In vitro reconstitution of the spinach chloroplast cytochrome b(6) protein from a fusion protein expressed in Escherichia coli

ARTICLE *in* BIOCHIMICA ET BIOPHYSICA ACTA · AUGUST 2002

Impact Factor: 4.66 · DOI: 10.1016/S0167-4838(02)00369-2 · Source: PubMed

2 AUTHORS:

Jarosław Króliczewski
University of Wroclaw
22 PUBLICATIONS **76** CITATIONS
SEE PROFILE

Andrzej Szczepaniak
University of Wroclaw
36 PUBLICATIONS **498** CITATIONS
SEE PROFILE

ELSEVIER

Biochimica et Biophysica Acta 1598 (2002) 177–184

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.bba-direct.com

In vitro reconstitution of the spinach chloroplast cytochrome b_6 protein from a fusion protein expressed in *Escherichia coli*

Jarosław Króliczewski, Andrzej Szczepaniak *

The Institute of Biochemistry and Molecular Biology, University of Wrocław, Tamka 2, 50-137 Wrocław, Poland

Received 6 August 2001; received in revised form 27 March 2002; accepted 30 May 2002

Abstract

We have developed a strategy for overproduction of spinach apocytochrome b_6 as a fusion protein to maltose-binding protein (MBP) in *Escherichia coli*, using the expression vector pMal-c2. The fusion protein was purified to virtual homogeneity by gel filtration chromatography and the method of insertion of hemes into fusion protein was elaborated. The ambient and low-temperature absorption spectra of the reconstituted cytochrome b_6 were similar to those of cytochrome b_6 spectra in isolated proteins or cytochrome b_6f complexes and are typical for bis-histidine ligated *b*-type cytochromes. Optical circular dichroism (CD) spectra of the visible region further confirmed the appropriate binding of hemes by the apocytochrome b_6 protein. We found that the incorporation of hemes was required for the refolding of the cytochrome b_6 protein into the more compact structure found in the native cytochrome protein. Heme staining experiments suggested that the two hemes in the reconstituted cytochrome b_6 protein are bound with different affinities. The reconstituted cytochrome b_6 protein was cleaved by Xa factor proteolysis from fusion protein and separated for characterization. The procedure presented in this work for reconstitution of hemes into the cytochrome b_6 protein should provide an important tool for structure/function studies of membrane-bound cytochrome proteins.

Keywords: Membrane protein; Cytochrome b_6; Heme insertion; Folding; Denaturation

1. Introduction

In spite of the enormous efforts associated with the study of membrane protein folding and structure, the current rate of progress seems particularly sluggish when compared to that of the water-soluble proteins. In vitro refolding has for long been a routine for soluble protein. However, until recently, it has rarely been tried for membrane protein [1,2]. Lately, the β-barrel protein OmpA [3] and phospholipase A [4] were refolded from the polypeptides expressed as inclusion bodies, and it was the refolded material that was used for crystallisation. The research was aimed at obtaining functional thylakoid membrane protein, cytochrome b_6, by refolding polypeptide expressed as inclusion bodies.

The cytochrome b_6f complex is one of three integral membrane protein complexes in the oxygenic photosynthetic membrane that participate in electron transport, proton translocation and generation of the transmembrane proton electrochemical potential. It occupies a central position with respect to the other two integral protein systems, the Photosystem I and II reaction centre complexes [5,6]. These functions are analogous to those carried by the cytochrome bc_1 complex of the respiratory electron chain and of purple bacteria [7]. They all possess three redox carriers: cytochrome b_6 or cytochrome *b* containing two *b* hemes, cytochrome *f* or cytochrome c_1 containing one *c* heme, and the Rieske protein containing one [2Fe–2S] cluster. The cytochrome b_6 is approximately half the size of the mitochondrial and bacterial cytochromes, its 215–216 residues encompassing only the heme-binding domain. The four transmembrane α-helices (A–D) of the cytochrome b_6 correspond to the N-terminal domain of the larger mitochondrial cytochrome *b*. The subunit IV, with three transmembrane α helices, corresponds with the C-terminal part [8] and may contain one or more functions, specified by this part of the mitochondrial, cytochrome *b* including the quinone-binding function [5]. The bis-histidine heme ligation of the two *b* hemes occurs through four histidines, absolutely conserved in all of the approximately 110 bc_1 and b_6f sequences that are available in the data bank, and results in one heme near each side of the membrane bilayer. Two histidines are located on helix B and two on

* Corresponding author. Tel.: +48-71-3752926; fax: +48-71-3752608.
E-mail address: aszczep@bf.uni.wroc.pl (A. Szczepaniak).

0167-4838/02/$ - see front matter © 2002 Elsevier Science B.V. All rights reserved.
PII: S0167-4838(02)00369-2

helix D, the hemes bridging these two transmembrane α helices, resulting in one heme on the positive (p) side and one on the negative (n) side of the membrane. The two hemes are perpendicularly oriented to the membrane with their edges separated by ~12 Å [6]. Another different feature of cyt b_6, in comparison to cyt b (bc_1), is that the two histidine residues on helix D are separated by 14 residues in cyt b_6 and 13 in cyt b (bc_1) [9]. Most data indicate that the α band peaks (λ_m) of the two hemes are more similar in cyt b_6 ($\Delta\lambda_m \sim 0.5$ nm), as compared with cyt b of the bc_1 complex ($\Delta\lambda_m \sim 4-5$ nm); the redox data indicate less heterogeneity in the midpoint potential of the two b-hemes (Ref. [6] and references within). The resolutions of these differences await the elucidation of the structure–function relationship in these cytochromes at the molecular level.

In vitro reconstituting of a protein with heme can be helpful in understanding the feature of its assembly. While many water-soluble proteins can be refolded in vitro from a denatured state into their native structure with refolding occurring spontaneously [10], at present—for membrane proteins containing a prosthetic group—this has only been achieved for a few proteins: bacteriorhodopsin [11] and light-harvesting complexes [12–14]. On the other hand, in vitro association of a heme only with a water-soluble protein has been achieved. The heme has been incorporated into soluble apoprotein of hemoglobin, mioglobin [15] cytochrome b-562 [16], synthetic peptides [17,18] or the soluble domain of cytochrome b_5 [19], and cytochrome b-560 from bovine heart mitochondrial succinate–ubiquinone reductase [20].

To our knowledge, this is the first example of a successful refolding and incorporation of hemes into recombined integral membrane b type cytochrome. The site-directed mutation within the cyt b_6 may help study the function of highly conserved residues in this protein. Here, the construction of a cytochrome b_6 expression vector, pMal-c2b6, the conditions for high expression of maltose-binding protein–apocytochrome b_6 fusion protein (MBP–apocyt b_6) in *Escherichia coli* TB1, and the isolation of pure recombinant MBP–apocyt b_6 fusion protein is reported. The reconstitution of recombinant MBP–apocyt b_6 with hemes and the properties of reconstituted cytochrome b_6 are also discussed.

A preliminary and abridged version of this work has been presented [21].

2. Materials and methods

2.1. Construction of the expression plasmids

The gene for the spinach apocytochrome b_6 was amplified by PCR from the isolated chloroplast DNA [22]. The direct primer 5′ GCC AGT ACT **ATG AGT** AAA GTC TAT GAT TGG3′ carrying *Sca*I site (underline), exon I (bold) and first 15 bases of exon II and reverse one 5′G GGG ATC CTA TTA TAA GGG ACC AGA AAT ACC-3′ were designed from the known sequence of gene *petB* from chloroplastic DNA of spinach [23].

The PCR product was cleaved by *Sca*I and *Bam*HI (New England Biolabs) and purified using PCR purification kit (Qiagen). The purified product was cloned into the *Xmn*I and *Bam*HI sites of the expression vector pMal-c2 (New England Biolabs) by a standard T4 DNA ligase procedure, resulting in the expression of the target gene product as a fusion protein to MBP. The recombinant plasmid, designated pMal-c2b6, was checked by restriction analysis and verified by DNA sequencing.

2.2. Recombinant apocytochrome b_6 expression and purification

E. coli TB1 cells, transformed with pMal-c2b6, were grown aerobically at 37 °C in 1 l of Luria-Bertani medium containing 100 μg/ml ampicillin and inoculated with 10 ml overnight cultures. The production of fusion protein (MBP–apocytochrome b_6) was induced by addition of 0.5 mM IPTG (isopropyl thio-β-D-galactoside) at an OD_{550} of 0.6. After shaking for 4 h, cells were harvested by centrifugation (5000×g 15 min), resuspended in 30 ml of TEN buffer (20 mM Tris–HCl, pH 7.8, 10 mM EDTA, 1 mM PMSF, 100 mM NaCl) and then frozen overnight.

After thawing, the solution was sonicated (6×15 s, 65 W microtip sonifier cell disrupter, Barnsted) and the lysate was centrifuged at 10000×g, 20 min. The pellet fraction of inclusion bodies (IB), which were resuspended in 20 ml of buffer TEN supplemented with RNAse and DNAse (both 10 μg/ml, Sigma), was stirred at room temperature (RT) for 30 min and next centrifuged at 5000×g, 60 min, 4 °C. Insoluble materials were washed several times in TENT buffer (50 mM Tris–HCl, pH 7.8, 1 mM EDTA, 1 mM PMSF, 200 mM NaCl 2% v/v Triton X-100). Triton was removed by centrifugation (4300×g, 20 min), washed twice in TEN buffer (20 min shaking at 37 °C), and further centrifugation (5000×g, 30 min) was done, yielding a white pellet of IB contained MBP–apocytochrome b_6 fusion protein.

The final pellet was solubilized in 10 ml of freshly made Urea buffer (7 M Urea in TEN buffer) incubated for 30 min at 37 °C under mild shaking and recentrifuged (5000×g, 10 min). The supernatant containing the fusion protein was purified to virtual homogeneity by gel filtration chromatography in the TEN buffer, pH 7.8, containing 7 M urea on a Superdex 75 FPLC column (Pharmacia). Fractions containing the fusion protein were collected, analysed by SDS-PAGE [24], concentrated using a Centricon-30 filter (Amicon), frozen, and stored at −70 °C.

2.3. Cytochrome b_6 folding and reconstitution

The hemin was obtained from Fluka AG. Fresh stock solutions of heme (0.1 mg/ml) were made by adding the hemin to 50% ethanol, followed by a careful dropwise

addition with stirring of 1 M NaOH until the entire hemin dissolved and subsequently diluted in the buffer R2 (50 mM Tris–HCl, pH 8.0, 50 mM NaCl, and 10 mM sodium dithionite). This solution was then passed through a 0.2 μm filter into Eppendorf tube. Concentrations of the heme were determined spectrophotometrically, using an extinction coefficient of ε_{385}=56 mM^{-1} cm^{-1} in buffer R2 [25].

The refolding experiments were carried out in buffer R1 (50 mM Tris–HCl, pH 8.0, 50 mM NaCl, 0.3% SDS, 10 mM sodium dithionite and 7 M urea). The soluble MBP–cytochrome b_6 was prepared by adding a 1.5-fold molar excess of the heme to MBP–apocytochrome b_6 in buffer R1. After incubating for 30 min at room temperature, the mixture was dialysed in a few steps against the buffer R1, containing a decreased concentration of urea (from 7 to 0 M). The folding mixture was centrifuged at 6000×g for 15 min to remove precipitated protein, loaded on a Sephadex G-25: PD 10 column (Pharmacia), and concentrated using a Centricon-30 filter (Amicon) to remove free heme.

2.4. Difference spectra measurements

Visible absorption spectra were recorded at the room temperature in a 1 cm optical-path length cuvette (1 ml), using a Cary 1 spectrophotometer with a spectral band width of 0.5 nm, and a scan speed of 600 nm/min. The spectra were usually recorded in 50 mM Tris–HCl, pH 8.0, 50 mM NaCl, 0.3% SDS. For the absorbency difference spectra, the complex was oxidised by ferricyanide and reduced by an addition of a concentrated dithionite solution. Low-temperature spectra, over liquid nitrogen at fixed redox potentials, were measured in a M40 spectrophotometer (Carl Zeiss Jena), equipped with low temperature accessory, using 2 mm cuvettes, calibrated in each case to ±0.2 nm with the 656.1 nm emission line from an internal D_2 lamp. Samples of cytochrome b_6 fused protein were taken at fixed redox potential and were quickly frozen in liquid nitrogen before low difference spectra were recorded.

2.5. Circular dichroism (CD) measurements

CD spectra of proteins were recorded on a Jasco J-715 spectropolarimeter at RT using a 1-mm path length cell in 10 mM sodium phosphate buffer, pH 7.8, 10 mM NaCl, 0.3% SDS. Data points were collected every 0.2 nm from 185 to 300 nm, averaging 10 scans per sample, and samples from 300 to 700 nm averaged 5 scans per sample. The data was analysed using the least-squares algorithms with the reference data from Yang et al. [26–28]. The spectra were noise-reduced using the instrument algorithm and corrected for the buffer contributions.

2.6. Protein concentration measurements

MBP concentration was determined directly from the absorbance at 280 nm with an excitation coefficient of ε=68 750 M^{-1} cm^{-1} [29]. The concentration of fused protein was determined by using the BCA reagent (Sigma) according to the manufacturer's instructions with BSA as a standard.

2.7. Heme staining

The samples were dissolved immediately prior to electrophoresis in 3% SDS, 50 mM Tris–HCl and 10% glycerol at 37 °C for 15 min or 95 °C for 1 min. Heme proteins staining was performed according to Thomas et al. [30].

2.8. SDS-PAGE and Western blot

Proteins were separated on a 15% acrylamide gel containing 8 M urea. The Western blot was carried out as described by Szczepaniak and Cramer [31].

2.9. Cleavage with factor Xa

Cleavage was carried out at w/w ratio of 0.5% of the amount of fusion protein in 10 mM Tris–HCl, pH 8.0, 10 mM NaCl, 0.05% SDS buffer. The reaction mixture was incubated for 24 h at 4 °C. The complete cleavage was checked by SDS-PAGE.

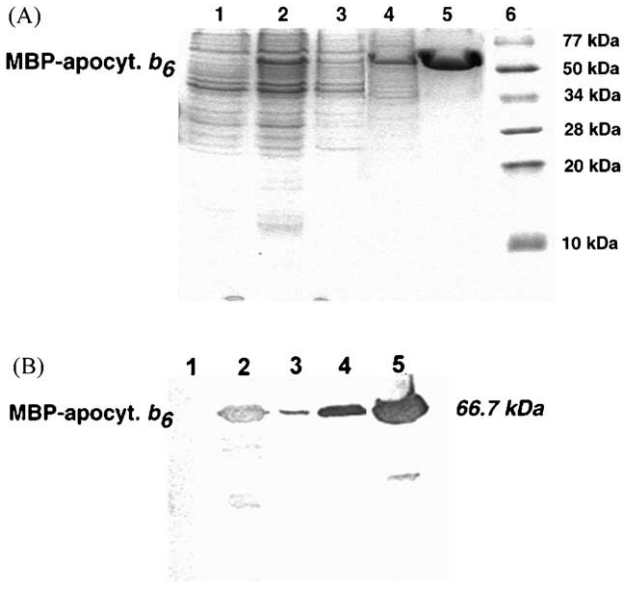

Fig. 1. (A) SDS-PAGE of the expressed MBP–apocytochrome b_6 fusion protein. Lane 1 shows the total protein of *E. coli* TB1 cells carrying the plasmid pMal-c2b_6 before induction; lane 2, total protein after induction with IPTG; lane 3, cytoplasmic fraction; lane 4, insoluble fraction of *E. coli*; lane 5, isolated inclusion bodies; lane 6, molecular weight standard. (B) Western blot analysis of the purification of expressed MBP–apocytochrome b_6 fusion protein. Lane 1 shows the total protein of *E. coli* TB1 cells carrying the plasmid pMal-c2b_6 before induction; lane 2, total protein after induction with IPTG; lane 3, cytoplasmic fraction; lane 4, insoluble fraction of *E. coli*; lane 5, isolated inclusion bodies.

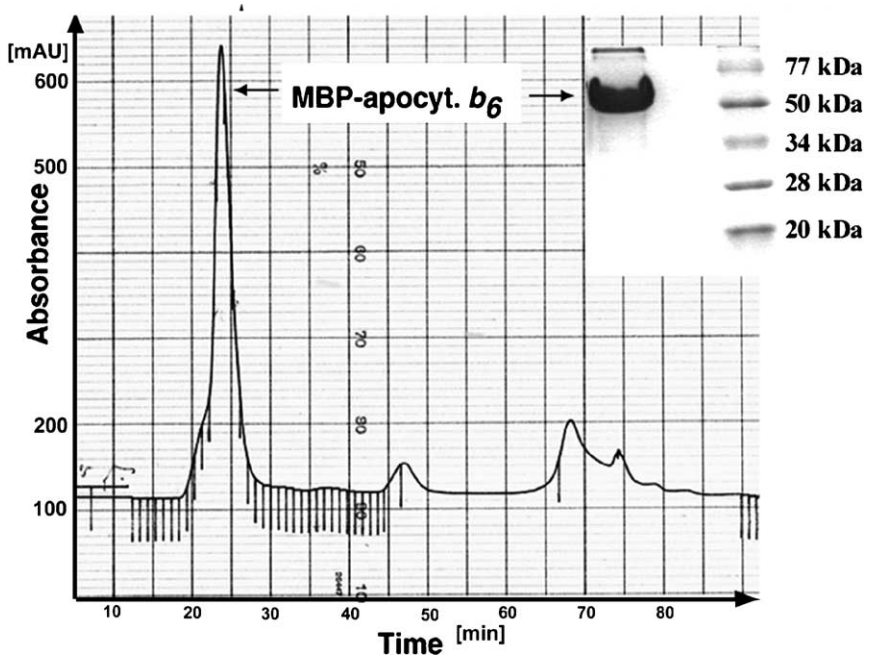

Fig. 2. Elution pattern from the one chromatographic purification of MBP–cytochrome b_6 fusion protein from IB solubilized in 7 M urea by size exclusion chromatography on a Pharmacia Superdex 75 column. The proteins from all peaks were identified by SDS-PAGE and Western blot analysis. The insert shows SDS-PAGE analysis of purified protein from the peak A and molecular weight standard.

2.10. Separating the protein from MBP after Xa cleavage

The reconstituted cyt b_6 fusion protein was separated from MBP and Factor Xa by gel filtration chromatography using HiLoad 16/60 Superdex 75 column (Amersham Pharmacia Biotech) on AKTA purifier FPLC system. The fractions containing the target protein free of MBP were collected, concentrated and characterized.

3. Results

3.1. The construction of the expression vector for apocytochrome b_6, expression and purification of the fusion protein

On the basis of the known sequence of the *petB* gene from spinach [23], the direct and reverse primer have been designed. Full-length cDNA encoding cytochrome b_6 from spinach chloroplasts was amplified using PCR and was fused to gene encoding bacterial protein (MBP), using the vector pMal-c2. *E. coli* (strain TB1) transformed with the constructed plasmid pMal-c2b6 produces, upon the induction with IPTG, a protein of the expected molecular weight (Fig. 1A; lanes 2, 4, 5 apparent molecular weight, 67 kDa), which reacts with antibodies raised against the COOH-terminal decapeptide of spinach cytochrome b_6 (Fig. 1B) and antibodies raised against MBP (not shown). The expression products, apocytochrome b_6 fused to MBP (MBP–apocyt b_6), are accumulated in the bacteria in an insoluble, readily sedimented form, the so-called 'inclusion bodies'. The induction *E. coli* cells harbouring plasmid pMal–c2b6 produced inclusion bodies that contained MBP–apocyt b_6 with some contaminating host cell proteins. Typical inclusion bodies obtained from 1 l of bacterial culture contained 105–130 mg protein, of which approximately 80% was fusion protein (Fig. 1A). The inclusion bodies were solubilized in a buffer containing 7 M urea and fusion protein was purified on a Superdex 75 column. After a one-step gel filtration, the preparation contained more than 97% of MBP–apocyt b_6

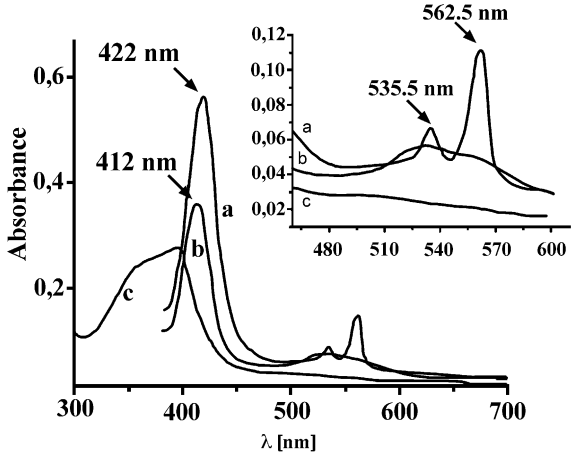

Fig. 3. Absorption spectra of the oxidised (a) and reduced (b) hemes inserted into MBP–apocytochrome b_6 fusion protein. A spectrum of free protoporphyrin IX (c) in R1 buffer is shown for comparison.

Table 1
Spectroscopic properties of the heme bound to MBP and reconstituted cytochrome b_6 (in MBP–cytochrome b_6 fusion protein)

Absorption peaks	MBP		Fusion protein	
	Oxidized[a]	Reduced[a]	Oxidized[a]	Reduced[a]
Soret band	401±0.6 nm	413±0.7 nm	412±0.8 nm	422±1.1 nm
α and β band	510±0.7 nm; 540±1.3 nm	510±1.4 nm; 540±0.9 nm	533.5±0.8 nm broad peak	535±0.6 nm; 562.5±0.7 nm

[a] Values are the average of 10 measurements and presented as means±S.D.

fusion protein as judged from SDS-PAGE. The fusion protein can be cleaved with the factor Xa protease in TEN buffer containing 0.03% SDS (not shown). However, 80% of the protein was precipitated during the incubation with protease, resulting in a low yield of apocytochrome b_6. Therefore, all the reconstitution experiments described in this paper have been performed with MBP–apocyt b_6 typically generating on elution pattern from the one Superdex chromatographic step as shown in Fig. 2.

3.2. Reconstitution of cytochrome b_6 from MBP–apocytochrome b_6 fusion protein

Fig. 3 shows generation of cytochrome b_6 from MBP–apocyt b_6 fusion protein after addition of the hemin. After the hemin dialysis and removal of precipitated protein by centrifugation, 60% of the protein was still in soluble form. The maximum absorption peak (Soret band) of the oxidised form of heme shifts from 398 to 412 nm, with increasing absorption intensity. The spectrum also displays a broad band around 535 nm. When dithionite was added to reduce hemes, the sample now showed a α-band absorbance at 563 nm, a β-band peak at 535 nm, and Soret peak at 422 nm. The ratio of the absorbance of 5.7 at the γ- and α-bands of the reduced reconstituted cytochrome b_6 is also very close to that of 6.0 found for natural *b*-type cytochromes [17] and indicates that the two hemes are in a well-defined orientation between two histidines. The width of the α-band at half-height is 10–11 nm. These spectral characteristics are close to those of cytochrome b_6 in an isolated active cytochrome b_6f complex preparation [32,33]. Thus, the cytochrome b_6 spectral signature is restored to MBP–apocyt b_6 fusion protein by heme addition in the presence of SDS after removing urea by dialysis. The absorption spectroscopy measurement of reconstituted MBP–cytochrome b_6

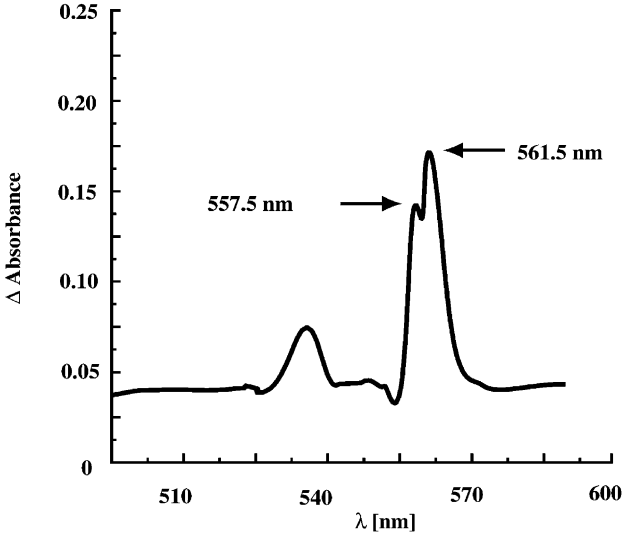

Fig. 4. Low-temperature redox difference spectrum of a reconstituted cytochrome b_6. Difference spectrum of reconstituted cytochrome b_6 obtained as described under Materials and methods. Ferricyanide and dithionite were used respectively as oxidant and reductants.

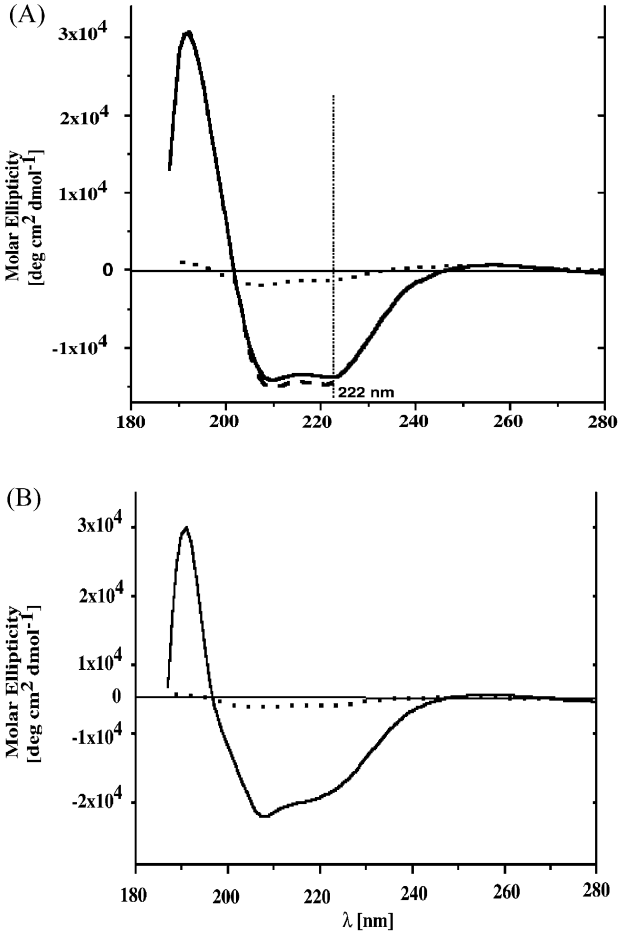

Fig. 5. (A) CD spectra of the denatured MBP–apocytochrome b_6 (doted line), MPB–apocytochrome b_6 after renaturation (solid line) and MBP–cytochrome b_6 with the heme in the oxidised state (dash line) in potassium phosphate buffer at room temperature. (B) CD spectra of renatured MBP (solid line) and denatured MBP (doted line) in potassium phosphate buffer at room temperature.

Table 2
Secondary structure analysis calculated from the formula of Yang et al. [28] of the renatured MBP, renatured MBP–apocytochrome b_6, reconstituted MBP–cytochrome b_6 and cytochrome b_6 after cleavage and separation from fusion protein

Structure	MBP–apocyt b_6[a]	MBP–cyt b_6[a]	MBP[a]	cyt b_6
α-helix	28.2±1.3%	32.2±0.7%	25±1.0%	43.5±1.2
β-sheet	26.0±0.9%	27.7±1.3%	27.4±0.6%	9.3±0.3
β-turn	5.6±0.4%	6.0±0.5%	3.5±0.2%	4.1±0.7
Random coil	40.2±1.4%	34.1±0.8%	44.1±1.5%	43.1±0.9

[a] Calculated values are the average of six measurements and presented as means±S.D.

has shown that more than 85% of proteins have a good refolded and reconstituted structure.

It is important to establish that the histidine ligands of reconstituted cytochrome b_6 in MBP–apocytochrome b_6 are provided by the apocytochrome b_6 and not by the MBP moiety. This is achieved by comparing the absorbance spectra (not shown) and peaks of heme-ligated MBP with those of heme-ligated MBP–apocytochrome b_6 (Table 1). Since, after the addition of heme to purified MBP, under conditions identical with those for MPB–apocytochrome b_6, the cytochrome b_6 spectrum is absent (data not shown), it is concluded that the bis-histidine ligands of reconstituted cytochrome b_6 are provided by the apocytochrome b_6 moiety of the fusion protein.

3.3. Low-temperature spectra

The cytochrome b_6, either within the cytochrome b_6f complex or in an isolated form, can be separated into two distinct spectral forms, as derived from the higher resolution spectra obtained by low-temperature spectroscopy. The reconstituted MBP–cytochrome b_6 fusion protein has an asymmetric α-band at 561 nm in the low-temperature difference spectrum (Fig. 4). This α-band shows two spectral species with peaks at 557.5 and 561.5 nm.

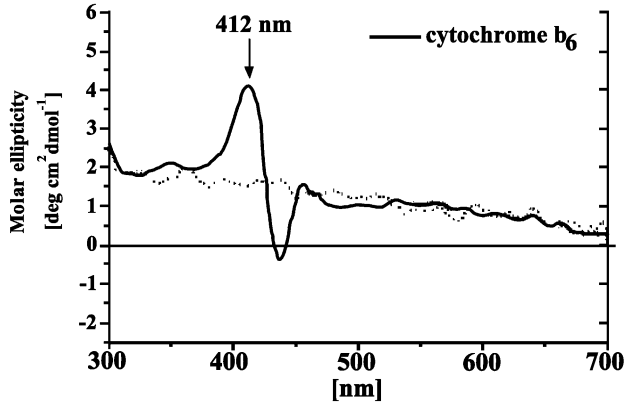

Fig. 6. CD spectra of the reconstituted and cleaved by factor Xa cytochrome b_6 (solid line) and MPB–apocytochrome b_6 after renaturation (dotted line) in potassium phosphate buffer at room temperature.

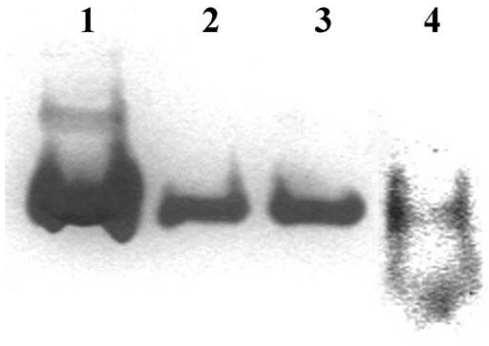

Fig. 7. Reconstituted cytochrome b_6 heme staining after boiling at various time treatments. TMBZ staining was after urea SDS-PAGE. Samples were not heated (line 1), heated at 100 °C for 10 s (line 2), heated at 100 °C for 30 s (line 3) and heated at 100 °C for 60 s (line 4).

3.4. Circular dichroism

Fig. 5A shows the room temperature CD spectra of MBP–apocytochrome b_6 in 7 M urea (dashed line), MBP–apocytochrome b_6 after renaturation (dotted line) and reconstitution of MBP–cytochrome b_6 (solid line). Fig. 5B shows the CD spectra of MBP in 7 M urea and MBP after renaturation with the methods used for renaturation of MBP–apocytochrome b_6. The mean residue molar ellipticity at 222 nm was −11680, −13734, and −14459 for renatured MBP, renatured MBP–apocytochrome b_6 and reconstituted MBP–cytochrome b_6, respectively (the results were significantly different at $P \leq 0.01$). From these values, a helicity of 25%, 28% and 32% can be estimated if 100% helicity corresponds to a value of −35700° cm^2 dmol^{-1}, as calculated from the formula of Yang et al. [28]. From Table 2, the α-helix content of apocytochrome b_6 moiety of the

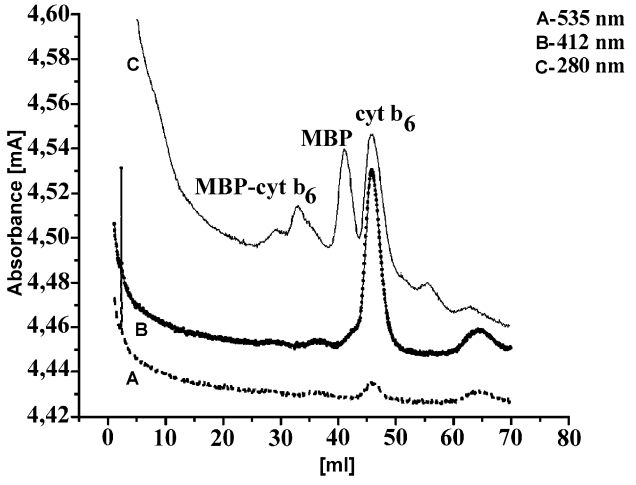

Fig. 8. Elution pattern from the chromatographic purification of cytochrome b_6 from the cleavage mixture by size exclusion chromatography on a Pharmacia Superdex 75 column. The proteins from peaks containing cyt b_6 were concentrated, identified by VIS spectroscopy and Western blot analysis.

renatured fusion protein in the absence and presence of bound heme can be estimated as 37% and 48%, respectively.

CD spectroscopy is a powerful tool for structure analysis of the heme proteins. This is because free heme is a symmetrical molecule and therefore displays no CD spectrum. The protein induces an asymmetry in electronic absorption of the heme, resulting in the observed optical activity [34]. The visible CD spectrum of reconstituted MBP–cytochrome b_6 displays Cotton effects in the Soret band region (Fig. 6).

3.5. Heme stain

Since cytochrome b_6 can be stained by heme after electrophoresis [35,36], while the related cytochrome *b* cannot, this peroxidase activity of the reconstituted MBP–cytochrome b_6 was tested. The samples of MBP–cytochrome b_6 were dissolved in 2% SDS and treated by boiling for 10, 30 and 60 s (Fig. 7, lanes 7, lanes 2, 3 and 4, respectively) and analysed for heme content after SDS-PAGE. The unheated sample was a control (Fig. 7, lane 1). The MBP–cytochrome b_6 was heavily stained with TMBZ under denaturing (2% SDS) conditions. After 10 and 30 s of boiling, only part of the heme was released (about 50%), suggested that part of the *b* heme in reconstituted cytochrome is strong-associated with the polypeptide.

3.6. Separating the protein from MBP after Xa cleavage

The reconstituted MBP–cyt b_6 was cleaved by Factor Xa and MBP was removed from the cleavage mixture by gel filtration. After reconstitution, the fusion protein solved in 0.05% SDS was cleaved very ‘‘efficiently’’, i.e., no uncleaved fusion protein or precipitates were observed. The hemes were detected only in cyt b_6 fraction and substoichiometrical amount in MBP fraction (Fig. 8). The isolated cyt b_6 shows the same spectra properties as that in native protein (Fig. 9).

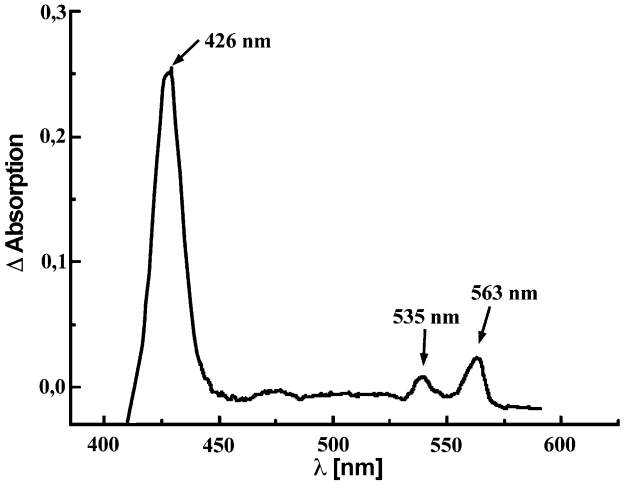

Fig. 9. Difference absorption spectra of reconstituted cytochrome b_6 purified by gel filtration after cleaved of MBP–cytochrome b_6 fusion protein by factor Xa.

4. Discussion

The most common method for refolding membrane protein uses a detergent to induce the native conformation. The detergent is usually added to the unfolded protein in denaturant, which is removed by dialysis. It is this approach that refolds cytochrome b_6 from overproduced apocytochrome b_6 fused to the MBP.

It can be observed that the plant chloroplast apocytochrome b_6, synthesised in bacteria as a fusion protein with MBP, can be reconstituted with hemes to yield cytochrome b_6. The confidence that reconstituted cytochrome b_6 is quite similar to the cytochrome isolated from thylakoids comes from the similarity of oxidised and reduced absorption spectra. The spectra in the α and β region of the MBP–cyt b_6 and cut off and isolated cyt b_6 from the reconstituted fusion protein are quantified similar to previously published spectra of the cyt b_6 [32,33] [M. Rögner, unpublished data]. The γ peak for MBP–cyt b_6 is shifted to short wavelengths: 412 nm for oxidised and 422 nm for reduced cytochrome. This shift is most probably caused by the presence of MBP and for substoichiometrical amount of heme bound to MBP. After cutting off and isolating the absorption, γ peak is shifted to 415 and 426 nm (Fig. 9), which are similar to those of isolated cytochrome b_6 from cyanobacteria [M. Rögner, personal communication]. A second line of evidence comes from the low-temperature differences spectra, which are similar to those of isolated cytochrome [33] or spectra of cytochrome b_6 in the isolated b_6f complex [32]. A CD spectrum in the visible region provides a very sensitive measure of heme binding. This is because a free heme is a symmetrical molecule, and therefore displays no CD spectra. The protein induces an asymmetry in the electronic absorption of the heme resulting in the observed optical activity [34]. Appearance of optical activity in CD spectra in the visible region confirms appropriate binding of heme by apocytochrome b_6.

This is the first report on proteins refolding and incorporation of hemes into a hydrophobic integral membrane protein. The incorporation of hemes into soluble *b*-type cytochrome or soluble synthetic peptides was shown earlier where the hemes were inserted into folded polypeptides [17–20]. In the present case, the addition of hemes before refolding of the apoprotein was necessary. The folding is induced by dialysis of the urea-solubilized MBP–apocytochrome b_6 to buffer with SDS. As shown by CD, the disordered structure of urea-solubilized heme free form of apocytochrome b_6 in the presence of SDS is converted into a partially folded structure with 37% α helical content. The incorporation of hemes is required for the refolding urea-solubilized apocytochrome b_6 into more compact structure found in native cytochrome b_6 containing 48% α helix.

The appropriate reconstitution of cytochrome b_6 was confirmed by separation of cytochrome moiety from the reconstituted fusion protein. The CD spectrum of this separated and reconstituted cytochrome b_6 shows high helical content (see Table 1) close to that of native cytochrome b_6.

At least four nuclear gene products designed, CCB1, CCB2, CCB3 and CCB4, have been shown to be specifically involved in the heme attachment to cytochrome b_6 [37]. These observations indicated that heme binding to cytochrome b_6 is catalysed in vivo by specific enzymatic machinery. Our data shows that in vitro apocytochrome b_6 and hemes spontaneously form a complex in the absence of any other proteins except MBP.

The hemes of cytochrome b_6 can be visualised after SDS-PAGE (without the boiling of samples) by heme staining. In contrast, the hemes of mitochondria and bacterial cytochrome *b* are not detected after electrophoresis [38,39]. Here it is demonstrated that hemes of reconstituted cytochrome b_6 could be visualised by heme staining after electrophoresis. This feature is similar to that of the native cytochrome b_6. The heme bound to cytochrome b_6 is only partially resistant to denaturing conditions (boiling of samples in 2% SDS) that destroy the noncovalent interaction between the *b* hemes and apoprotein, suggesting that one of the two *b* hemes of holocytochrome b_6 is tightly bound to the polypeptide. At least one heme binding in cytochrome b_6 from *Chlamydomonas* resisted boiling SDS, strongly suggest that one heme, most probably b_L, may be covalently attached to cytochrome [37]. The method of heme insertion into MBP–apocytochrome b_6 would not form such covalent binding. In spite of that, we can observe sequential liberation of hemes dependent upon time of boiling.

The experimental procedure, presented in this work, should allow for the introduction of mutations or deletions in the cytochrome b_6 protein sequence, and thus to determine the importance of particular amino acids or segments for feature of the cytochrome and the assembly of the heme–protein complex.

Acknowledgements

This work was supported by a grant from the State Committee for Scientific Research (grants no. 6 P04C 019 13 and 6 P04C 004 20) to A.S. and from the Deutsche Forschungsgemeinschaft to A.S. We thank Prof. A. Trebst and Prof. U. Johanningmeier for help in the early stage of this project.

References

[1] S.K. Buchanan, Curr. Opin. Struct. Biol. 9 (1999) 455–461.
[2] J.H. Kleinschmidt, L.K. Tamm, Biochemistry 35 (40) (1996) 12993–13000.
[3] A. Pautsch, J. Vogt, K. Model, C. Siebold, G.E. Schulz, Proteins 34 (1999) 167–172.
[4] N. Dekker, T. Merck, J. Tommassen, H.M. Verheij, Eur. J. Biochem. 232 (1995) 214–219.
[5] W.A. Cramer, P.N. Furbacher, A. Szczepaniak, G.-S. Tae, in: C.P. Lee (Ed.), Current Topics in Bioenergetics, vol. 16, Academic Press, Orlando, FL, 1991, pp. 179–222.
[6] W.A. Cramer, G.M. Soriano, M. Ponomarev, D. Huang, H. Zhang, S.E. Martinez, et al., Annu. Rev. Plant Physiol. Plant Mol. Biol. 47 (1996) 477–508.
[7] T. Kallas, in: D.A. Bryant (Ed.), The Molecular Biology of Cyanobacteria, Kluwer Academic Publishing, Dordrecht, The Netherlands, 1994, pp. 259–317.
[8] W.R. Widger, W.A. Cramer, R.G. Herrmann, A. Trebst, Proc. Natl. Acad. Sci. U. S. A. 81 (1984) 674–678.
[9] W.R. Widger, W.A. Cramer, in: L. Bogorad, I.K. Vasil (Eds.), Cell Culture and Somatic Cell Genetic of Plants, vol. 7B, Academic Press, London, 1991, pp. 149–176.
[10] C.R. Matthews, Annu. Rev. Biochem. 62 (1993) 653–683.
[11] E. London, H.G. Khorana, J. Biol. Chem. 257 (1982) 7003–7011.
[12] F.G. Plumley, G.W. Schmidt, Proc. Natl. Acad. Sci. 84 (1987) 146–150.
[13] H. Paulsen, U. Rumler, W. Rudiger, Planta 181 (2) (1990) 204–211.
[14] E. Giuffra, D. Cugini, R. Croce, R. Bassi, Eur. J. Biochem. 238 (1) (1996) 112–120.
[15] M.S. Hargrove, D. Barrick, J.S. Olson, Biochemistry 35 (1996) 11293–11299.
[16] C.R. Robinson, Y.F. Liu, J.A. Thomson, J.M. Sturtevant, S.G. Sligar, Biochemistry 36 (51) (1997) 16141–16146.
[17] D.E. Robertson, R.S. Farid, C.C. Moser, J.L. Urbauer, S.E. Mulholland, R. Pidikiti, et al., Nature 368 (1994) 425–432.
[18] H.K. Rau, W. Haehnel, J. Am. Chem. Soc. 120 (3) (1998) 468–476.
[19] L.D. Gruenke, J. Sun, T.M. Loehr, L. Waskell, Biochemistry 36 (23) (1997) 7114–7125.
[20] G.Y. Lee, J. Zhu, L. Yu, C.A. Yu, Biochim. Biophys. Acta 1363 (1) (1998) 35–46.
[21] J. Króliczewski, U. Johanningmeier, A. Szczepaniak, in: G. Garab (Ed.), Photosynthesis: Mechanism and Effects, Proceedings of the XIth International Congress of Photosynthesis, vol. III, Kluwer Academic Publishing, Budapest, Hungary, 1998, pp. 1560–1564, five volumes.
[22] R.G. Herrmann, in: M. Edelman, R.B. Hallick, N.-H. Chua (Eds.), Methods in Chloroplast Molecular Biology, Elsevier, Amsterdam, 1982, pp. 259–279.
[23] W. Heinemeyer, J. Alt, R.G. Herrmann, Curr. Genet. 8 (1984) 543–549.
[24] U.K. Laemmli, Nature 227 (1970) 680–685.
[25] J. Ozols, P. Strittmatter, J. Biol. Chem. 239 (1964) 1018–1023.
[26] C.T. Chang, C.-S.C. Wu, J.T. Yang, Anal. Biochem. 91 (1978) 13–31.
[27] Y.-H. Chen, J.T. Yang, K.H. Chau, Biochemistry 13 (1974) 3350–3359.
[28] J.T. Yang, C.-S.C. Wu, H.M. Martinez, Methods Enzymol. 130 (1986) 208–269.
[29] J.-M. Betton, M. Hofnung, J. Biol. Chem. 271 (1996) 8046–8052.
[30] P.E. Thomas, D. Ryan, W. Levin, Anal. Biochem. 75 (1) (1976) 168–176.
[31] A. Szczepaniak, W.A. Cramer, J. Biol. Chem. 265 (29) (1990) 17720–17726.
[32] R.D. Clark, G. Hind, Proc. Natl. Acad. Sci. U. S. A. 80 (1983) 6249–6253.
[33] E. Hurt, G. Hauska, FEBS Lett. 153 (1983) 413–419.
[34] M.-C. Hsu, R.W. Woody, J. Am. Chem. Soc. 93 (1971) 3515–3525.
[35] R.M. Wynn, J. Bertsch, D.B. Bruce, R. Malkin, Biochim. Biophys. Acta 935 (1988) 115–122.
[36] C. Lemaire, J. Girard-Bascou, F.-A. Wollman, P. Bennoun, Biochim. Biophys. Acta 851 (1986) 229–238.
[37] R. Kuras, C. deVitry, Y. Choquet, J. GirardBascou, D. Culler, S. Buschlen, et al., J. Biol. Chem. 272 (51) (1997) 32427–32435.
[38] E.A. Berry, L.S. Huang, V.J. DeRose, J. Biol. Chem. 266 (14) (1991) 9064–9077.
[39] P.O. Ljunghdal, J.D. Pennoyer, D.E. Robertson, B.L. Trumpower, Biochim. Biophys. Acta 891 (1987) 227–241.