzymes in the Calvin-cycle. However, little is known about Trx-regulation in the lumen. It is only recently that a Trx-like protein was found within the lumen, leading to a new field of exploration [7]. In that study, it was shown that the protein HCF164 can reduce Cys residues of the PSI subunit PsaN. A recent large-scale proteomics survey has revealed several additional potential Trx targets within the thylakoid lumen [8]. Since that study was limited to soluble proteins, little is known of the integral membrane proteins exposed to the lumen, such as PsaF. An immunophilin FKBP13 found within the lumen has been shown to be *deactivated* through Trx-mediated reduction, which suggests a clear distinction between the lumen and stroma in the mode-of-action in Trx-mediated signaling [9,10].

We show in this study that Trx from *Escherichia coli* can reduce the existing disulfide bridge in PsaF′. Furthermore, we observe chemical-shift changes in $^{1}H–^{15}N$ HSQC spectra of Pc which indicate that the binding affinity towards PsaF′ is significantly lower when the latter is in its thiolated form. In addition, we report that oxidized Pc (Pc^{ox}), can oxidize the thiols in PsaF′ and Trx. Thus, Pc may act as an agent of activation of PsaF and other thiolated proteins within the lumen as recently suggested [11].

2. Materials and methods

2.1. Chemicals

Primers were purchased from MWG, 2-(4′-maleimidylanilino) naphthalene-6-sulfonic acid (MIANS) was obtained from Invitrogen Molecular Probes. All other chemicals were of at least pro-analysis quality.

2.2. Protein samples

Expression and purification of the lumen-exposed domain of PsaF from spinach (PsaF′) was conducted essentially as reported elsewhere [6]. At the N-terminal end of PsaF a TEV site (ENLYFQG) was introduced after the factor-Xa site in the original construct by using a two step PCR procedure. The primer design was as follows: First PCR: forward 5′-gaa aac ctg tat ttt cag ggc **gac att gca ggg cta aca cc**-3′, reverse 5′-cta **gtg tct ctg atc acc act cac tat** c-3′. Second PCR: forward 5′-*ggt att gag ggt cgc* **gaa aac ctg tat ttt cag ggc gac**-3′, reverse 5′-*aga gga gag tta gag cc* **cta gtg tct ctg atc acc act** c-3′. Here, additional sequences compatible with the original pET32 Xa/LIC cloning procedure are shown in italics and overlaps are indicated in bold. Proteolysis by TEV was made in a buffer supplemented with 1 mM GSH/0.1 mM GSSG. The tertiary fold and integrity of the disulfide bond between C8 and C63 was verified by thermofluorescence and MS FT-ICR as described [6].

Expression and purification of ^{15}N-labelled spinach Pc was as described elsewhere [12] with a few modifications (Farkas and Hansson, submitted). *E. coli* Trx with a His tag at the Trx C-terminal (Trx-6His) was obtained from the Trx-6His-PsaF′ fusion protein as a side product in the PsaF′ purification protocol [6]. The protein was further purified on a Sephacryl S100 column (Pharmacia) in 10 mM DTT, 50 mM NaH_2PO_4 (pH 6.5) and 150 mM NaCl. A final buffer of 10 mM KH_2PO_4 (pH7.5), 90 mM NaCl and <1 μM DTT was attained using a PD-10 column (GE Healthcare) and a spin column (cutoff 5 kDa, Sartorius Stedim Biotech), reaching a final protein concentration of 185 μM. All buffers were bubbled with N_2 prior to use. The Trx-6His construct (18 kDa) is significantly larger than Pc (10.5 kDa) and yields unambiguous bands on a high-resolution Criterion Tris-Tricine gel (BioRad). The Trx-6His concentration was determined using $\varepsilon_{280} = 15470\ M^{-1}cm^{-1}$ [13]. The procedure described here results in a relatively pure sample with only slight contaminations of C-terminal-truncated forms of Trx-6His with apparent molecular masses of ~16 kDa on SDS–PAGE as verified by MS.

2.3. Optical spectroscopy

UV–vis spectra were acquired on a Shimadzu UV-1601 spectrophotometer. Time-dependent absorption changes were monitored at 597 nm using 1 s increments. All measurements were made at 20 °C. First, a spectrum of 14 μM Pc^{ox} in 10 mM KH_2PO_4 (pH 7.5) and 90 mM NaCl was recorded. This was followed by time-course measurements after additions of PsaF′, Trx^{red}-6His or both at 1:1, 1:3 and 1:1:3 stoichiometries, respectively. Prior to adding PsaF′ and Trx^{red}-6His to the Pc sample, PsaF′ and Trx^{red}-6His were pre-incubated for 15 min to ensure a complete reduction of PsaF′.

2.4. Fluorescence electrophoresis

The thiol-reactive fluorophore MIANS was used to trap and detect reduced Cys residues. Samples were prepared using 60 μM PsaF′, 60 μM Trx^{red}-6His and 120 μM Pc at 20 °C according to the reaction scheme in Fig. 3a. Trx^{red}-6His and PsaF′ were incubated for 15 min in 10 mM KH_2PO_4 (pH 7.5) and 90 mM NaCl prior to adding Pc, after which the incubation was extended for another 15 min and followed by the addition of 0.6 mM MIANS. After additional 20 min incubation the reactions were terminated with 6 mM L-Cys. The added L-Cys binds to free MIANS which avoids a labeling of the Pc copper ligand C84 in the following denaturation step. Samples for SDS-PAGE were prepared in a 2 × SDS loading buffer lacking reducing agents and heated for 2 min at 95 °C. DTT and β-mercaptoethanol were avoided since separate experiments showed a lower intensity of MIANS fluorescence in their presence. The Tris-Tricine gel was run in the dark according to [14]. UV image and MIANS fluorescence were recorded using a Gel Doc 2000 (BioRad) UV table and imaging system prior to fixation and Coomassie staining. Reduced Pc (Pc^{red}) was obtained by adding a 10-fold excess of sodium ascorbate prior to the reaction setup.

2.5. NMR spectroscopy

$^{1}H–^{15}N$ HSQC spectra of 100 μM ^{15}N-Pc in 10 mM KH_2PO_4 (pH 7.5), 90 mM NaCl, 1 mM sodium ascorbate and 10% D_2O were recorded on a Varian INOVA 800 MHz spectrometer at 25° C. The data were acquired using 1024 complex points in the ^{1}H dimension and 256 complex points in the ^{15}N dimension with sweep widths of 13 and 3 kHz, respectively. Spectral assignments for Pc^{red} were taken from [15]. Data processing was done with the software nmrPipe [16]. Visualization and fitting of Lorentzian line shapes was done with Sparky [17].

3. Results and discussion

3.1. NMR spectroscopy

The binding of both the oxidized and thiolated forms of PsaF′ to Pc was investigated by NMR. The interaction was monitored by studying the magnitude of chemical-shift changes in the $^{1}H–^{15}N$ HSQC spectrum of Pc upon addition of PsaF′ in the absence or presence of 5 mM DTT. The combined chemical-shift perturbations, as defined by $\Delta\delta_{HN} = (\Delta\delta_H{}^2 + \Delta\delta_N{}^2)^{1/2}$ (in digital points), are shown in Fig. 1a. Residues which are significantly affected (that is, the combined shift exceeds two points) upon the addition of PsaF′ are: D42, E43, D44, I46, M57, E60, L62, K81, S85, K95, and V96. Upon