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Heterologous expression and *in vitro* assembly of the transmembrane cytochrome b_6

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Abstract

Folding and assembly studies with α-helical membrane proteins are often hampered by the absence of high-level expression systems as well as by missing suitable *in vitro* refolding procedures. Experimental constraints and requirements for heterologous expression and *in vitro* assembly of cytochrome b_6 have been examined and conditions for *in vitro* reconstitutions of the protein have been optimized. Cytochrome b_6 can serve as an excellent model system for *in vitro* studies on the dynamic interplay of an apo-protein and heme cofactors during assembly of a transmembrane *b*-type cytochrome. *In vitro* assembled cytochrome b_6 binds two hemes with different midpoint potentials and both ferri as well as ferro heme bind to the apo-cytochrome. However, the ferro cytochrome appears to be less stable than the ferri form.

Keywords: Assembly; Cytochrome b_6; Heme; Midpoint potential

Protein synthesis and integration into a biological membrane are coupled processes *in vivo*, and integration of the protein into a membrane is mediated by large transmembrane channels or translocons [1,2]. This might conspire to make *in vitro* studies of membrane protein folding and stability a daunting enterprise. Fortunately, studies with bacteriorhodopsin have indicated about twenty years ago that folding of α-helical membrane proteins can be conceptually reduced to a few steps encapsulated in the Two-Stage-Model of α-helical membrane protein folding [3,4]. In this model inserting of individual helices into the membrane (Stage I) occurs prior to helix-helix association to form higher ordered oligomeric structures (Stage II). However, the model does not include influences of soluble domains and/or of bound cofactors. In general, little is known about the importance of cofactors for membrane protein folding, assembly, and stability. Mainly based on studies with bacteriorhodopsin, a third stage has been suggested, in which a cofactor is bound to a preformed transmembrane helix bundle [5]. In line with such a model it has recently been shown that heme binding to the transmembrane *b*-type cytochrome b_{559}' occurs at a late step and helix–helix interactions are an independent earlier step during assembly of the holo-cytochrome [6,7]. Several transmembrane *b*-type cytochromes are involved in charge transfer across membranes and are crucial for both respiratory and photosynthetic electron transfer. In contrast to *c*-type cytochromes, *b*-type cytochromes contain a non-covalently bound heme group, which is usually ligated by two histidine residues as the fifth and sixth ligands of the iron atom [8]. In general, transmembrane *b*-type cytochromes could serve as simple model systems

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to study assembly of cofactor containing membrane proteins.

Cytochrome b_6 is a core subunit of the cytochrome b_6f complex and the four helix bundle protein binds two heme molecules non-covalently [9]. The two heme molecules are ligated by conserved histidine residues, which are located in helices B and D of the protein. The structure of the cytochrome b_6f complex has been resolved recently by X-ray crystallography and, unexpectedly, a third heme molecule (heme c_x, or heme c_i) was found to be covalently bound to cytochrome b_6 [10,11]. Cytochrome b_6 from spinach can be heterologously expressed in *Escherichia coli* and the protein can be reconstituted *in vitro* [12,13]. Therefore, cytochrome b_6 could serve as an excellent model system for *in vitro* studies to analyze the role of the protein moiety and of the heme cofactors during assembly of a transmembrane *b*-type cytochrome. Folding and assembly studies with α-helical membrane proteins are often hampered by the absence of high-level expression systems as well as by missing suitable *in vitro* refolding procedures. Here we report an analysis of the experimental constraints and requirements for assembly of cytochrome b_6 *in vitro*. We have optimized conditions for *in vitro* reconstitutions and we show that the cytochrome assembles with both ferri and ferro heme. Nevertheless, we found that the ferro cytochrome is less stable than the ferri form.

Materials and methods

Expression and purification of cytochrome b_6

For large scale expression of spinach cytochrome b_6 the *petB* gene was amplified by PCR from genomic spinach DNA using the primers NTPetBspol (5′ atgagtaaagtctatg attggttcgaag 3′) and CTPetBspol (5′ cccaagcttttataagggacca ctgata 3′). After restriction digestion with HindIII the PCR product was ligated to the plasmid pRSET-H10Mal, which was restriction digested with HindIII and XmnI, finally resulting in the expression plasmid pRHMb6. Correct insertion of the PCR fragment was verified by DNA sequencing (MWG Biotech, Martinsried, Germany). Side directed mutagenesis was done using the Quick change mutagenesis kit (Stratagene, Amsterdam, The Netherlands) according to the manufactures instructions.

For protein production the expression plasmid pRHMb6 was transformed into *E. coli* HMS174 (DE3) cells. 10 mL LB medium with 100 μg/mL ampicillin was inoculated with a single colony and grown over night at 37 °C. 1 L LB medium with 100 μg/mL ampicillin was inoculated with the over night culture and at an $OD_{600} = 0.6$ protein expression was induced by addition of 0.5 mM IPTG to the growth medium. Cells were harvested after 3 h. For protein purification cells were broken by sonication in buffer (50 mM Hepes pH 7.5, 10 mM EDTA) and inclusion bodies were sedimented by centrifugation (10000*g*, 15 min, 4 °C). The supernatant was further separated by ultracentrifugation (190000*g*, 1 h, 4 °C) finally resulting in a soluble protein fraction and membranes.

The pellet, which contained the inclusion bodies, was washed twice with buffer containing 1% Triton X-100 to remove residual membrane fragments. Finally proteins were solubilized in 50 mM Tris pH 8.0, 50 mM NaCl buffer containing 50 mM SDS and incubated for 30 min at 60 °C. After incubation non-solubilized material was removed by centrifugation and the supernatant was further purified. 45 mM KCl was added to the protein solution to precipitate the bulk of SDS and to reduce the SDS concentration to 5 mM. The solution was incubated for 10 min on ice and precipitated KDS was removed by centrifugation. The supernatant was incubated with a Ni-NTA agarose matrix (Qiagen, Hilden, Germany) for 1 h at 4 °C. Afterwards, the affinity matrix was washed three times with buffer containing 2 mM SDS, and apo-cytochrome b_6 was finally eluted with buffer containing 2 mM SDS and 500 mM imidazole.

Folding and reconstitution

After purification in SDS buffer the protein was either dialyzed against buffer with SDS or the detergent was exchanged. To change the detergent (compare Table 1) another detergent was added to the protein solution and the remaining SDS was removed by addition of a 10-fold excess of KCl to precipitate KDS. After incubation on ice the sediment was removed by centrifugation and the remaining protein fraction was dialyzed over night against buffer containing the appropriate detergent. The following detergents were used: sodium dodecylsulfate (SDS)[2]; *t*-octylphenoxypolethoxyethanol (Triton X-100); *n*, *n*-dimethyldodecylamine-*n*-oxide (LDAO); *n*-octyl-β-D-glucopyranoside (OGP); *n*-dodecyl-*n*,*n*-dimethyl-3-ammonio-1-propanesulfate (SB-12); *n*-dodecyl-β-D-maltoside (DDM); 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS); sodium cholate; sodium deoxycholate; Nonylphenyl-polyethylenglycole (NP-40); polyethylenglycole sorbitane monolaurate (Tween 20). All detergents were purchased from Fluka (Buchs SG, Switzeland) or Sigma–Aldrich (Taufkirchen, Germany).

For reconstitution, hemine chloride (Fluka, Buchs SG, Swizerland) was dissolved in 50% ethanol and after addition of a few drops of a concentrated NaOH solution the solution was further diluted into buffer containing the appropriate detergent. After filtration of the solution the heme concentration was determined spectroscopically using an extinction coefficient of 56 mM^{-1} cm^{-1}. For cytochrome formation the heme was directly added to apocytochrome b_6 and cytochrome formation was followed by UV/Vis spectroscopy. Visible absorbance spectra were recorded on a Perkin-Elmer Lambda 35 instrument at 25 °C with a spectral bandwidth of 0.5 nm and a scan speed of 240 nm/min. The protein was measured either air-oxidized or after reduction with 5 mM sodium dithionite.

[2] *Abbreviations used:* SDS, sodium dodecylsulfate; LDAO, *n*,*n*-dimethyldodecylamine-*n*-oxide; OGP, *n*-octyl-β-D-glucopyranoside; MBP, Maltose binding protein; DDM, β-dodecyl-maltoside.