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Biochimica et Biophysica Acta 1506 (2001) 55–66

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Isolation of membrane protein subunits in their native state: evidence for selective binding of chlorophyll and carotenoid to the b_6 subunit of the cytochrome b_6f complex

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Received 9 October 2000; received in revised form 18 April 2001; accepted 23 April 2001

Abstract

Cytochrome (cyt) *b–c* complexes play a central role in electron transfer chains and are almost ubiquitous in nature. Although similar in their basic structure and function, the cyt b_6f complex of photosynthetic membranes and its counterpart, the mitochondrial cyt bc_1 complex, show some characteristic differences which cannot be explained by the high resolution structure of the cyt bc_1 complex alone. Especially the presence of a chlorophyll molecule is a striking feature of all cyt b_6f complex preparations described so far, imposing questions as to its structural and functional role. To allow a more detailed characterization, we here report the preparation of native subunits cyt b_6 and IV starting from a monomeric cyanobacterial cyt b_6f complex. Spectroscopical and reversed-phase HPLC analyses of the purified cyt b_6 subunit showed that it contained not only two *b*-type hemes, but also one chlorophyll *a* molecule and a cyanobacterial carotenoid, echinenone. Evidence for selective binding of both pigments to this subunit is presented and their putative function is discussed. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Cytochrome b_6 subunit; Chlorophyll; Echinenone; High performance liquid chromatography; *Synechocystis* 6803

1. Introduction

The cytochrome (cyt) b_6f complex functions as a plastoquinol-plastocyanin oxidoreductase in photosynthetic membranes of higher plants, green algae and cyanobacteria [1]. By transferring electrons, it builds up a proton gradient across the membrane which is finally used for the generation of ATP. In cyanobacteria, the cyt b_6f complex plays this central role both in photosynthetic and respiratory electron transport chains and is therefore indispensable for growth.

The complex consists of four large (PetA–D) and four small subunits (PetG, L, M, N), of which one

Abbreviations: CMC, critical micellar concentration; ferricyanide, potassium hexacyanoferrate(III); Hecameg, 6-*O*-(*N*-heptylcarbamoyl)-α-D-glucopyranoside; HPLC, high performance liquid chromatography; LH, (bacterial) light harvesting complex; LHC2, (plant) light harvesting complex 2; Mega 9, nonanoyl-*N*-methylglucamide; MES, 4-morpholinoethanesulfonic acid; OGP, *n*-octyl-β-D-glucopyranoside; OGTP, *n*-octyl-β-D-glucothiopyranoside; PAGE, polyacrylamide gel electrophoresis; PIC A, tetrabutylammonium phosphate; RT, room temperature; SB12, sulfobetaine 12 (=zwittergent 3-12=*n*-dodecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate); SDS, sodium dodecyl sulfate

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PII: S0005-2728(01)00184-0

(PetL) is absent in cyanobacteria. Recently, an additional subunit (PetO or subunit V) was shown to be part of the cyt b_6f complex in *Chlamydomonas*. However, although this protein seems to be present in higher plants, a homologue of the corresponding gene could not be found in *Synechocystis* [2]. A cyt b_6f monomer contains one copy of each subunit. The cyt b_6 subunit, which binds two *b*-type hemes, and subunit IV together form the binding sites for oxidized and reduced quinol. Quinol oxidation is linked to the release of protons into the thylakoid lumen. According to the widely accepted Q-cycle model, transfer of the two electrons is obligatorily branched between the low potential heme b_L on the cyt b_6 subunit and the Rieske iron–sulfur protein (ISP). Heme b_L transfers an electron to the high potential heme b_H on the opposite side of the membrane, which in turn reduces oxidized quinone, thus completing the cycle. The reduction of quinone on the stromal side of the membrane is coupled with the uptake of two electrons resulting in an overall H^+/e^- ratio of 2:1 for the whole cycle. The Rieske ISP carries a [2Fe2S] cluster and transfers one electron to cyt *f*, a polypeptide carrying a *c*-type cytochrome, which then reduces plastocyanin.

Presently, structural information on the cyt b_6f complex is only available to a resolution of about 8 Å [3,4]. However, the soluble domains of cyt *f* and the ISP have been crystallized yielding structures with ≤ 2 Å resolution [5,6]. Also, the structure of the mitochondrial cyt bc_1 complex has been resolved to ≤ 3 Å [7,8]. These cyt bc_1 complexes are central components of electron transport chains in mitochondria and photosynthetic bacteria and show similarity in structure and function to their cyt b_6f counterparts, but differ in detail. One major difference is the presence of a single chlorophyll molecule in cyt b_6f complexes which was first reported for a cyanobacterial complex [9] and later on confirmed also for cyt b_6f complexes from green algae and higher plants [10,11]. It is very unlikely that this chlorophyll (Chl) molecule is a contamination, as the chloroplast cyt b_6f complex shows very low exchange rates with radiolabeled Chl [10]. Also, the fixed stoichiometry of one Chl per monomer and the defined orientation of the pigment within the complex shown by linear dichroism spectroscopy [9,12,33] confirm a specific binding.

As to the localization of this pigment, a dimeric cyt b_6f preparation of *Chlamydomonas reinhardtii* was shown to lose its chlorophyll molecule upon monomerization [13], suggesting a binding site at the interface connecting the monomers. In contrast, a monomeric cyt b_6f complex isolated from *Synechocystis* reproducibly showed one chlorophyll per monomer which in no case was lost during the isolation procedure [14]. Although this organism was reported to contain dimeric b_6f complexes, too [15], they seem to be very labile and easily dissociate into monomers during isolation, apparently without any detectable loss of the chlorophyll molecule.

Besides Chl, carotenoids have also been reported in cyt b_6f complexes [14,16]. While most of them seem to contain β-carotene, *Synechocystis* seems to bind the rare carotenoid echinenone in substoichiometric amounts [14]. In order to elucidate further the localization and function of these pigments – especially by spectroscopy – the isolation of individual cyt b_6f subunits in sufficient amounts without losing their native structure would be very helpful.

In this report, we present a gentle method for the separation of the cyt b_6f complex into its subunits while preserving their native state. This method allowed especially the isolation, purification, and characterization of the cyt b_6 subunit which was found to exclusively bind both pigments, i.e. the chlorophyll and the echinenone. Beyond this application on the cyt b_6f complex, the procedure presented here may also be useful for the investigation of hydrophobic subunits of other intrinsic membrane protein complexes.

2. Materials and methods

2.1. Preparation of isolated b_6f subunits

Cyt b_6f complex was isolated from a PS1-minus *Synechocystis* mutant strain (obtained from Dr. W. Vermaas) as described in [12] and incubated in 20 mM MES–NaOH, pH 6.5, 0.5% (w/v) zwittergent sulfobetaine 12 (SB12; Calbiochem) for 30 min at 30°C under dim light. Samples were diluted 4-fold with low salt HPLC buffer (20 mM MES, pH 6.5, 0.025% *n*-dodecyl-β,D-maltoside (β-DM) and protease inhibitors: 10 μM tosyllysylchloromethylketone

(TLCK), 100 μM phenylmethylsulfonyl fluoride (PMSF)) and loaded on an anion exchange chromatography column (Bio-Rad Q-1). This column proved to be superior in comparison with the following columns which have also been tested: sulfopropyl-Sepharose (Pharmacia), ethyl (POROS ET, PE Biosystems), HA/P hydroxyapatite (Bio-Rad) and quaternary amine (POROS HQ/M, PE Biosystems). Proteins were eluted with a two-step linear NaCl gradient (0→100 mM, 3.5 min and 100 mM→1 M NaCl, 9.5 min) at a flow rate of 3 ml/min (=1.9 column volume/min). UV/VIS spectra of the fractions were recorded online by the Photodiode Array Detector 996 (Waters, USA).

2.2. Optimization of pH

To check the influence of high pH values on the cyt b_6f preparation, samples were incubated at pH 6.5, 10.5 or 12 for 30 min on ice and analyzed on native gels with the pH 6.5 sample as control. Standard buffer (buffer A) composition was: 20 mM MES–NaOH, pH 6.5, 10 mM $MgCl_2$, 10 mM $CaCl_2$, 10 mM $MgSO_4$ and 0.03% β-DM; addition of 0.75 vol 200 mM 3-(cyclohexylamino)propanesulfonic acid, 10 mM $MgCl_2$, 10 mM $CaCl_2$ and 0.03% β-DM raised the pH to 10.5–10.7, and addition of 0.1 vol. 1 M NaOH raised the pH to about 12. Samples were incubated on ice for 30 min and analyzed by Deriphat–PAGE.

2.3. Detergent screening

Various detergents have been tested for their ability to dissociate a cyt b_6f preparation into their (native) subunits. They have been purchased from the following suppliers: Biomol (β-dodecyl maltoside, *n*-octyl-β-D-glucopyranoside (OGP)), Sigma (Triton X-100, Na-cholate), Calbiochem (SB12, nonyl glucoside, nonanoyl-*N*-methylglucamide (Mega 9), *n*-octyl-β-D-glucothiopyranoside (OGTP)) and Vegatec (6-*O*-(*N*-heptylcarbamoyl)-α-D-glucopyranoside (Hecameg)).

Stock solutions of the respective detergents were prepared in 20 mM MES–NaOH, pH 6.5, 10 mM $MgCl_2$ and 10 mM $CaCl_2$. Respective aliquots were added to the samples (dissolved in buffer A) up to the desired concentration. Incubations were done at 0°C, room temperature (RT) and 30°C for 15–60 min, followed by analysis of the samples by Deriphat–PAGE.

2.4. SDS–PAGE and immunoreactions

SDS–PAGE analyses of the fractions were performed according to [17] on a 16% acrylamide gel. The resulting bands were visualized by silver staining according to [18]. Identification of the bands was achieved by blotting onto polyvinylidene fluoride membranes (Millipore) according to [19] followed by immunoreaction with PetA, B, C and D antibodies. The following antibodies were used: anti-PetA, raised against *C. reinhardtii* protein, dilution 1/2000 (kind gift of Dr. C. de Vitry, Paris), anti-PetB, raised against *Arabidopsis thaliana* protein, dilution 1/1000 (kind gift of Dr. K. Meyerhoff, Düsseldorf), anti-PetC and anti-PetD, raised against spinach proteins, dilutions 1/3000 and 1/500, respectively (kind gifts of Dr. R. Berzborn, Bochum).

2.5. Deriphat–PAGE

The gentle detergent Deriphat-160 has been widely used for the separation of pigment-binding proteins in polyacrylamide gels [20]. This gel system was therefore often referred to as 'native gel' or 'green gel'. In this work, Deriphat-based electrophoresis was used for the estimation of the degree of solubilization of cyt b_6f subunits by various detergents in different concentrations. The stacking gel contained 3% acrylamide/bisacrylamide (3% T, 3% C), the separating gel 8% acrylamide/bisacrylamide. The gel buffer was identical for both gels, containing 25 mM Tris–HCl/50 mM glycine, pH 8.5 and 0.125% (v/v) Deriphat (Henkel). The running buffer consisted of 12.5 mM Tris–HCl/100 mM glycine pH 8.5. For the cathode buffer, 0.2% (v/v) Deriphat was added. The samples were diluted 1/1 with 25 mM Tris–HCl/50 mM glycine pH 8.5 and 30% (v/v) glycerol and applied directly to the gel. Electrophoresis was performed at 10°C and at a constant voltage of 80–100 V for 2–3 h.

2.6. Heme staining

Bands in Deriphat gels were visualized by heme

staining. The gels were incubated with 1.9 mM 3,3′,5,5′-tetramethylbenzidine hydrochloride (TMBZ, Sigma) in 30% (v/v) methanol and 175 mM sodium acetate, pH 5.0 for 1 h in the dark. Addition of 300 μl 35% (v/v) hydrogen peroxide resulted in a blue precipitate due to the reaction of the hemes with TMBZ. Prior to subsequent silver staining, the gel was destained by incubation with solution 1 (175 mM sodium acetate, pH 5.0, 30% (v/v) isopropanol and 70 mM Na_2SO_3) for 2×15 min at RT, followed by washing for 2×15 min at RT with solution 2 (175 mM sodium acetate, pH 5.0, 30% (v/v) isopropanol).

2.7. Two-dimensional electrophoresis

For characterization of the bands obtained by native PAGE, lanes were cut out prior to staining and a subsequent SDS–PAGE was performed. In order to denature and mobilize the proteins in the gel slices, they were incubated for 20 min at RT under gentle shaking in freshly prepared 'solubilization buffer' (2.5% (w/v) SDS, 7.5% (v/v) glycerol, 25 mM Tris–HCl, pH 6.8 and 1% (v/v) β-mercaptoethanol). The gel slices were then placed horizontally onto a Laemmli SDS gel and the electrophoresis was performed as described above.

2.8. Quantification of cytochromes

Cytochrome content was determined from difference redox spectra of the HPLC fractions in 20 mM MES–NaOH, pH 6.5, 0.025% β-dodecyl maltoside and different concentrations of NaCl according to the eluting fraction. Spectra were recorded on a Beckman DU 7400 spectrophotometer with a measuring beam half-bandwidth of 1.2 nm. Samples were oxidized with 1 mM potassium hexacyanoferrate(III) (ferricyanide) and reduced stepwise by addition of 20 mM sodium ascorbate (cyt *f*) and saturating amounts of solid sodium dithionite (cyt b_6). Heme quantification was done according to [21] with extinction coefficients of 18 mM^{-1} cm^{-1} for cyt *f* at 556 nm and 14 mM^{-1} cm^{-1} for cyt b_6 at 563 nm, relative to the isosbestic wavelength at 540 nm.

2.9. Protein intrinsic fluorescence

Fluorescence emission spectra of the aromatic amino acid tryptophan are highly dependent on their environment, showing a strong blue shift in a hydrophobic environment. As the cyt b_6 subunit contains seven tryptophan residues, intrinsic Trp fluorescence was used to probe for native or denatured structure of the isolated cyt b_6 polypeptide. Samples were excited at 293 nm, and fluorescence emission spectra were recorded between 305 and 405 nm on an Aminco Bowman 2 luminescence spectrometer (SLM, USA).

2.10. Pigment identification and quantification

Pigments were extracted from untreated cyt b_6f preparations and the various HPLC fractions by addition of ice-cold acetone to a final concentration of 90% (v/v) and intensive vortexing. All steps were carried out under dim light. The solubilized pigments were separated from denatured protein by centrifugation (10 min, 12 000×*g*) followed by filtering through a polyamide membrane (0.45 μM diameter, Spartan, Schleicher&Schuell). Routinely, a second extraction step was done to check for complete extraction.

Reversed-phase analysis of pigments was performed on a Spherisorb ODS 2 column (CROM) according to [14]. At a flow rate of 0.6 ml/min, the fractions were eluted with a stepwise gradient of buffer 1 (38.5% (v/v) acetone, 46.5% (v/v) methanol, 5% (v/v) H_2O, 10% (v/v) 5 mM tetrabutylammonium phosphate buffer (PIC A; Waters)) and buffer 2 (100% ethyl acetate). Eluted peaks were recorded online by the Photodiode Array Detector 996 (Waters). The pigments were identified by comparison with calibrated standards and published spectra [22]. Relevant peak areas were quantified by integration.

3. Results

3.1. 'Native' separation of the cyt b_6f complex subunits

The general aim of these experiments was the development of strategies for the isolation and purification of cyt b_6f subunits in their native state, in order to be able to characterize in detail their associated cofactors and pigments. For this purpose, a

well characterized monomeric cyt b_6f complex of the cyanobacterium *Synechocystis* sp. 6803 containing one chlorophyll *a* molecule and 0.7 molecules echinenone per cyt *f* [14] was used as starting material.

In contrast to whole thylakoid membranes from spinach, where the separation of cyt b_6f subunits from the membrane-embedded complex had been observed at high pH values [23], the isolated cyanobacterial cyt b_6f complex did not show any dissociation when incubated at pH 10.5 or pH 12 for 30 min on ice as judged by native gel electrophoresis analysis (data not shown).

Alternatively, various detergents were tested for their capability to solubilize individual subunits. Relevant criteria were efficient separation as well as a minimal loss of cofactors from the polypeptides. In initial screens, subunit integrity was checked by native gel electrophoresis followed by heme staining. Table 1 shows that a whole range of detergents – marked '+' – are very efficient in the separation of subunits as judged by native PAGE. However, most of them led to the formation of a brown precipitate after incubation with the respective detergent which was indicative of denatured protein; therefore, these detergents were labeled '+' in the column 'denaturation of subunits' and classified as too destructive. The zwitterionic detergent SB12 yielded both pure and mostly intact cyt b_6, that could be separated to yield pure cyt b_6 and subunit (SU) IV in a single HPLC step (see below). The optimal concentration of SB12 was determined to be 0.5% (w/v), corresponding to about 4 times the critical micellar concentration (CMC) of this detergent under the conditions used.

3.2. Chromatographic separation of b_6f subunits

After treatment with SB12, the polypeptides were separated by an HPLC step. Judging from absorbance spectra, subunit separation, cofactor and pigment composition of the isolated subunits, the UNO-Q anion exchange matrix was best suited among various materials tested (see Section 2).

Fig. 1 shows a typical elution profile from the anion exchange column under conditions optimized for separation of subunit b_6 and SU IV. The resulting fractions were analyzed by redox difference spectroscopy in order to quantify the cytochrome content of the subunits. In addition, SDS–PAGE followed by immunodetection of the bands was used for identification of the polypeptides and reversed-phase chromatography for identification and quantification of the pigments. Fig. 1B shows the analysis of the resulting peaks by SDS–PAGE which was followed by immunoblotting. The combination of both methods identified peak 1 as the purified cyt b_6 subunit with an apparent molecular mass of 24 kDa. Additional small peptides (<3 kDa) present in this fraction were also recognized by the cyt b_6 antibody and therefore most likely represent proteolytic degradation products of cyt b_6. They were completely removed by filtration through 10 kDa membranes without any detectable change in the absorbance spectrum of this fraction. The homogeneous subunit b_6 was then characterized in more detail (see below).

Peak 2 was identified as subunit IV. It contained traces of heme which may be responsible for the observed heterogeneous elution behavior. This free heme contamination could easily be removed by fil-

Table 1
Detergents tested for the dissociation of a cyt b_6f complex into its subunits

Detergent	CMC (%)	Tested detergent concentrations (%)	Separation of subunits	Denaturation of subunits
β-Dodecyl maltoside	0.018	1/2.5	−/−	−/−
Triton X-100	0.03	0.075/0.2	−/±	−/−
Na-cholate	0.4	1.5	−/−	−/−
SB12	0.12	0.25/0.5/1	−/+/+	−/−/−
Nonyl glucoside	0.2	0.5	+	±
Hecameg	0.65	1.5	+	+
Mega 9	0.84	1.5	+	+
OGTP	0.28	0.4/0.8	±/+	+/+
OGP	0.7	1/1.5/2.5	±/±/+	+/+/+

Suitability was judged by most effective separation of subunits at minimal protein denaturation and minimal loss of cofactors.

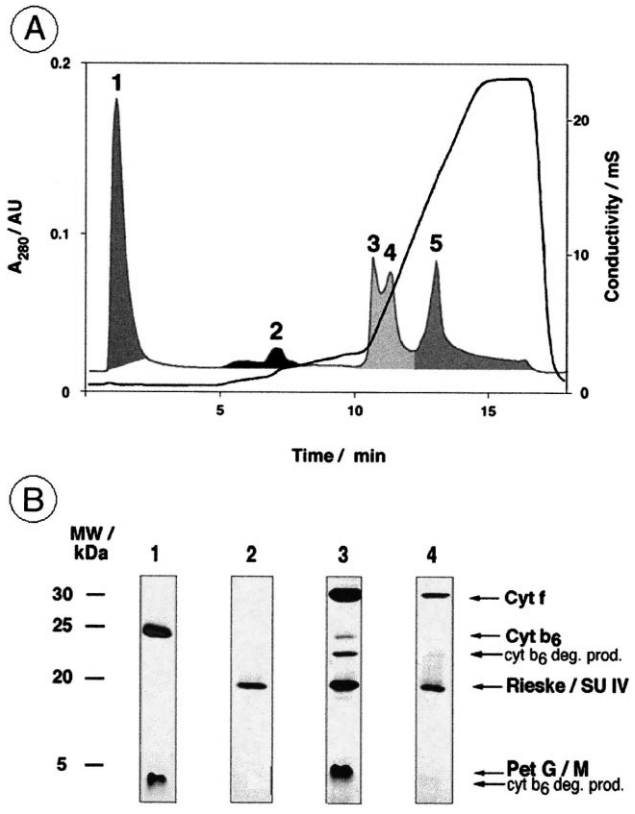

Fig. 1. Purification of cyt b_6f subunits by HPLC. (A) Elution profile of the dissociated cyt b_6f complex from the Bio-Rad Q-1 anion exchange column at a flow rate of 3 ml/min and at 10°C; absorbance at 280 nm. Peaks eluted by a stepwise NaCl gradient (0–100 mM and 100–1000 mM) are numbered 1–5. (B) SDS–PAGE analysis of fractions 1–4 from the HPLC run in A. Bands are visualized by silver staining and have been probed by immunoblotting (not shown). The combination of both methods could identify fractions 1–4 as follows: (1) cyt b_6 (24 kDa), cyt b_6 degradation products (<3 kDa); (2) SU IV (19 kDa); (3) cyt f (31 kDa), cyt b_6 (24 kDa), cyt b_6 degradation product (22 kDa), Rieske ISP (19 kDa), SU IV (19 kDa), and small unidentified proteins (3–5 kDa); (4) cyt f, Rieske ISP. Fraction 5 did not contain any detectable protein but most likely represented the detergent SB12.

tration through a 10 kDa membrane, yielding a homogeneous SU IV preparation. As determined by SDS–PAGE and immunoblotting, fraction 3 contained cyt f (31 kDa), traces of cyt b_6 (24 kDa), the Rieske ISP and residual SU IV (co-migrating at 19 kDa). The band at 24 kDa, corresponding to cyt b_6, has almost disappeared at the expense of a new one at about 22 kDa. As the latter is also cross-reacting with the cyt b_6 antibody, it may be a degradation product of the cyt b_6 subunit, in accordance with the presence of small degradation products in fraction 1. Peak 3 also shows bands at 3–5 kDa which most likely represent the small subunits of the cyt b_6f complex, PetG and PetM. This fraction was furthermore referred to as 'residual cyt b_6f complex'. Another fraction eluting at higher salt concentration (320 mM NaCl) was identified as a cyt f/ISP aggregate [5]. Peak 5 contains no detectable protein but represents the detergent SB12 eluting from the column.

3.3. Characterization of the native cyt b_6 subunit

As shown above, major parts of this subunit could be purified to homogeneity by HPLC, yielding sufficient material for a more detailed characterization. Fig. 2A shows absorbance spectra of isolated, reduced and oxidized cyt b_6, in 0.03% β-dodecyl maltoside. Positions of peaks and shoulders clearly indicate the presence of hemes (peaks at 416 nm and 425 nm, respectively, 530–535 and 563 nm), chlorophyll (peaks at 430–435 and 670 nm) and some carotenoid (shoulder at 460–500 nm). The absorbance maxima of the pigments in reduced and oxidized states differ by about 10% for chlorophyll (at 670 nm) and 3–5% for echinenone (at 460–500 nm), being higher in the oxidized state. The significance of this observation is still unclear.

From these spectra, the cytochrome content of the isolated cyt b_6 subunit was determined to 1.8 (±0.2) b-hemes per mol cyt b_6 protein, whereas no cyt f could be detected, confirming the purity of this preparation. Quantitatively, about 90% (±10%) of the native heme content of the cyt b_6f complex was retained in the cyt b_6 subunit. The observed difference absorbance maxima of 533 nm and 563 nm (see Fig. 2 insert) are identical to dithionite-minus-ascorbate-induced redox difference spectra of the whole cyt b_6f complex, showing that the protein environment of the cytochromes remained unchanged during the isolation procedure.

Based on these spectroscopic data, we obtained a ratio of Chl a to echinenone of 1.2:1 (±0.1) in various preparations of the cyt b_6 subunit. This was reproducibly smaller than in the entire cyt b_6f complex (1.4:1±0.1), suggesting an easier loss of Chl a

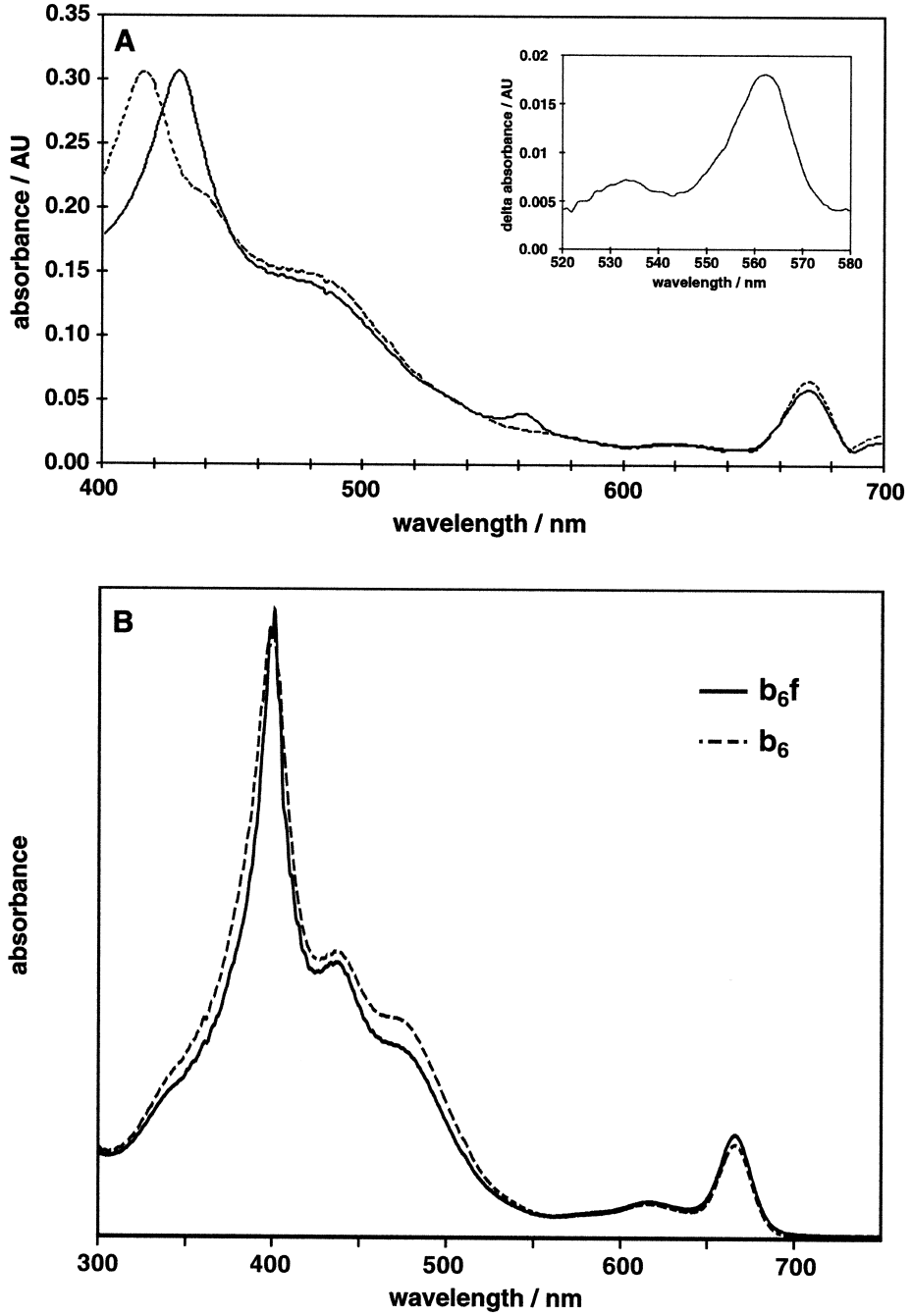

Fig. 2. (A) Absorbance spectra of the reduced (continuous line) and oxidized (dotted line) cyt b_6 subunit at RT. Samples were oxidized with ascorbate and reduced with dithionite. Maxima can be attributed to oxidized heme (416 nm), reduced heme (425 nm, 533 nm, 563 nm), chlorophyll (430–435 nm and 670 nm) and carotenoid (460–500 nm). (Insert) Redox difference spectrum (oxidized with ferricyanide and reduced with dithionite) of the *b*-type hemes with maxima at 533 and 563 nm. (B) Absorbance spectra of methanolic extracts from the isolated cytochrome b_6f complex (full line) and the cytochrome b_6 subunit (dotted line) for comparison: maxima can be attributed to chlorophyll (about 435 nm and 666 nm) hemes (around 400 nm) and carotenoid (460–500 nm). Spectra were normalized to the Soret band of the *b*-type hemes.

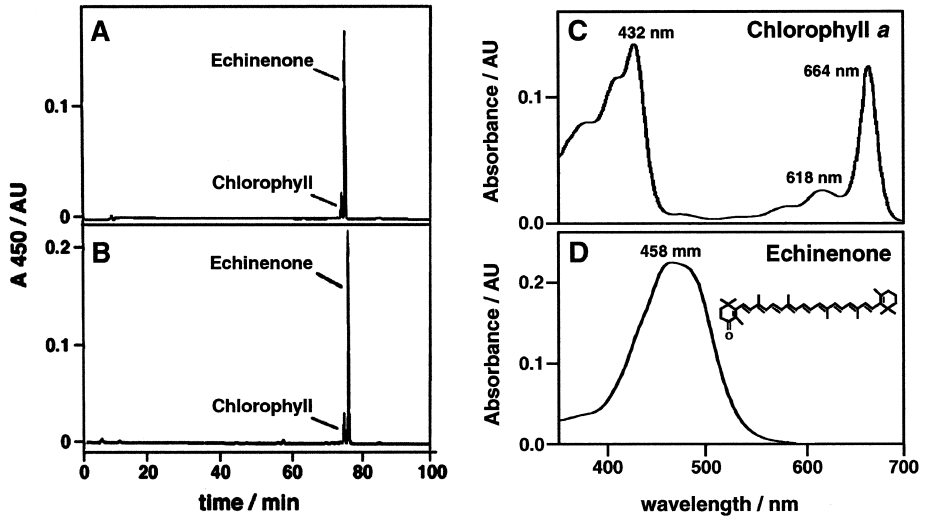

Fig. 3. Pigment analysis of the isolated cyt b_6f complex and the purified cyt b_6 subunit by reversed-phase HPLC. Column: Crom Spherisorb ODS 2, flow rate 0.5–0.9 ml/min at RT. Pigments were eluted by stepwise addition of ethyl acetate (buffer 2) to buffer 1 (38.5% (v/v) acetone, 46.5% (v/v) methanol, 5% (v/v) H_2O, 10% (v/v) 5 mM PIC A). (A) Elution profile of an acetonic extract from the isolated cyt b_6f complex (starting material for the cyt b_6 isolation). (B) Elution profile of an acetonic extract from the isolated cyt b_6 protein. (C) Absorbance spectra of chlorophyll *a* and (D) of echinenone, both recorded online from the peak fractions of run B.

relative to echinenone during the cyt b_6 isolation procedure. Although during the preparation of the whole cyt b_6f complex only substoichiometric amounts of Chl were retained per b_6f complex (0.6–0.8), 80–85% of the Chl remains bound to the isolated cyt b_6 subunit even after the additional detergent treatment. Fig. 2B shows absorbance spectra of methanolic extracts from the whole cyt b_6f complex and from the isolated cyt b_6 subunit for comparison. While only small changes in the stoichiometry between carotenoid (absorbance at 460–500 nm) and chlorophyll (absorbance at 666 nm) can be observed, it is obvious that chlorophyll in the isolated b_6 subunit is easier lost relative to carotenoid.

A thorough pigment analysis was performed with all fractions obtained by anion exchange chromatography (see Fig. 1A) and with the isolated cyt b_6f complex that was used as starting material for the separation experiments. Fig. 3 shows elution profiles from a reversed-phase HPLC column, obtained with the isolated cyt b_6f complex (Fig. 3A) and the isolated cyt b_6 subunit (Fig. 3B), respectively. Spectra of the peak fractions of both runs were recorded online by a diode array detector: the spectra of the isolated cyt b_6 subunit clearly identify a chlorophyll *a* (Fig. 3C) and an echinenone (Fig. 3D); identical spectra have been obtained for the whole cyt b_6f complex. As no other pigments could be observed in these runs nor in those performed with the other fractions shown in Fig. 1A (data not shown), we conclude from these results that Chl *a* and echinenone are exclusively bound to the cyt b_6 subunit.

Intactness of the isolated cyt b_6 protein was also checked by intrinsic fluorescence spectroscopy, using the fluorescence of the seven Trp residues of this subunit as an internal indicator of its tertiary structure. Upon selective excitation of the Trp fluorescence, the emission spectrum shows a peak at 326 nm (Fig. 4A, top). This indicates a very hydrophobic environment of the fluorescent Trp residues and confirms a non-denatured state of this subunit, as fully denatured protein should show a Trp peak at about 350–360 nm, similar to free Trp (shown for comparison in Fig. 4A, bottom). Fig. 4B shows a model of the b_6 subunit with four intrinsic and one amphiphilic α-helix. Also the approximate positions of all seven Trp residues of this subunit are indicated. While four of the Trp residues are located in a rather hydrophobic environment, three seem to be exposed to a more hydrophilic environment; however, the lack of a fluorescence peak around 360 nm indicates that these three residues are either shielded by a hydrophobic environment or quenched by their environment (see Section 4).

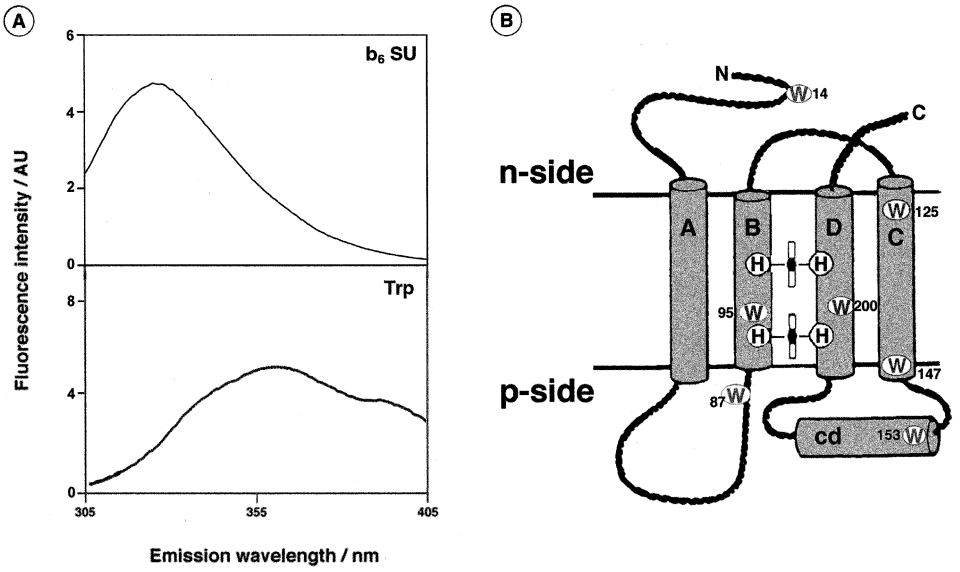

Fig. 4. (A) Fluorescence emission spectrum of the isolated cyt b_6 subunit upon excitation at 293 nm at RT (top); for comparison, also the emission spectrum of free Trp using identical conditions is shown (bottom). (B) Model for the localization of tryptophan residues (W) in the cyt b_6 subunit (*Synechocystis* sp. PCC 6803 numbering). 'H's indicate the positions of the heme-liganding histidines. Modified from [25].

4. Discussion

In this work, we present the dissociation of a cyanobacterial cyt b_6f complex by a moderate detergent treatment into its isolated subunits in their native state. This gentle purification procedure of the cyt b_6 protein allowed to perform a detailed characterization of this protein which is an apparent advantage in comparison with experiments where subunits had been recovered by cutting out gel slices from a polyacrylamide gel [15]. In those samples which included also a cyanobacterial cyt b_6 subunit from *Synechocystis* sp. PCC 6803, most of the hemes were lost during the preparation, and the remaining part could not be reduced any more, indicating the severe damage of this cyt b_6 subunit.

According to the preparation scheme presented here, a single HPLC step is sufficient to isolate both pure cyt b_6 subunit and subunit IV. The detergent treatment conditions were chosen carefully in order to minimize the amount of denatured protein on the one hand and to maximize the dissociation of the complex on the other. The fact that the isolated cyt b_6 subunit still contained about 90% of the non-covalently bound *b*-type cytochromes which showed the same redox properties as in the untreated cyt b_6f complex indicates the gentleness of this approach.

The observed interaction of the cyt b_6f complex with detergents also allows to draw conclusions on the overall structure of the complex. While the detergent of choice, SB12, seems to be quite efficient in dissociation of the complex, the individual subunits remain intact, i.e. in a native state. This may indicate that the individual subunits, especially the cyt b_6 subunit, are quite tightly packed and stabilized by intramolecular interactions rather than by intermolecular interactions between different subunits. This view is supported by the observation that lipids are especially crucial for cyt b_6f activity as shown for *Chlamydomonas* [24]. If the space between the subunits was also partly filled by lipids, this would reduce hydrophobic protein–protein interactions and facilitate solubilization of individual subunits as observed in our experiments.

4.1. Structural information on the cyt b_6 subunit

Intrinsic fluorescence of the tryptophans in the cyt b_6 protein backbone yielded information on the protein structure. As deduced from the amino acid sequence and the topology model shown in Fig. 4B,

four out of the seven tryptophan residues are localized in the membrane facing a rather hydrophobic environment (W95, 125, 147, and 200 according to *Synechocystis* numbering). Of the three remaining tryptophans, one (W14) is close to the N-terminus, in a hydrophilic environment, one (W 87) is localized on loop *ab*, probably just outside the membrane surface, and one (W153) is part of the extramembranous helix *cd*. The data obtained by intrinsic fluorescence indicate a predominantly hydrophobic environment of the tryptophan residues. Although it is not possible by this technique to address individual tryptophan residues, these experiments do suggest a densely packed structure of the cyt b_6 subunit, which may result in a partly hydrophobic environment even of the extramembranous domains. This is in accordance with the structural data obtained by two-dimensional crystallography [3,4] and also in line with the observed detergent–protein interactions (see above).

4.2. Localization of the pigments in the cyt b_6f complex

The binding of chlorophyll *a* to the cyt b_6f complex has been a widely confirmed observation [9–12,25,26], and it could be shown that this molecule is apparently not necessary for the activity of the complex [10]. In addition to Chl *a*, carotenoids have been found in many cyt b_6f preparations in substoichiometric amounts [16]. The pigment analyses performed in this study clearly indicate that chlorophyll *a* is bound to the native cyt b_6 subunit. This is in agreement with traces of chlorophyll remaining on the cyt b_6 subunit after separation from the other subunits by mild polyacrylamide electrophoresis [15]. However, while the stoichiometry of this association was approximately only 10%, the results presented here show a specific binding of the chlorophyll molecule to the native cyt b_6 subunit with only a moderate loss during the isolation procedure. Based on sequence homologies between PetG and PetM in comparison with the α-subunit of bacterial light harvesting (LH) 2 complexes, pigment binding to the small cyt b_6f subunits has been suggested [10]. However, since no small subunit was present in the cyt b_6 preparation and since all chlorophyll *a* detected in the whole cyt b_6f complex has been quantitatively recovered in the cyt b_6 subunit, this can now be ruled out – at least for *Synechocystis*. Also, another hypothesis based on kinetic measurements of the reduction kinetics of cyt *f* combined with the rise time of an electrochromic band shift of a Chl *a* suggesting a binding of the Chl close to cyt *f* [27] now seems unlikely.

The protein structure of the mitochondrial cyt bc_1 complex shows that the four transmembrane helices corresponding to cyt b_6 form the contact area between the monomers within the dimer [6]. Therefore, the reported loss of chlorophyll *a* upon monomerization of a dimeric cyt b_6f complex from *C. reinhardtii* [10] is also in good accordance with a localization of Chl on the cyt b_6 subunit. There may be differences, however, between this green algae and *Synechocystis* regarding the tightness of chlorophyll binding to the cyt b_6f complex; in addition, *Chlamydomonas* shows an unusually tight binding of heme b_L to the cyt b_6 subunit [28] which was not observed with our cyanobacterial preparation (U. Boronowsky, S.-O. Wenk, unpublished results). These differences may reflect a different immediate protein environment of heme b_L and the chlorophyll molecule – which are probably close to each other – in these two organisms, and it will be interesting to compare their molecular structures.

Our results also clearly show the binding of an additional pigment to the isolated cyt b_6 subunit, the carotenoid echinenone. A comparison of the absorbance spectra and the pigment analyses of all protein-containing fractions obtained from anion exchange HPLC indicates that the cyt b_6 subunit exclusively binds all pigments of the complex. The ratios of Chl *a* to echinenone remain largely unchanged during the preparation, indicating that (i) the binding of both pigments to cyt b_6 is selective and (ii) echinenone is at least as tightly bound to the subunit as chlorophyll *a* under the given conditions.

The physiological role of these pigments still remains highly speculative and has been extensively discussed before [10]. While it was suggested that they only represent an evolutionary relict of unknown function [26], the presence of Chl *a* has been shown not to be essential for the PQH_2-plastocyanin oxidoreductase activity of cyt b_6f in *Chlamydomonas* [10]. As the bleaching of chlorophyll did not impair this function, a structural role of the pigment

seems likely [10] which may be combined with an impact on cyt b_6f assembly as observed with *Chlamydomonas* mutants [10]. A merely structural role of the chlorophyll molecule would be conceivable if the formation of long-lived, excited Chl states was made unlikely by a special protection mechanism. This could include either quenching by protein side chains or by a carotenoid molecule, as has recently been suggested by an observed triplet energy transfer from Chl to β-carotene in a cyt b_6f complex [29]. The very low number of destructive Chl triplet states observed for a cyanobacterial cyt b_6f complex indeed indicates a very efficient protection mechanism for this protein [12].

Experiments with recombinant cyt b_6 from *Synechocystis* sp. PCC 6803 overexpressed in *Escherichia coli* [30] also support a possible structural role of the pigments. When the cyt b_6 subunit was purified from inclusion bodies and refolding in the presence of various cofactor combinations was attempted, the stability of the heme–protein interaction increased in the presence of chlorophyll. In contrast, the addition of β-carotene had no effect, which could be due to a selectivity of this polypeptide for echinenone. In analogy to the light harvesting complex of higher plants, LHC2, echinenone could also have the function of bridging α-helices in an 'X'-like shape [31]. Similar structural roles of carotenoids have been shown for the stabilization of bacterial LH complexes and the PS2 core complex from green algae and higher plants (reviewed in [32]).

4.3. Implications for studies on membrane protein complexes

We have established a procedure for the isolation of native subunits from a membrane protein complex. The same protocol was also successfully used for the purification of the recombinant subunits b_6 and subunit IV overproduced in *E. coli* [30], indicating that this method could be generally applied – with minor modifications – for the semi-preparative purification of hydrophobic subunits in a native or semi-refolded state.

Although the detergent used for dissociation into individual subunits may have to be optimized in each case, the presented procedure opens up new possibilities for the study of structure, folding and assembly of individual subunits of membrane protein complexes. Since handling of whole membrane protein complexes is still difficult as reflected by the very low number of well characterized examples, the study of individual subunits in the native state could precede the investigation of whole complexes. This is especially true for studying a process such as binding and integration of cofactors which often is extremely difficult to monitor in the presence of other processes occurring at the same time in these (mostly) multisubunit complexes. Moreover, individual 'native' subunits can also be useful references for the correct folding of recombinant subunits overproduced in hosts like *E. coli* or yeast, and they are needed to estimate the implication of directed mutations for structure and function. Finally, the (mostly) limited size of these individual subunits – about 23 kDa in case of the cyt b_6 subunit – also makes them amenable for structural studies alternative to X-ray diffraction such as NMR, which can be performed in solution, thus avoiding possible crystallization artifacts. A combination of the described techniques may help in speeding up membrane protein characterization in general.

Acknowledgements

The support of this project by the DFG (SFB 480, project C1) and the Human Frontier Science Program is gratefully acknowledged.

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