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Proteomic characterization and three-dimensional electron microscopy study of PSII–LHCII supercomplexes from higher plants ☆

Cristina Pagliano [a,*], Jon Nield [b], Francesco Marsano [c], Tillmann Pape [d], Simone Barera [c], Guido Saracco [a], James Barber [a,d,**]

[a] Applied Science and Technology Department, BioSolar Lab, Politecnico di Torino, Viale T. Michel 5, 15121 Alessandria, Italy
[b] School of Biological and Chemical Sciences, Queen Mary University of London, London E1 4NS, UK
[c] Department of Science and Technological Innovation, University of Piemonte Orientale, Viale T. Michel 11, 15121 Alessandria, Italy
[d] Department of Life Sciences, Faculty of Natural Sciences, Imperial College London, London SW7 2AZ, UK

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ABSTRACT

In higher plants a variable number of peripheral LHCII trimers can strongly (S), moderately (M) or loosely (L) associate with the dimeric PSII core (C_2) complex via monomeric Lhcb proteins to form PSII–LHCII supercomplexes with different structural organizations. By solubilizing isolated stacked pea thylakoid membranes either with the α or β isomeric forms of the detergent n-dodecyl-D-maltoside, followed by sucrose density ultracentrifugation, we previously showed that PSII–LHCII supercomplexes of types $C_2S_2M_2$ and C_2S_2, respectively, can be isolated [S. Barera et al., Phil. Trans. R Soc. B 67 (2012) 3389–3399]. Here we analysed their protein composition by applying extensive bottom-up and top-down mass spectrometry on the two forms of the isolated supercomplexes. In this way, we revealed the presence of the antenna proteins Lhcb3 and Lhcb6 and of the extrinsic polypeptides PsbP, PsbQ and PsbR exclusively in the $C_2S_2M_2$ supercomplex. Other proteins of the PSII core complex, common to the $C_2S_2M_2$ and C_2S_2 supercomplexes, including the low molecular mass subunits, were also detected and characterized. To complement the proteomic study with structural information, we performed negative stain transmission electron microscopy and single particle analysis on the PSII–LHCII supercomplexes isolated from pea thylakoid membranes solubilized with n-dodecyl-α-D-maltoside. We observed the $C_2S_2M_2$ supercomplex in its intact form as the largest PSII complex in our preparations. Its dataset was further analysed *in silico*, together with that of the second largest identified sub-population, corresponding to its C_2S_2 subcomplex. In this way, we calculated 3D electron density maps for the $C_2S_2M_2$ and C_2S_2 supercomplexes, approaching respectively 30 and 28 Å resolution, extended by molecular modelling towards the atomic level. This article is part of a Special Issue entitled: Photosynthesis Research for Sustainability: Keys to Produce Clean Energy.

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1. Introduction

Photosystem II (PSII) is one of the key protein complexes of the light reactions of photosynthesis, carrying out the conversion of solar energy into electrochemical potential energy required to drive the water splitting reaction which it catalyses, together with the production of reducing equivalents needed for driving CO_2 fixation. In plants and green algae, the PSII core complex has associated with it membrane-bound light-harvesting antenna complexes (LHCII), to form large macromolecular complexes called PSII–LHCII supercomplexes. The LHCII complexes, functioning as peripheral solar energy collectors, absorb most of the sunlight subsequently directed to the photochemical reaction centre (RC) of PSII.

In plants and green algae the PSII core complex is mainly embedded in the stacked regions of the thylakoid membranes where it is organized as a dimer, each monomer consisting of several proteins including: 1) D1 and D2, making up the photochemical RC; 2) CP47 and CP43, acting as inner antenna proteins; 3) several low molecular mass subunits (LMM subunits, <10 kDa), accounting for more than half of the entire

Abbreviations: BN-PAGE, blue native polyacrylamide gel electrophoresis; Chl, chlorophyll; 1D/2D SDS-PAGE, mono-dimensional/bi-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis; 3D, three-dimensional; α-DM, n-dodecyl-α-D-maltoside; β-DM, n-dodecyl-β-D-maltoside; FEG, field emission gun; FSC, Fourier shell correlation; LC-ESI-MS/MS, liquid chromatography electrospray ionization tandem mass spectrometry; LHC, light harvesting complex; LMM, low molecular mass; MALDI-TOF/TOF, matrix-assisted laser desorption/ionization-time of flight/time of flight; MS, mass spectrometry; OEC, oxygen evolving complex; PS, photosystem; PTM, post translational modification; RC, reaction centre; TEM, transmission electron microscopy

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* Corresponding author. Tel.: +39 131 229301; fax: +39 131 229344.
** Correspondence to: J. Barber, Imperial College London, Department of Life Sciences, Faculty of Natural Sciences, London SW7 2AZ, UK. Tel.: +44 208 747 1165; fax: +44 207 594 5267.
E-mail addresses: cristina.pagliano@polito.it (C. Pagliano), j.nield@qmul.ac.uk (J. Nield), francesco.marsano@mfn.unipmn.it (F. Marsano), t.pape@imperial.ac.uk (T. Pape), simone.barera@mfn.unipmn.it (S. Barera), guido.saracco@polito.it (G. Saracco), j.barber@imperial.ac.uk (J. Barber).

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complex and playing a role in stabilizing the binding of cofactors to the PSII core; and 4) the extrinsic polypeptides PsbO, PsbP, PsbQ and PsbR, forming the oxygen evolving complex (OEC) on the lumenal side of the membrane (for a recent review see [1]). Up to now the highest resolution structure available for the plant PSII core complex has been obtained by electron crystallography [2,3], which led to the assignment of the major subunits and location of their transmembrane helices. Moreover, crystal structures have been determined for the isolated extrinsic polypeptides PsbP [4] and PsbQ [5,6].

The most abundant PSII-associated LHCII complex, called "major", consists of homo- or hetero-trimers of Lhcb1, Lhcb2 and Lhcb3 polypeptides, usually occurring in a ratio of about 8:3:1 [7–9], whose high-resolution structures have been solved by X-ray crystallography [10,11]. According to these studies, all LHCIIs have three membrane-spanning regions connected by both stromal and lumenally-exposed loops and bind a total of 14 chlorophyll (Chl) molecules (8 Chl *a* and 6 Chl *b*) plus 4 carotenoid molecules. In addition, there are three "minor" LHCII antenna polypeptides, termed Lhcb4 (CP29), Lhcb5 (CP26) and Lhcb6 (CP24), which usually occur in monomeric form. So far, among the minor LHCII antenna proteins, the three-dimensional (3D) structure is available at high resolution only for Lhcb4 [12], revealing three transmembrane α-helices with 13 Chls binding sites (8 assigned as Chl *a* sites, 4 as Chl *b* sites and 1 putative mixed site occupied by both Chl *a* and Chl *b*) and 3 carotenoid binding sites.

A variable number of LHCII can associate with the dimeric PSII core complex to form different types of PSII–LHCII supercomplexes, named according to their composition [13]. The dimeric PSII core complex (C_2) strongly binds two copies of the monomeric Lhcb4 and Lhcb5 and two LHCII trimers (S-trimer) in order to form the C_2S_2 supercomplex [14], which can be regarded as a basic building block of PSII *in vivo*. Larger PSII–LHCII supercomplexes, containing two extra copies of the monomeric Lhcb6 with two additional LHCII trimers (M-trimer) moderately bound to the dimeric PSII core complex via Lhcb4 and Lhcb6, are known as $C_2S_2M_2$ and have been found to represent the basic organization of the PSII in *Arabidopsis thaliana* thylakoid membranes [13,15]. Occasionally even larger supercomplexes have been observed in isolated spinach thylakoids fragments, with one or two additional LHCII trimers (L-trimer) even more loosely bound to the dimeric PSII core complex via Lhcb6, and are known as $C_2S_2M_2L_{1-2}$ [16].

Note that the classification of LHCII trimers within the PSII–LHCII supercomplexes in strongly (S), moderately (M) or loosely (L) bound to the PSII dimeric core complex is based on susceptibility to solubilization by detergent. Thus the typology and composition of the isolated supercomplexes reflect the mildness of the detergent(s) used and the overall conditions of solubilization. By solubilizing isolated stacked pea thylakoid membranes either with the α or β isomeric forms of the detergent n-dodecyl-D-maltoside (DM), followed by sucrose density ultracentrifugation, we isolated PSII–LHCII supercomplexes with different molecular masses, shown to be respectively of types $C_2S_2M_2$ and C_2S_2, demonstrating the milder detergent action of α-DM with respect to β-DM [17].

In order to gain insights into the primary and tertiary structure of the isolated $C_2S_2M_2$ and C_2S_2 PSII–LHCII supercomplexes, we applied extensive multiple approaches of mass spectrometry (MS), combining bottom-up and top-down methods. Bottom-up MS techniques involve approaches where the intact protein is enzymatically cleaved to peptides before measurements via tandem MS; top-down MS targets intact proteins rather than peptides for analysis, with the aim to define the protein primary structure by providing highly accurate structural assignment of MS/MS fragments. In this way, we obtained a detailed overview of the proteins in the isolated PSII–LHCII supercomplexes of different organization, revealing the presence of the antenna proteins Lhcb3 and Lhcb6 and of the extrinsic polypeptides PsbP, PsbQ and PsbR exclusively in the $C_2S_2M_2$ supercomplex. Other proteins of the PSII core complex, common to the $C_2S_2M_2$ and C_2S_2 supercomplexes, including the LMM subunits, were also detected and characterized. Conversely, the LHCII-like PsbS protein was not detected in either the $C_2S_2M_2$ or C_2S_2 supercomplex.

To date, the only 3D structure available of a PSII–LHCII supercomplex has been obtained at 17 Å resolution by cryo-transmission electron microscopy (cryo-TEM) and single particle analysis of C_2S_2 isolated particles containing only one LHCII trimer (S-trimer) per RC core and lacking the minor antenna Lhcb6 [18–20]. For the supercomplex of type $C_2S_2M_2$ only 2D projection maps obtained by TEM analysis of negatively stained single particles derived either from fully or partially solubilized thylakoids are available [15–17,21]. In this paper we show 3D electron density maps, derived from negatively stained samples, for the $C_2S_2M_2$ supercomplex as well as for its C_2S_2 subcomplex from pea (*Pisum sativum*), with resolutions respectively of 30 and 28 Å, subsequently extended by molecular modelling towards atomic level.

2. Material and methods

2.1. PSII–LHCII supercomplexes isolation

Stacked thylakoid membranes were isolated from pea plants according to [22]. By solubilizing thylakoid membranes either with α-DM or β-DM, followed by sucrose density gradient ultracentrifugation, PSII–LHCII supercomplexes of different size were isolated, attributable to the $C_2S_2M_2$ and C_2S_2 organization, respectively, as described in our previous paper [17]. Sucrose bands, containing PSII–LHCII supercomplexes, were carefully removed using a syringe and, if necessary, concentrated by membrane filtration with Amicon Ultra 100 kDa cut-off devices (Millipore) and then stored at −80 °C. The Chl concentration was determined spectrophotometrically after extraction in 80% (v/v) acetone according to [23].

2.2. Gel electrophoresis and western blotting

PSII–LHCII supercomplexes were analysed in native conditions by using the blue-native polyacrylamide gel electrophoresis (BN-PAGE) system according to [24], with a 3–12% acrylamide separating gel and a 4% acrylamide stacking gel. Prior to loading, samples were supplemented with a one-sixteenth volume of the loading buffer (750 mM ε-amino caproic acid, 5% (w/v) Coomassie G250) and incubated for 10 min on ice. After centrifugation at 21,000 ×*g* for 10 min, the supernatants were loaded onto the 20 cm gradient gel and run for 7 h at a constant voltage of 70 V, using as anode buffer a solution made of 50 mM Bis-Tris–HCl pH 7.0 and as cathode buffer a solution made of 50 mM Tricine, 15 mM Bis-Tris–HCl pH 7.0, and 0.02% (w/v) Coomassie G250. After two-thirds of the run, the cathode buffer containing Coomassie G250 was replaced by a buffer with the same composition but devoid of Coomassie G250 and run overnight at a constant voltage of 60 V. For molecular mass markers, a mixture of lyophilized standard proteins (Amersham, high molecular mass calibration kit (code 17-0445-01), GE Healthcare) was used. For bi-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (2D SDS-PAGE), bands corresponding to $C_2S_2M_2$ and C_2S_2 PSII–LHCII supercomplexes resolved on BN-PAGE were cut out and equilibrated in a buffer made of 66 mM Na_2CO_3, 2% (w/v) SDS and 0.66% (v/v) 2-mercaptoethanol at 25 °C for 30 min and subjected to 15% acrylamide SDS-PAGE containing 6 M urea using Laemmli's system [25].

Mono-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (1D SDS-PAGE) was performed on a linear gradient gel (18–22% acrylamide) containing 6 M urea using Kashino et al.'s system [26], to improve the resolution of LMM subunits.

The proteins separated in 1D or 2D SDS-PAGEs were either stained by 0.25% (w/v) Coomassie R250 for 1 h in a solution made of 50% (v/v) methanol and 10% (v/v) acetic acid, and destained by a solution made of 25% (v/v) methanol and 7.5% (v/v) acetic acid, or transferred onto nitro-cellulose membrane and immunodetected with a specific antiserum (Agrisera, catalog number AS09533) against the

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