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Early folding events during light harvesting complex II assembly *in vitro* monitored by pulsed electron paramagnetic resonance

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ABSTRACT

Efficient energy transfer in the major light harvesting complex II (LHCII) of green plants is facilitated by the precise alignment of pigments due to the protein matrix they are bound to. Much is known about the import of the LHCII apoprotein into the chloroplast *via* the TOC/TIC system and its targeting to the thylakoid membrane but information is sparse about when and where the pigments are bound and how this is coordinated with protein folding. *In vitro*, the LHCII apoprotein spontaneously folds and binds its pigments if the detergent-solubilized protein is combined with a mixture of chlorophylls *a* and *b* and carotenoids. In the present work, we employed this approach to study apoprotein folding and pigment binding in a time-resolved manner by using pulsed electron paramagnetic resonance (EPR). Intra-molecular distances were measured before folding, after 255 ms and 40 s folding time in the absence of cryoprotectant, and in the fully folded and assembled LHCII. In accordance with earlier results, the most of the folding of the three membrane-spanning alpha helices precedes their apposition into the final tertiary structure. However, their formation follows different kinetics, partially extending into the final phase of LHCII formation during which much of the condensation of the pigment-protein structure occurs, presumably governed by the binding of chlorophyll b. A rough timetable is proposed to sort partial events into the LHCII formation process.

1. Introduction

About 30% of sequenced genes encode membrane proteins [1], and the crucial role of these proteins in many metabolic processes makes them an interesting and challenging field of study [2]. Folding pathways of membrane proteins are clearly different from those of water soluble proteins [3]. Folding studies with membrane proteins are complicated by the fact that their natural environment is a double layer of amphiphilic lipid molecules [4]. Many membrane proteins have been folded *in vitro* in detergent micelles replacing the lipid bilayer [5]. Different experimental and theoretical approaches have been used for studying membrane protein folding, such as hydrogen–deuterium exchange NMR studies [6], ultrafast resonance energy transfer [7], mass spectrometry [8] or ϕ-value analysis [9], in which the impact of a mutation on the free energy of a protein chain is studied. All of these experiments are performed *in vitro*. But even in detergent micelles as an artificial environment imitating the lipid bilayer, only a few membrane proteins can be folded into their native structure, with bacteriorhodopsin [10], lactose permease a [11] and the erythrocyte anion exchanger (AE1) [12] being prominent examples [13].

The major light harvesting complex II (LHCII) of green plants is another membrane protein commending itself for folding studies. It is probably the most abundant membrane protein on earth and plays a key role in photosynthesis, where its main purpose is the collection of solar energy to be transmitted to the reaction centers [14]. The recombinant apoprotein, amenable to site-directed mutations, spontaneously folds upon mixing it with its pigments, for instance in a stopped-flow apparatus [15]. With a folding time of ~5 min [16], it is slow in folding compared to other membrane proteins and much slower than water-soluble proteins [17,18]. This is presumably due to the large number of cofactors [14 chlorophyll (Chl), 4 carotenoid, and 2 lipid molecules] bound to the LHCII apoprotein. Ligand binding has been seen to slow down protein folding, for instance in bacteriorhodopsin where formation of a first intermediate is completed after 20 ms, while the binding of retinal extends the folding time to up to tens of seconds [8,19]. The

Abbreviations: EPR, electron paramagnetic resonance; Chl, chlorophyll; LHCII, light harvesting complex II; DEER, doube electron electron resonance; ESEEM, electron echo envelope modulation; LHCP, light harvesting complex protein; FQ, freeze quench; *w*/*v*, weight per volume; LDS, lithium dodecyl sulfate; OG, octyl-glucoside; *v*/v, volume per volume; PG, dipalmitoyl phosphatidylglycerol; DM, n-dodecyl-β-D-maltoside; AA, amino acid; H1, H3, H4, transmembrane helices of light harvesting complex II; GuHCl, guanidinium hydrochloride; s/n, signal to noise; SI, supplementary information; sim, simulation; MMM, multiscale modeling of macromolecular systems.

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pigments in LHCII bind in a highly cooperative manner [20], likely slowing down the folding and assembly process into the minute time range.

In former time-resolved studies of LHCII assembly the intrinsic Chl fluorescence of the complex was utilized [15,16,21,22]. In these studies, reconstitution of LHCII with subsets of the pigments indicated that Chl *a* binding (with an apparent time constant of less than 1 min) precedes the one of Chl *b* (with an apparent time constant of several min). Time resolved CD measurements revealed that the secondary structure formation spans the entire folding time, but is tightly coupled with the pigment binding with similar time constants.

To have a closer look at the protein structure, pulsed electron paramagnetic resonance (EPR) spectroscopy has been used after site-specifically spin-labeling the LHCII apoprotein [23–25]. In these experiments, the protein folding and complex assembly process was stopped by rapid manual freezing of the mixture at chosen time points, and subsequently analyzing partly or fully assembled complexes by double-electron–electron-resonance (DEER) and electron-spin-echo-envelope-modulation (ESEEM) measurements. From these studies, which span time ranges from 40 s to 15–30 min, it was concluded that the formation of the superhelix by intertwining transmembrane helices H1 and H4 is a late event in the folding of LHCII that takes place in the 5 min range while formation of helix H3 may be much faster. In the present study, the time-resolved EPR measurements of LHCII assembly *in vitro* have been extended to times as short as 255 ms. In addition, further spin label positions in the protein have been used to obtain more detailed information about other structural units (H1, H3 and H4) in the protein folding pathway.

2. Material and methods

2.1. Protein preparation and site-directed spin labeling

The LHCII apoprotein (LHCP) versions used in this study were derivatives of Lhcb1*2 (AB80 [26],) from *Pisum sativum*, having its native cysteine at position 79 replaced with serine. In each LHCP version used for DEER measurements, two amino acids were exchanged with cysteine. In LHCP versions 59/90 (carrying cysteines in positions 59 and 90 for spin pair labeling), 90/196 and 106/160 the exchanged amino acids were serine or valine, and isoleucine in version 124/143. Versions 59/90, 90/196 and 106/160 where described earlier [23,27], version 124/143 and 174/196 were constructed as described there with the QuickChange Lightning Kit (Agilent).

Protein expression was performed over night; proteins were spin labeled with a 20-fold molar excess of 3-(2-iodacetamido)-PROXYL (Sigma, free radical) per labeling position as described elsewhere [27].

2.2. Rapid-mixing/freeze-quench setup

Samples for studying LHCP folding were produced using an experimental setup combining a commercial rapid mixer (SFM300, BioLogic, Calix, France) with a home-built freeze-quench apparatus. The SFM300 allows reliable mixing of the two solutions and very accurate control of the aging time with limited sample consumption. For this work we used the interrupted-flow mode in which the starting materials (apo LHCII and pigment solutions) are mixed and pushed to an aging chamber where the flow is stopped for a period of time controlled from the software. After the desired time span the aged sample is pushed throw an outlet nozzle on the silver rods of the freeze-quencher (FQ). The setup is similar to the one described in [28] except for the freezing method. While in the fully commercial apparatus the mixture is sprayed onto cold isopentane, we constructed a device inspired in the paper by Tanaka et al. [29] where the sample is flushed against two rotating silver rods semi-immersed in nitrogen at 77 K (see Fig. 1). This cold-block freezing method yields to faster freezing times [30–32]. The resulting frozen flakes

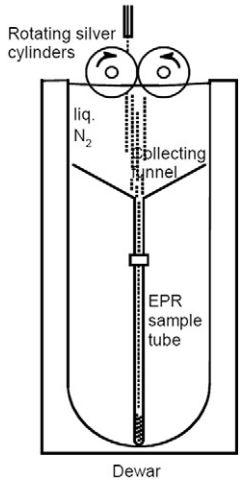

Fig. 1. Schematic drawing of the FQ apparatus. The sample is sprayed on two rotating silver wheels driven by a chain motor. The wheels are in contact with the liquid nitrogen contained in the dewar below them. Immersed in the liquid nitrogen there is a funnel connected to an EPR tube where the sample is collected.

of sample are collected by gravity in a funnel connected to a 3 mm OD EPR tube.

2.3. Stopped-flow calibration

Consistency of the aging times of the stopped-flow/freeze-quench setup was checked by calibration of the SFM300 settings by using the reaction of azide with metmyoglobin [33]. Pseudo first-order conditions were used in the calibration reaction. For calibration, one syringe was loaded with 0.1 mM metmyoglobin (Sigma) in 50 mM HEPES, 100 mM $NaNO_3$ at pH 7, and the other one with 1 mM azide in the same buffer. Upon mixing, the azide molecule replaces the water molecule as the sixth ligand of the heme iron with known kinetic rates (27). Azide binding changes the spin state of myoglobin from high- ($S = 5/2$, $g_{eff} = 6$ and 2) to low-spin ($S = ½$, $g_{eff} = 2.60$, 2.17 and 1.84). Both states are easily distinguishable by Continuous Wave-EPR. Quantification of the relative amount of high-and low-spin myoglobin from the Continuous Wave-EPR spectra, together with the reaction rate measured from stopped-flow experiments at exquisitely the same temperature and concentrations, leads to the time spanned from the mixing to the freezing, the actual reaction time. The calibrated reaction times of 255 ms, 300 ms, 400 ms, 500 ms, and 600 ms were all found to be in good agreement (within ±31 ms) with the reaction time settings of the mixer apparatus.

2.4. Rapid-freeze-quench reconstitutions

For the production of LHCII reconstitution samples with different aging times, *i.e.* at different time points of the folding process, LHCP was solubilized in 1% (*w*/*v*) lithium-dodecylsulfate (LDS), 12.5% sucrose, 100 mM Li-borate, pH 8.1 at a concentration of 90 μM. 3.3 mM pigments were solubilized in 5% (*w*/*v*) octyl-β-D-glucopyranoside (OG), 1.5% (*v*/*v*) Triton X-100, 0.03 (*w*/*v*) % Na-deoxycholate, 0.26% (*w*/*v*) dipalmitoyl phosphatidylglyceriol (PG), 12.5% (*w*/*v*) sucrose and 100 mM Li-borate, pH 8.1. In all buffers, H_2O was replaced with D_2O in order to increase the relaxation times of the spin probe. The LHCP and pigment solutions were then filled into two syringes of the SFM300. The reconstitution was triggered by rapidly mixing 110 μl of each of the two solutions in the SFM300, and stopped after either 255 ms or 40 s by spraying the mixture on rotating silver drums cooled with liquid nitrogen. The pulverized frozen powder containing the reaction mixture was collected by using a funnel into an EPR tube (outer diameter 3 mm). Note that, unlike in our previous work [23], no cryoprotectant was used in freeze-quench reconstitution. That