Journal of Chromatography, 512 (1990) 219–226
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 1812

Purification of membrane protein complexes isolated from a cyanobacterial thylakoid membrane by high-performance liquid chromatography

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ABSTRACT

Reaction centers of photosystem I, photosystem II and a genetically altered photosystem II and the cytochrome b_6f complex have been isolated from the thylakoid membrane of the cyanobacterium *Synechocystis* PCC 6803 and purified by two high-performance liquid chromatography steps. For comparison, both steps (*i.e.* an anion-exchange and a hydroxyapatite column chromatography) have been performed on different columns, including 5-μm-particle-size material. Protein complexes purified according to the optimized high-performance liquid chromatography procedure retained their biological activity and were characterized for homogeneity and apparent molecular mass by gel high-performance chromatography.

INTRODUCTION

The photosynthetic electron transport chain, located in the thylakoid membrane of cyanobacteria, algae and higher plants, consists of three major membrane-spanning protein complexes —the photosystem I reaction center (PS I RC), the photosystem II reaction center (PS II RC) and the cytochrome b_6f complex— which are interconnected by mobile electron carriers. The overall process finally leads to the oxidation of water at the PS II RC and the reduction of $NADP^+$ at the ferredoxin/NADP oxidoreductase. For further elucidation of the complicated structure–function relationships, these three membrane-spanning multisubunit complexes are needed in an isolated and highly purified form. Purification of these complexes from wild-type and specific mutants facilitates their characterization by spectroscopic methods, and should finally lead to a complete resolution of the tertiary structure by crystallization and subsequent X-ray diffraction. In addition to the need for a pure and functionally active starting material, crystallization benefits from a strict homogeneity in the size of the complexes, since smaller-sized particles are easier to analyze than large aggregates.

Until now, few attempts have been made to isolate and characterize PS I and PS

0021-9673/90/$03.50

II RC by high-performance liquid chromatography (HPLC)[1–5], and no HPLC procedure has been published for the purification of the b_6f complex. In this report, a two-step HPLC procedure for the purification of all three components from the cyanobacterium *Synechocystis* PCC 6803 is described. As a demonstration of the versatility of this separation method, the purification of a modified PS II RC present in low amount in the membrane, will also be described.

EXPERIMENTAL

Growing of cells and extraction of isolated membranes

Cells from a glucose-tolerant and phycocyanin-deficient strain of the cyanobacterium *Synechocystis* PCC 6803 (characterized in ref. 6) were grown photo-autotrophically according to ref. 7. In addition, cells from a mutant with a deleted *psb*C gene, which codes for the chlorophyll-binding 43 000-dalton protein (CP-43 subunit) of the PS II RC (a kind gift of D.A. Chisholm), were grown photoheterotrophically in the presence of 5 m*M* glucose. Membranes from both strains were isolated as reported in ref. 6, extracted by 1% dodecyl β-D-maltoside (β-DM), loaded on a sucrose density gradient and centrifuged overnight (150 000 *g*, 16 h 4°C) in the presence of 0.04% β-DM. Out of three bands, the middle (green) band was collected and diluted with buffer A (20 m*M* 4-morpholineethanesulfonic acid (MES) (pH 6.5)–10 m*M* $CaCl_2$–10 m*M* $MgCl_2$–0.5 *M* mannitol–0.03% β-DM) to reduce the residual sucrose concentration to less than 100 m*M*. After concentration to less than 50 ml in a stirred cell (Amicon 8400, equipped with a YM 100 membrane, molecular mass cut-off 100 000 dalton; W. R. Grace & Co.), up to 1 ml was injected for analytical HPLC runs. For (semi-)preparative runs, the sample was applied via a 50 ml superloop (Pharmacia-LKB), which was kept in the oven compartment of the chromatograph.

Apparatus and columns

All HPLC steps were performed on a Hewlett-Packard 1090 M liquid chromatograph, equipped with a diode-array detector and an oven compartment (connected to an external thermostat). Samples were collected in a Gilson FC 203 fraction collector connected to a thermostat (LKB).

For anion-exchange chromatography the following columns were used: TSK DEAE-5PW (75 × 8 mm I.D.; TosoHaas, U.S.A.), Mono Q HR 5/5 (50 × 5 mm I.D.) and HR 10/10 (100 × 10 mm I.D.; Pharmacia-LKB, Sweden), and a LiChrospher 1000 TMAE glass cartridge (50 × 10 mm I.D.; Merck, F.R.G.). Hydroxyapatite HPLC was performed on a MAPS HPHT analytical cartridge (30 × 4.6 mm I.D.; Bio-Rad, U.S.A.), a HAP5-50 column (50 × 7.5 mm I.D.; Toa Nenryo Kogyo K.K., Japan) and a MHAP5-10 (100 × 21 mm I.D.; Toa Nenryo Kogyo K.K., Japan/SynChrom, U.S.A.). Size-exclusion HPLC was performed on a TSK 4000 SW-column (300 × 7.5 mm I.D.; Toyo Soda, Japan); column calibration was carried out, using common standard calibration proteins from Pharmacia. Buffer exchange was achieved via gel chromatography on Econo-Pac 10DG disposable desalting columns (Bio-Rad).

RESULTS

Restrictions on the conditions for separation of the PS I RC, PS II RC and cytochrome b_6f complexes by HPLC

Conditions for separating PS II RC from the other two complexes by anion-exchange chromatography are flawed by the lability of the PS II RC under both acidic and alkaline conditions and the loss of subunits from PS II RC at high-salt concentrations and at elevated temperature. Furthermore, during all steps of purification, detergent at 3–5 times the micellar concentration and an appreciable amount of sugar (0.5–1.0 *M*) must be present to prevent (irreversible) aggregation of these hydrophobic complexes. β-DM proved to be the mildest detergent for the preservation of the activity of both the isolated PS I and PS II RC, and mannitol, having lower viscosity than sucrose at the same concentration, the best-suited sugar for HPLC.

All separations were performed at 10°C, a reasonable compromise between an increase in column backpressure at lower temperatures, and an increase stability of

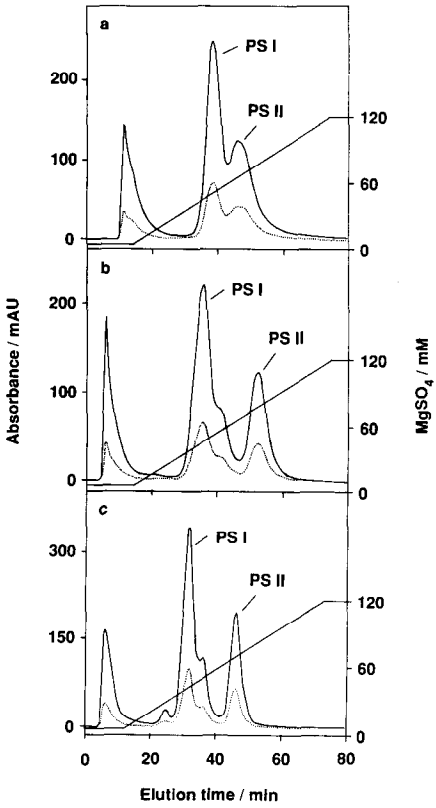

Fig. 1. Chromatogram of an extract of *S.* 6803, pre-purified by sucrose-density-gradient centrifugation, on different HPLC anion-exchange columns: (a) TSK DEAE-5PW column, (b) Mono Q HR 5/5 column and (c) LiChrospher 1000 TMAE glass cartridge. Buffer 20 m*M* MES (pH 6.5)–10 m*M* $MgCl_2$–10 m*M* $CaCl_2$–0.5 *M* mannitol–0.03% (w/v) β-DM; gradient of 5 to 120 m*M* $MgSO_4$; flow-rate 0.4 ml/min (a + b) and 0.8 ml/min (c). Absorbance was recorded at 280 nm (dotted line) and 435 nm (solid line).

the proteins at lower temperatures. Immediately after separation, all fractions were kept at 4°C. Instability at higher salt concentrations, especially of PS II RC, was reduced by desalting the respective protein fractions by gel chromatography and concentrating all samples immediately after separation.

Separation of PS I and PS II RC by anion-exchange HPLC

Three anion-exchange columns were tested for their ability to separate PS I from PS II RC under the conditions described above. Fig. 1 shows elution profiles of the membrane extract (prepurified on a sucrose density gradient) obtained with a linear gradient of $MgSO_4$. All columns show the same sequence of elution of three main components —free pigment (carotene/chlorophyll), the PS I RC, and the PS II RC. The TSK DEAE-5PW column (Fig. 1a) showed a poorer separation of PS I and PS II RC than the Mono Q HR 5/5 column (Fig. 1b). Although run at a higher flow-rate (due to the larger bed volume) and with a larger amount of sample, the LiChrospher TMAE-column (Fig. 1c) yielded the best resolution. Both Mono Q and the LiChrospher column show a minor peak of PS I RC, following the main peak; this peak, containing PS I RC, mixed with a contaminating protein (data not shown), was discarded.

It should be mentioned, that a gradient of monovalent (*i.e.* NaCl) or trivalent (*i.e.* sodium phosphate) ions yielded a similar elution pattern to the $MgSO_4$ gradient on the Mono Q column.

This step was scaled up on a Mono Q HR 10/10 column, applying up to *ca.* 100 mg protein in a volume of up to 50 ml by a "superloop". Additionally, instead of a linear gradient, a multi-stage gradient was applied, yielding a better separation of the PS I and PS II RC peaks.

It should also be mentioned that, relative to the amount of PS I RC, the preparation shown in Fig. 1 (from the phycocyanin-deficient mutant) contains more than double the amount of PS II RC found in preparations from wild-type cells and considerably more PS II RC than most of the engineered mutants with defects in PS II function. This fact stresses the importance of this first HPLC purification step and the requirement of quantitative separation of PS I and PS II RC prior to spectroscopic characterization, as the PS I signal may mask the PS II signal. As an example, Fig. 2 shows a separation of PS I and PS II RC from a mutant in which the gene for one

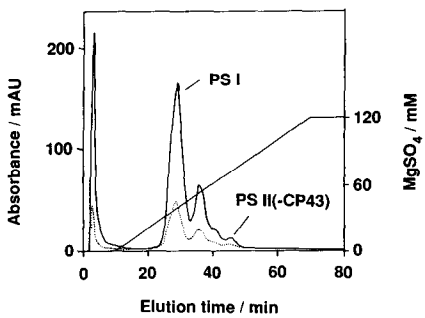

Fig. 2. Chromatogram of an extract from a *S.* 6803 mutant, lacking the CP43 subunit in the PS II RC, from a Mono Q HR 5/5 column; conditions as for Fig. 1b. Absorbance was recorded at 280 nm (dotted line) and 435 nm (solid line).

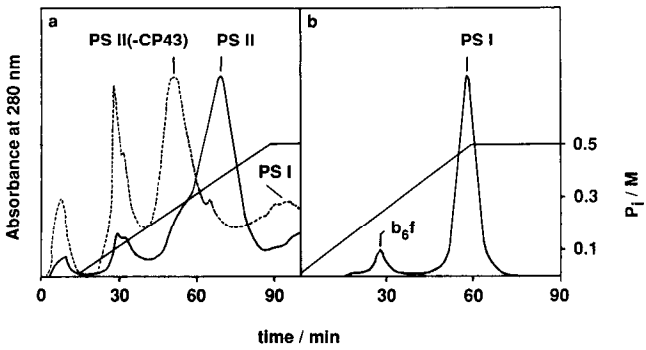

Fig. 3. Chromatogram of PS II(−CP43)RC and PS II RC (a), and for PS I RC and cytochrome b_6f complex (b) from an analytical HPLC hydroxyapatite column (HAP5-50); starting buffer 10 m*M* sodium phosphate (pH 6.8)–10 m*M* $MgCl_2$–13 μM $CaCl_2$–0.5 *M* mannitol–0.03% (w/v) β-DM; gradient up to 0.5 *M* sodium phosphate; flow-rate 0.4 ml/min.

subunit of the PS II RC, the *psb*C gene, has been deleted. Conditions of the separation are identical to those of Fig. 1b. This gene deletion leads to a severely decreased amount of PS II RC in the thylakoid membrane of this mutant and the loss of photo-autotrophic growth. Although the amount of PS II(−CP43)RC extracted from the thylakoid membrane is only about 1% of the amount of extracted PS I RC (on a chlorophyll basis), a higher purity of this impaired PS II RC is still possible by this first HPLC step. However, analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) still revealed the presence of several contaminants among the subunits of the PS I and PS II RCs. To achieve extremely pure preparations, a second HPLC step with different elution characteristics is necessary.

Further purification of the complexes by hydroxyapatite HPLC

Prior to the hydroxyapatite step, the PS I and PS II RC peak fractions of the anion-exchange step were passed twice through an Econo-Pak gel column. With this step, the $MgSO_4$ and Ca^{2+} concentrations were reduced to < 10 m*M* and 13 μM, respectively, and MES buffer was exchanged for sodium phosphate buffer (see Experimental). The low calcium concentration turned out to be necessary for the prevention of frequent high-pressure-instrument shutdowns, caused by the formation of salt crystals.

Due to the different sequence of elution from a new ceramic spherical hydroxyapatite column, HAP5-50, the PS II RC could be further purified; the main contaminants were an unpigmented protein, eluted at lower phosphate concentrations than PS II RC (presumably ATPase), and residual PS I RC (see Fig. 3a). Similarly, PS II(−CP43)RC, eluted at somewhat lower phosphate concentrations than PS II RC, could be further purified from these two contaminants.

Further purification of PS I RC on this column (Fig. 3b) by applying a linear gradient of 10 to 500 m*M* sodium phosphate revealed the presence of another protein, which could be identified as the b_6f complex (see below). Repeating the anion-exchange chromatography step on this purified complex showed that it was eluted at the same $MgSO_4$ concentration as the PS I RC (data not shown).

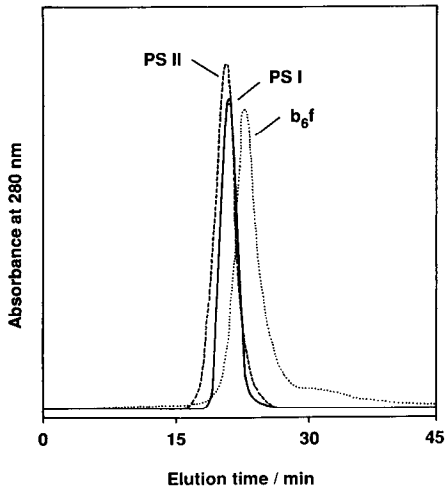

Fig. 4. HPLC gel chromatography of purified PS I RC, PS II RC and cytochrome b_6f complex on a TSK 4000 SW column; buffer 20 m*M* MES (pH 6.5)–10 m*M* $MgCl_2$–30 m*M* $CaCl_2$–0.5 *M* mannitol–0.03% (w/v) β-DM; flow-rate 0.5 ml/min.

The hydroxyapatite purification step could be scaled up by using a larger column of the same type, *i.e.* MHAP5-10. This column was observed to exhibit stable operation at reasonable flow-rate (0.8–1.6 ml/min). In contrast, the hydroxyapatite cartridge, MAPS HPHT, showed frequent pressure fluctuations, and the increase in backpressure with increasing analysis time was much greater than with the ceramic spherical type of HAP5-50 and MHAP5-10. The elution profiles were similar for all three columns.

Characterization of the isolated protein complexes

The homogeneity and molecular mass of the isolated protein complexes was determined by gel chromatography HPLC. The elution profiles of the PS I RC, PS II RC, and the b_6f complex from a TSK 4000 SW column, shown in Fig. 4, yielded apparent molecular masses (including the detergent shell) of $(300 \pm 20) \cdot 10^3$ dalton, $(310 \pm 20) \cdot 10^3$ dalton and $(180 \pm 20) \cdot 10^3$ dalton, respectively. The PS II(−CP43)RC from the *psb*C deletion mutant showed a molecular mass slightly lower than that of the PS I RC. The contribution of the detergent shell in all three cases is *ca.* 50 000 dalton, as estimated from electron micrographs of a similar PS II RC (ref. 3). By SDS-PAGE and immunoblotting, the PS I RC was determined to contain 9 protein subunits (*ca.* 60, *ca.* 60, 18.5, 18.5, 16, 15, 10.5, 9.5 and $6.5 \cdot 10^3$ dalton), the PS II RC 5 subunits (43, 37, 33, 29 and $10–11 \cdot 10^3$ dalton) and the b_6f complex 4 subunits (38, 24, 19 and $15 \cdot 10^3$ dalton); subunits below 5 000 dalton may not have been resolved in these gels[6]. Analysis by SDS-PAGE and immunoblotting also confirmed that the CP43-apoprotein was absent from the PS II RC isolated from the deletion strain[8]. In addition, determination of the chlorophyll-per-reaction-center ratio yielded values of 70 Chl/PS I, 40 Chl/PS II, and 33 Chl/PS II(−CP43), based on light-flash-induced-charge separation. These values are among the lowest reported for these isolated complexes. A more detailed biophysical and biochemical characterization of these complexes, including a detailed subunit analysis, will be given elsewhere[6,8].

DISCUSSION

HPLC purification of comparatively labile hydrophobic protein complexes imposes severe operating conditions on columns, if the protein is to be kept in its native state, *i.e.* retain its biological activity. The presence of detergent, high sugar and high salt concentrations contribute to increasing column backpressure, as does the lower temperature ($< 10°C$) at which chromatography must be performed. Therefore, it is worth mentioning that all the columns tested here are usable under these conditions; biological activity of the isolated membrane proteins is retained.

However, comparing the resolution of separation and the stability of experimental conditions major differences between the columns tested became obvious. Within the group of anion-exchange columns, the LiChrospher 1000 TMAE yielded the best separation of PS I and PS II RC and removed some unwanted proteins. Several effects may contribute to the superior resolution of this recently developed column in comparison to the TSK DEAE-5PW and the Mono Q columns:

(1) The ion-exchange groups of the LiChrospher column are not bound to the matrix via short spacer groups, but sit on "tentacle"-like polymer chains, which are claimed to move freely. This should minimize non-specific interactions between the protein and the matrix and avoid irreversible deformations, which may occur if the ionic groups of the ion exchanger were rigidly fixed on the surface.

(2) The support material of hydrophilic silica beads carries untreated silanol groups on their surface, which may cause a difference in the interaction with the proteins.

(3) The smaller size of the particles, 5 μm, may also lead to a much higher mass transfer rate (greater peak sharpness) than with the other ion exchangers, which contain 10-μm particles. This is especially decisive for separating larger macromolecules, which have poorer diffusion.

On the other hand, differences in the chromatograms between the TSK DEAE-5PW and the Mono Q column may be due to different matrices and/or different charge densities of the anion-exchange groups. Considering that a separation of PS I and PS II RC isolated from the same organism was not achieved by conventional anion-exchange chromatography on a TSK DEAE-Toyopearl 650S column[9], the use of HPLC for this first step constitutes a considerable progress in the purification of these two photosystem core complexes.

As in the first HPLC step, the hydroxyapatite step was improved by the use of the 5μm spherical particles of the HAP5-50 column[10] rather than the larger, irregularly shaped particles of the MAPS HPHT cartridge: Besides better resolution, the column used exhibited much more stable chromatographic conditions and lower back-pressure, even after scaling up by a factor of more than 10.

This second HPLC step is very important for obtaining PS I and PS II RCs free of any contaminating proteins. In the case of the PS I RC, the b_6f complex is the only "contaminant" in the main peak of the Mono Q column. As both protein complexes are eluted from the anion-exchange column together, hydroxyapatite chromatography is required for their separation. Much higher yields of the cytochrome b_6f complex can be obtained, if instead of the middle (green) band, the upper yellow-brown band of the sucrose density gradient is taken as the starting material (data not shown). The molecular mass determined by gel HPLC suggests that this is the first

reported isolation of a monomeric cytochrome b_6f complex. The benefit of this additional purification for PS I RC is reflected by the fact that preliminary crystallization attempts using this monomeric particle have been successful[11].

In addition, this hydroxyapatite chromatography is especially valuable for further purifying PS II RC from mutants with low levels of PS II RC in site-directed mutations. In the case of the CP43-less mutant shown above, the amount of extracted PS II(−CP43)RC is only 1% of that of PS I RC (on a chlorophyll basis). The fact that PS II RC is eluted before PS I RC from the hydroxyapatite column (in contrast to the first HPLC step) enables purification of a PS II(−CP43)RC free of PS I RC contamination, despite its low concentration in this mutant. High purity was a prerequisite for absorbance change measurements, which showed that this "minimal core complex" was still capable of a stable charge separation[8].

It should be mentioned that this HPLC method, for the first time, enables the characterization of isolated complexes from genetically engineered PS II RCs. For example, it was shown recently by electron paramagnetic resonance and optical spectroscopy of PS II RCs isolated according to this method that a mutant in which tyrosine 161 of the D1 polypeptide was replaced by phenylalanine had lost the PS II secondary electron donor, Z (ref. 12).

ACKNOWLEDGEMENTS

I am grateful to Bruce A. Diner, Peter J. Nixon, Jim G. Metz, J. Jack Kirkland and Barry E. Boyes for stimulating discussions.

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