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A simple and efficient method to prepare pure dimers and monomers of the cytochrome b_6f complex from spinach.

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Abstract

Using a single size-exclusion chromatography we were able to isolate highly pure dimers and monomers of the Cyt b_6f complex from spinach from a bulk preparation of that protein complex obtained with a standard procedure. At higher protein/detergent ratio during the chromatography most of the Cyt b_6f complex remained as dimers. In contrast, at lower protein/detergent ratio (around 15 times lower) most dimers became monomerized. As a bonus, this chromatography also allowed the elimination of potential Chl *a* contaminant to the Cyt b_6f preparations. SDS-PAGE protein analysis with 18% (w/v) acrylamide revealed the loss of the ISP subunit in our monomeric preparation. However, it fully retained the content of Chl *a*, a prerequisite to perform any spectroscopic study involving this unique pigment.

Introduction

The cytochrome (Cyt) b_6f is a photosynthetic membrane protein complex that mediates a linear electron transfer from the photosystem II (PSII) to the photosystem I (PSI) in oxygenic photosynthesis (Cramer et al. 2006; Baniulis et al. 2008). In certain circumstances, the Cyt b_6f can also be part of a cyclic electron transfer around the PSI (Iwai et al. 2010) and a redox sensor for the state transitions in chloroplasts (Vener et al. 1997; Vladkova 2016). During those processes electron transfer is coupled to proton translocation through the thylakoid membranes generating a proton-motive force to synthesize ATP. The electron transfer through the PSI is primarily used to reduce ferredoxin (Fd) and then $NADP^+$ to NADPH. The Cyt b_6f complex from spinach is made of four big protein subunits named Cyt *f* (31,935 Da), Cyt b_6 (24,887 Da), Rieske iron-sulfur protein (ISP) (18,936 Da) and subunit IV (17,312 Da), which showed a stoichiometry of 1:1:1:1, and four small subunits of Pet G (4,198 Da), PetM (3,972 Da), Pet L (3,478 Da) and PetN (3,197 Da) gene products. This makes a total protein mass

for the monomer of 107,913 Da in spinach (Baniulis et al. 2011). Note that Baniulis preparation also contained the FNR subunit bound to the complex, making a total protein molecular mass of 142,227 Da per monomer. The Cyt b_6f has been isolated from plants and algal *Chlamidomonas (C.) reinhardtii* as dimers (Huang et al. 1994; Pierre et al. 1995; Hauska 2005) and cyanobacteria as dimers or monomers (Bald et al. 1992; Dashdorj et al. 2005; Baniulis et al. 2011). The isolated dimeric form was enzymatically active but the monomeric form was normally inactive (Bald et al. 1992; Huang et al. 1994; Hasan et al., 2014).

The Cyt b_6f is a chromoprotein with multiples electron transfer active chromophores, including one *c*-type cytochrome (*f* subunit), two *b*-type cytochromes (b_6 subunit), and one 2Fe-2S cluster (ISP subunit). It also contains three other chromophores of unclear function: a chlorophyll (Chl) *a*, a carotenoid, and a *c*-type heme (Cyt c_n) (Baniulis et al. 2008). However, recent papers claimed that the Chl *a* mediates the photosynthetic state transition (Hasan et al. 2013; Vladkova 2016). The spectroscopic properties in terms of electronic absorption and fluorescence emission band maxima are remarkably different for spinach and *C. reinhardtii* in comparison with cyanobacteria (Dashdorj et al. 2005). The strong band red-shift in cyanobacteria (i.e., 3.5 nm in absorption) is most probably due to a higher number of aromatic amino acids nearby the Chl *a* (Peterman et al. 1998; Yan et al. 2008).

The sole Chl *a* pigment per monomer of Cyt b_6f makes this heteroprotein complex a unique system to study pigment-protein interactions and protein energy thermodynamics without interference from other pigments in contrast to what happen for any other photosynthetic pigment-protein complex. In that respect, some spectroscopic works have been published using the Cyt b_6f isolated from cyanobacteria (Petermen et al. 1998; Dashdorj et al. 2005; Yan et al. 2008). More recently, the

laboratory of Prof. V. Zazubovich (Najafi et al. 2015) has used the bulk dimeric form of the Cyt b_6f isolated from spinach to characterize the thermodynamics and energy landscape of this system using high resolution hole burning and site-selection fluorescence spectroscopy of the Chl *a* at cryogenic temperatures. A comparison of pure dimers and monomers seems to be necessary to reach more solid conclusions. The present technical report describes a simple, rapid and efficient method to prepare highly pure dimers and monomers. The use of an additional size-exclusion chromatography step in a FPLC system with various protein/detergent ratios was sufficient to obtain highly pure dimers and monomers. Both preparations were characterized by UV-VIS absolute and differential redox absorption spectroscopy and SDS-PAGE.

Materials and Methods

Isolation of spinach bulk cytochrome b_6f complex

The bulk Cyt b_6f from spinach was obtained as Romanowska (2011) with the exceptions that in the sucrose gradient centrifugation last step 15 mM *n*-dodecyl *β-D*-maltoside (*β*-DM) in buffer MES, pH 6.5, was substituted for 30 mM *n*-octyl *β-D*-glucoside (OGP) in TRIS/succinate , pH 6.5. The sucrose gradient was made directly in 13-mL centrifuge tubes filled up with 11 mL of a solution of 0.5 M sucrose in buffer 30 mM MES, pH 6.5, and 15 mM *β*-DM, frozen at -20 ^{o}C overnight, and then slowly thawed at 4 ^{o}C. Around 1-mL of sample was loaded onto the sucrose gradient and run at 280,000 x g for 20 hours in a SW 40 Ti rotor (Beckman). The intense brownish band that appeared around the middle of the gradient tube was removed from the top with a flat-tip syringe, and the absolute absorption spectra (Beckman DU 600 UV-VIS spectrophotometer; 1-cm pathlength cuvette) without and with dithionite were taken in the 750-350 nm range. To measure precisely the chromophore content, 2 mM ferricyanide was added to a new sample from the sucrose gradient to completely oxidize the cytochromes, and the

spectrum was taken in the 750-500 nm range, which constituted the blank. Then a few grains of sodium ascorbate were added to the same cuvette and the ascorbate *minus* ferricyanide differential absorption spectrum was obtained, which displayed the absorption spectrum of the Cyt *f*. Finally, a few grains of sodium dithionite were added to the same cuvette and the dithionite *minus* ascorbate differential absorption spectrum was run, which depicted the absorption spectrum of the Cyt b_6. From those absorption spectra, the concentration of each chromophore was calculated using extinction coefficients of 25 mM^{-1}. cm^{-1} at 554 nm and 21 mM^{-1} . cm^{-1} at 563 (Daniulis et al. 2011), for Cyt *f* and Cyt b_6, respectively, and 75 mM^{-1}. cm^{-1} at 668 nm for Chl *a* (Pierre et al. 1997). The obtained bulk preparation was concentrated using an Amicon-30 filter tube, distributed in aliquots, frozen in liquid nitrogen, and then kept at -80 ^{o}C until use.

Preparation of highly pure dimers and monomers of the Cyt b_6f complex

An aliquot of 200-µL with 1700 µg or 700 µg of bulk Cyt b_6f were injected into a FPLC apparatus (ÄKTA, GE Healthcare) connected to a size-exclusion chromatography column (Superdex-200 HR 10/30) pre-equilibrated with 25 mM TRIS-HCl, pH 7.8, 0.02% (w/v) *β*-DM or 30 mM MES, pH 6.5, 0.12% (w/v) *β*-DM for dimers or monomers, respectively. Samples were eluted with their corresponding buffers at a flow rate of 0.25 mL/min collecting fractions of 0.25 mL, and the chromatogram elution profile was obtained by measuring the absorption at 280 nm. The absorption spectra of the fractions were obtained in the 750-350 and 750-500 nm ranges, as explained above. From these spectra the concentration of the three chomophores (Chl *a*, Cyt b_6, and Cyt *f*) was calculated using the corresponding extinction coefficients. The central fractions of the main band of each chromatogram were pooled, concentrated using an Amicon-30 filter, distributed into aliquots, frozen at liquid nitrogen, and then kept at -80 ^{o}C. The

molecular weight calibration curve was performed using blue dextran (2,000 kDa), ferritine (440 kDa), catalase (232 kDa), and bovine seroalbumin (67 kDa).

Protein analysis

The protein content of the different Cyt b_6f preparations was determined by SDS-PAGE using 18% (w/v) acrylamide in a Miniprotean cuvette (Bio-Rad) using TRIS/glycine running buffer system. Proteins were denatured at room temperature for 1 hour with a buffer containing 15.5 mM TRIS, pH 6.8, 2% (w/v) SDS, 2.5% (w/v) glycerol, 0.02% (w/v) bromophenol blue, and 1.25% (w/v) β-mercaptoethanol. The electrophoresis was run at a constant voltage of 120 V for 4 h. Proteins were revealed by Coomassie blue staining. About 10 µg of protein were loaded per lane.

Results and Discussion

The comparison between dimers and monomers of the Cyt b_6f complex is sometimes necessary to have better information on structure, function, stability, detergent and/or lipid binding, reconstitution experiments, protein and chromophore contents, specific function of each protein subunit and chromophore, and so forth. This is especially true for spectroscopic studies on the sole Chl *a* present per monomer since energy transfer may occur between chromophores from different monomer moieties and also with potential contaminant Chls. To that end, we have developed a simple, rapid and efficient method that can provide with highly pure dimers and monomers starting with bulk Cyt b_6f complex preparation obtained from spinach using a standard method as described above. Figure 1 shows a picture of the Cyt b_6f brownish band migrating around the middle of the gradient tube. β-DM and MES were substituted for OGP and TRIS/succinate in the gradient since the former is well known to be a suitable detergent to stabilize membrane proteins in solution, and because it was the detergent/buffer

system used in the size-exclusion chromatography to obtain monomers. The brownish band was carefully removed from the top with a flat-tip syringe. The absolute absorption spectrum of the dithionite reduced bulk Cyt b_6f complex showed the typical absorption bands corresponding to Chl *a*, Cyt b_6, Cyt *f* , and *β*-carotene at 668.5, 563, 553.5, and 485 nm, respectively (not shown). The calculated chromophore stoichiometry, was normally close to 1:2:1 for Cyt *f*, Cyt b_6 and Chl *a*, respectively, but sometimes Chl *a* content was somewhat higher, indicating the presence of some pigment contamination. Typically about 15 mg bulk Cyt b_6f were obtained from 400 g spinach leaves.

As explained in Materials and Methods, around 1700 µg or 700 µg of bulk Cyt b_6f were injected into the FPLC system provided with a size-exclusion chromatography column (Superdex-200) pre-equilibrated with a buffer containing 0.02% (w/v) or 0.12% (w/v) *β*-DM, respectively. Each sample was eluted with its corresponding buffer and the chromatogram profile monitored at 280 nm. In the first chromatographic conditions (low detergent content), a main fastest moving band , a small band to the right of the main band, and two other slower moving small bands were observed (Fig. 2A). The main band corresponded to a molecular mass of around 298,000 Da and the small band to the right of the main band to around 147,000 Da. Based on the molecular masses of the four big protein subunits, the four small protein subunits, the chromophores, the detergent micelles, and some remaining membrane lipids bound to the protein complex, the main band should correspond to dimers and the small band to the right of the main band to monomers of the Cyt b_6f complex. The two slower moving small bands can be due to potential contaminants, detergent micelles with embedded pigments, and some degradation product due to the buffer/detergent action. In contrast, in the second chromatographic conditions (higher detergent content), the chromatogram profile

showed a small faster moving band, which corresponded to dimers, a main band that corresponded to monomers, and two more slower moving bands, which can be due to some potential contaminants, detergent micelles with embedded pigments, and some degradation products due to buffer/detergent action (Fig. 2B). The absolute and differential redox absorption spectra were taken of each pigmented fraction as explained in Materials and Methods. The two first fractions of the main band (Fig. 2A) showed spectra with Chl *a* maximum between 670-672 nm and with minor absorptivity in the cytochrome region (not shown). The rest of the main band fractions displayed absorption maxima in the presence of dithionite at 668-668.5, 563, 553.5, 485, 430.5, and 422 nm, corresponding to Chl *a*, Cyt b_6, Cyt *f*, *β*-carotene, and the Soret bands of Cyt b_6 and Cyt *f*, respectively (Fig. 3). The inset shows the differential redox spectra of Cyt b_6 and Cyt *f* in the 600-500 nm range. The stoichiometry Cyt b_6:Chl *a* of the central fractions of the main band was always 2:1. Those fractions were pooled, concentrated, distributed into aliquots, frozen in liquid nitrogen, and then kept at -80 ^{o}C, and it constituted our highly pure dimers of the Cyt b_6f complex. The main band obtained with the second chromatographic conditions (Fig. 2B) showed an almost identical absorption spectrum with maxima at 668 (Chl *a*), 563 (Cyt b_6), and, 553.5 (Cyt *f*), 485 (*β*-carotene), 430.5 (Cyt b_6), and 422 (Cyt *f*) nm (not shown). The stoichiometry Cyt b_6:Chl *a* of the central fractions of the main band was always 2:1. These fractions, which constituted our highly pure monomers, were pooled, concentrated with an Amicon-30 filter, distributed into aliquots, frozen at liquid nitrogen, and then kept-80 ^{o}C. It can be concluded that due to a higher detergent/protein ratio (about 15 times) in the second chromatographic conditions, most dimers were monomerized but with no loss of pigments. It can also be noticed that with these chromatographic conditions we were able to eliminate Chl-binding contaminant entities. About 60% and 50% of the total

injected protein into the FPLC system were normally recovered as highly pure dimers and monomers, respectively.

Figure 4 displays the SDS-PAGE protein analysis using 18% (w/v) acrylamide of the different obtained preparations. Samples from the bulk Cyt b_6f complex and highly pure Cyt b_6f dimers obtained as above depicted four main stained bands, but the monomeric preparation lost a band at around 20 kDa. According to previous publications (Huang et al. 1994; Hauska 2004; Romanowska 2011), the four main stained bands corresponded to Cyt *f*, Cyt b_6, ISP, and Subunit IV from higher to lower molecular weights. Note that the small protein subunits present in the Cyt b_6f complex were not seen in these electrophoretic conditions, they were lost in the running cuvette solvent due to the long electrophoretic running time necessary to optimize the separation of Cyt b_6 and ISP subunits. It is clear that our monomers missed the IPS subunit but importantly it fully retained the pigment contents.

Acknowledgements

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Figure Captions

Figure 1. Photograph picture of the bulk Cyt b_6f complex brownish band obtained from spinach at around the middle of a sucrose gradient tube.

Figure 2. Size-exclusion chromatography to obtain highly pure dimers and monomers of the spinach Cyt b_6f complex. A, around 1700 µg of Cyt b_6f were injected into the 200-µL loop of the FPLC system, and the elution buffer contained 0.02% (w/v) β-DM/30 mM TRIS, pH 6.5; B, around 700 µg of Cyt b_6f were injected into the loop of the FPLC system, and the elution buffer contained 0.12% (w/v) β-DM/30 mM MES, pH 6.5. The chromatogram profiles represent the absorption at 280 nm *versus* the elution volume.

Figure 3. Absorption spectra of pure dimeric Cyt b_6f. The absolute absorption spectra were obtained in the presence of dithionite in the 750-350 nm range with main bands at 668.5 (Chl *a*), 563 (Cyt b_6), 553.5 (Cyt *f*), 483 (β-carotene), 430.5 (Soret band of Cyt b_6), and 422 (Soret band of Cyt *f*). Inset: Ascorbate *minus* ferricynide differential absorption spectrum corresponding to Cyt *f* (full line); dithionite *minus* ascorbate differential absorption spectrum corresponding to Cyt b_6 (dashed line).

Figure 4. SDS-PAGE protein analysis in 18% (w/v) acrylamide of the different preparations of Cyt b_6f obtained from spinach. Lane 1, bulk Cyt b_6f as obtained from the sucrose gradient; lane 2, dimers obtained after the size-exclusion chromatography; lane 3, monomers obtained after the size-exclusion chromatography; lane MW, molecular weight markers. About 10 µg protein were loaded per lane. The four main protein subunits are described on the left side and the MW markers in kDa on the right side of

the panel. The minor contaminants corresponding to the higher MW bands are of unknown origin, and are partially eliminated with the chromatography. Such contaminants could be in part responsible for the slower moving bands shown in the chromatograms (Fig. 2).

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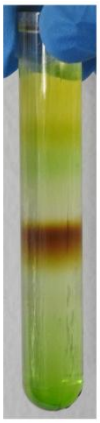

Figure 1

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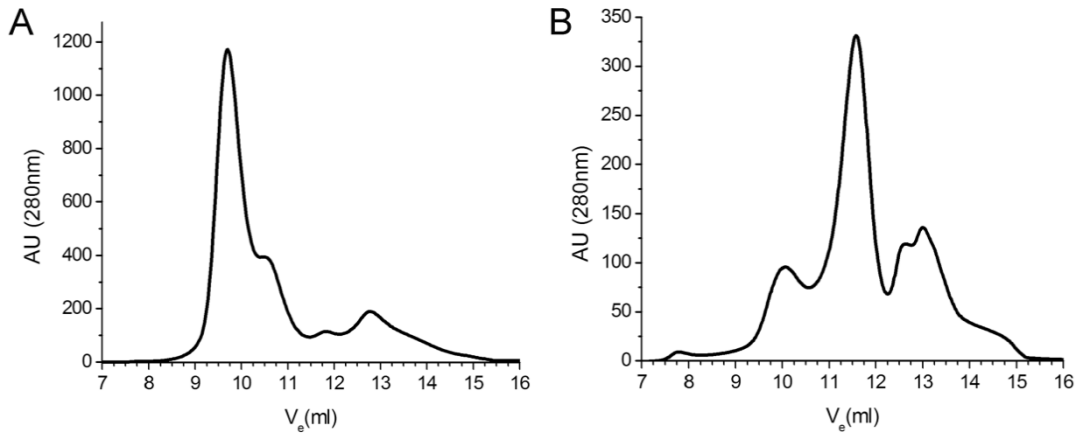

Figure 2

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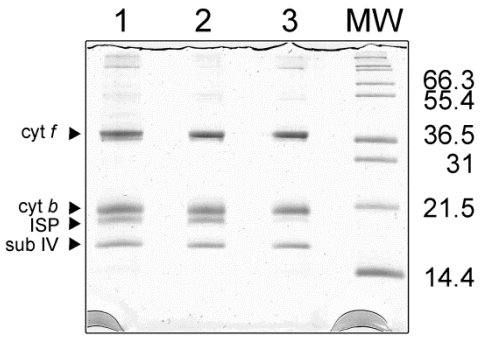

Figure 4