ACTA ABP BIOCHIMICA POLONICA

Vol. 58, No 3/2011
335–343
on-line at: www.actabp.pl

Regular paper

On the mode of integration of the thylakoid membrane protein cytochrome b_6 into cytoplasmic membrane of *Escherichia coli*

Jaroslaw Króliczewski[1], Beata Gubernator[1], Matthias Rögner[2] and Andrzej Szczepaniak[1]✉

[1]Laboratory of Biophysics, Faculty of Biotechnology, University of Wroclaw, Wrocław, Poland; [2]Lehrstuhl für Biochemie der Pflanzen, Ruhr-Universität Bochum, Bochum, Germany

In the stroma compartment, several pathways are used for integration/translocation of chloroplast proteins into or across the thylakoid membrane. In this study we investigated the mode of incorporation of the chloroplast-encoded cytochrome b_6 into the bacterial membrane. Cytochrome b_6 naturally comprises of four transmembrane helices (A,B,C,D) and contains two b-type hemes. In the present study, mature cytochrome b_6 or constructed deletion mutants of cytochrome were expressed in *E. coli* cells. The membrane insertion of cytochrome b_6 in this bacterial model system requires an artificially added presequence that directs the protein to use an *E. coli* membrane-insertion pathway. This could be accomplished by fusion to maltose-binding protein (MBP) or to the bacterial Sec-dependent signal peptide (SSpelB). The integration of mature cytochrome b_6 into the bacterial cytoplasmic membrane by the Sec pathway has been reported previously by our group (Kroliczewski *et al.*, 2005, *Biochemistry*, 44: 7570). The results presented here show that cytochrome b_6 devoid of the first helix A can be inserted into the membrane, as can the entire ABCD. On the other hand, the construct devoid of helices A and B is translocated through the membrane into the periplasm without any effective insertion. This suggests the importance of the membrane-anchoring sequences that are likely to be present in only the A and B part, and it is consistent with the results of computational prediction which did not identify any membrane-anchoring sequences for the C or D helices. We also show that the incorporation of hemes into the truncated form of cytochrome b_6 is possible, as long as the B and D helices bearing axial ligands to heme are present.

Keywords: membrane protein, cytochrome b_6, integration/translocation, signal sequence

Received: 07 December, 2010; revised: 26 May, 2011; accepted: 16 June, 2011; available on-line: 05 July, 2011

INTRODUCTION

Membrane proteins account for 20–25% of all open reading frames in sequenced genomes, and fulfill a wide range of central functions in cells (Wallim & von Heijne, 1998). Our knowledge regarding the assembly of this very important class of proteins is still poor and the functional and structural studies of membrane proteins have been strongly hampered due to the difficulties in overexpressing them in high yields. Little is also known about the mechanisms of targeting and insertion of chloroplast-encoded thylakoid membrane proteins despite their importance in chloroplast function (Rochaix, 1996; Choquet *et al.*, 1998). Chloroplasts are the site of photosynthesis and thylakoids are responsible for the crucial processes of light harvesting, electron transport and photophosphorylation. It is worth emphasizing that the chloroplast thylakoid is one of the most abundant membrane systems in nature. The structural and functional complexity of the chloroplast indicates that chloroplast proteins must be transported exactly to the correct compartment to reach their functional locations. Because chloroplasts are believed to have descended from prokaryotes, it can be postulated that the targeting and insertion pathways of the chloroplast-encoded proteins are likely to show strong similarities to those in the Gram-negative bacterium *Escherichia coli* (Schatz & Dobberstein, 1996). Indeed, several chloroplast homologues of *E. coli* targeting and insertion components have been identified (Schunemann, 2007; Cline & Dabney-Smitch, 2008; Aldridge *et al.*, 2009). The insertion of some thylakoid membrane proteins (cytochrome *f*, cytochrome b_6) expressed in *E. coli* into the bacterial cytoplasmic membrane has also been demonstrated (Rothstein *et al.*, 1985, Kroliczewski *et al.*, 2005).

Commonly, proteins are targeted to the membrane by hydrophobic amino-acid sequences which are either transmembrane segments (TMs) of membrane proteins or cleavable signal sequences (SSs). Typically, receptors located within the membrane recognize the hydrophobic amino-acid sequence and the membrane-embedded translocation machinery promotes the insertion into or transport across the membrane.

It is thought that in the chloroplast stroma, four transport pathways are used for the targeting of chloroplast proteins into or across the thylakoid membrane (Robinson *et al.*, 2001). These pathways are named the cpSec-dependent, cpSRP-dependent, ΔpH-dependent, and spontaneous (unassisted) pathways (Cline *et al.*, 1992; Sundberg *et al.*, 1997; Woolhead *et al.*, 2001; Yi & Dalbey, 2005; Aldridge *et al.*, 2009), and all of them have direct cognates in bacteria (Schatz & Dobberstein, 1996). The chloroplast cpSec pathway evolved from the secretory pathway involved in export of Sec-dependent proteins to the periplasm in bacteria. In *E. coli* the functional translocon of the Sec-dependent pathway contains at least three components: SecA, SecY and SecE. In

✉e-mail: andrzej.szczepaniak@ibmb.uni.wroc.pl

Abbreviations: BCA, bicinchoninic acid; BSA, bovine serum albumin; DTT, dithiothreitol; HMM, Hidden Markov Model; IPTG, isopropyl β-D-thiogalactopyranoside; LB, Luria–Bertani medium; MBP, maltose-binding protein; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; SPP, stroma processing peptidase; SS, signal sequence; SSpelB, Sec-dependent signal peptide; STS, signal anchor sequence; TCA, trichloroacetic acid; TM, transmembrane

post-translational transport, the SS of the preprotein interacts with SecA in the cytoplasm and the formed complex (SecA-preprotein) associates with the membrane components composed of SecY and SecE integral proteins which form the transmembrane channel (Aldridge *et al.,* 2009). The channel itself is a passive conduit for transported polypeptides and SecA is an ATPase that provides the driving force for translocation of the protein through the Sec channel pore. SecA is also a binding partner of SecY. Generally, it is accepted that the SecY core channel complex is a heterotrimer consisting of SecY, SecE and SecG subunits (Rapoport *et al*., 1996), which was confirmed by a crystal structure study of detergent-solubilized SecY complex from the archebacterium *Methanococcus jannaschii* (van der Berg *et al*., 2004). However, in *E. coli* only SecY and SecE subunits are required for cell viability while SecG is not.

In the chloroplast, homologues to SecA (cpSecA), SecY (cpSecY) and SecE (cpSecE) have been identified (Yuan *et al*., 1994; Roy & Barkan, 1998; Schuenemann *et al*., 1999), while a homologue to SecB has not been revealed. However, similar to bacteria, transport by the chloroplast Sec translocon requires the transported protein substrates to be in an unfolded conformation (Hynds *et al*., 1998; Marques *et al*., 2004). Because in the chloroplast the SecB homologue is absent, another as yet unknown stromal factor must be involved in stabilization of preprotein substrates in an unfolded state. In this context it seems that in the chloroplast the cpSec pathway in the cpSecA-cpSecYE configuration appears to operate much like the bacterial system in which the channel formed by SecYE is a passive conduit for unfolded protein substrates and the SecA ATPase is a pushing translocation motor.

The chloroplast-encoded cytochrome b_6 is an integral protein which consists of four transmembrane helices (A,B,C,D) that produce a proper environment for incorporation of two b-type hemes. Like many other integral proteins, cytochrome b_6 operates with an uncleaved signal for insertion into the biological membrane (Zhang *et al*., 2001). We decided to study this uncleaved signal for the insertion of cytochrome b_6 into a bacterial membrane by expressing the constructed cytochrome deletion mutants in *E. coli*. In a previous paper (Kroliczewski *et al*., 2005) we showed that the fusion of cytochrome b_6 to MBP directs the cytochrome to the Sec-dependent pathway and the cytochrome b_6 has a native structure in the bacterial cytoplasmic membrane with both NH_2 and COOH termini on the same, periplasmic side of the membrane. Dalbey and Kuhn have proposed that insertion of membrane proteins by Sec translocase requires that each hydrophobic anchor region is sensed during transport, further translocation of the protein is halted, and the anchor region is laterally released from the central channel region (Dalbey & Kuhn, 2004).

The data presented herein indicate that the insertion of mature cytochrome b_6 or its constructed deletion mutants into the cytoplasmic bacterial membrane is absolutely dependent on the presence of an N-terminal presequence artificially added which directs the protein to the *E. coli* membrane-insertion pathway. We also show that the proper folding and anchoring of the constructs into the bacterial membrane depends on its N-terminal part bearing helices A and B (first about 110 amino acids) and the incorporation of hemes is possible even into a truncated form of cytochrome b_6, as long as helices B and D bearing the axial ligands to heme are present.

MATERIALS AND METHODS

Prediction of signal sequences (SSs) and signal anchor sequences (STSs) in apocytochrome b_6. The amino-acid sequence of apocytochrome b_6 from spinach was used for the prediction of signal sequences (SSs) by the network tool SignalP ver. 3.0 (http://www.cbs.dtu.dk/services/SignalP). SignalP ver. 3.0 uses neural networks (NN) and hidden Markov models (HMM) to predict "secretory proteins" through analysis of protein N-terminal sequence (Nielsen & Krogh, 1998). These programs are among the most accurate methods for "secretory protein" prediction (Nielsen *et al*., 1999; Emanuelsson & von Heijne, 2001; Klee & Ellis, 2005) and the programs' HMMs have an uncommon ability to discriminate N-terminal SSs from N-terminal signal anchor sequences (STSs).

Construction of derivatives of the expression plasmid pMalp2b6. The recombinant plasmid pMal-c2b6 containing the gene (*petB*) for spinach apocytochrome b_6 (Kroliczewki & Szczepaniak, 2002) was used as a template for the creation of apocytochrome b_6 derivatives (Fig. 1). A fragment containing the appropriate part of the *petB* gene was amplified in each case from the plasmid using two primers. One of the primers was designed to introduce an *Xmn*I restriction site 5', and the second one to introduce a *Bam*HI restriction site 3' to the *petB* fragment. The primers were designed from the known sequence of *petB* gene from the chloroplast DNA of spinach (Heinemeyer *et al*., 1984). The PCR products were cleaved by double digestion with *Xmn*I (Promega) and *Bam*HI (Fermentas) and purified using a PCR purification kit (Macherey-Nagel). The purified products were cloned into the *Xmn*I and *Bam*HI sites of the pMal-p2 expression vector (New England Biolabs) by a standard T4 DNA ligase procedure. Resulting plasmids (Fig. 1) pMal-p2ABC, pMal-p2AB, pMal-p2A, pMalp2BCD, pMal-p2CD, pMal-p2C, pMal-p2BC and pMal-p2B were checked by restriction analysis and sequenced. Expression of the constructed vectors led to production of intact cytochrome b_6 or its fragments as fusion proteins with MBP. Between MBP and the cytochrome b_6 fragments an amino-acid sequence recognized by factor Xa was localized.

Construction of expression plasmids pET25b6 and pET25CD. Spinach apocytochrome b_6 *petB* gene or the part of the gene encoding helices C and D were amplified by PCR from isolated spinach chloroplast DNA (Herrmann, 1982). The forward primers 5'-GGCAGTGCCCATGGGTGTATCTTACCGGTGGGT-3' and 5'-CATGCCATGGATGTTGACTTGGGTTACAGGC-3' carrying the *Nco*I site (underlined) for the amplification of apocytochrome b_6 or the part encoding helices C and D (respectively) and the reverse one carrying *Bam*HI site (underlined) 5'-GGCAGTGCGGATCCCTATAAGGGACCAGAAATA-3' were designed from the known sequence of the *petB* gene from the chloroplast DNA of spinach (Heinemeyer *et al*., 1984). The amplified PCR products were purified, digested and then cloned into the pET25b vector (Novagen) treated with *Nco*I and *Bam*HI restriction enzymes. The resulting plasmids (Fig. 1) pET25b6 and pET25CD were checked by restriction analysis. Expression of the constructed vectors led to the production of intact cytochrome b_6 or its fragment as fusion proteins with SSpelB.

Expression of recombinant apocytochrome b_6 in *E. coli* CM 124-SecE depletion mutant. The SecE-

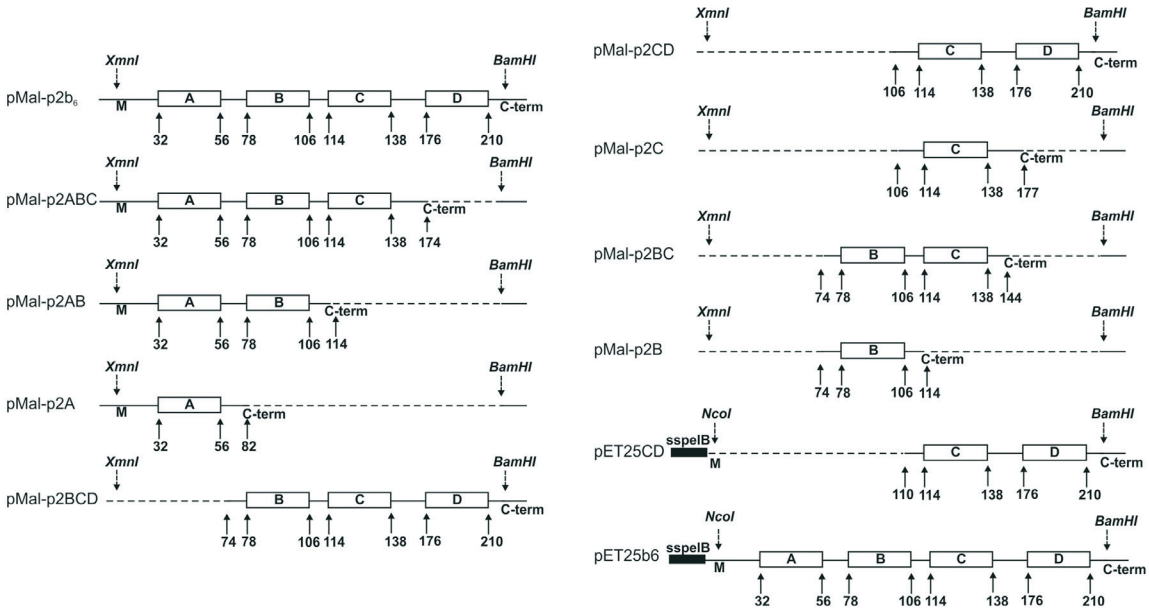

Figure 1. Diagram of *petB* gene constructs used to overexpress cytochrome b_6 mutants
DNA sequence corresponding to the transit peptide in pET25CD or pET25b6 is indicated by solid black box. Positions of membrane-spanning regions are indicated by open boxes. Numbers refer to the amino-acid sequence of apocytochrome b_6 where the initiating methionine (M) is 1. Relevant restriction sites are indicated by arrows. The deleted regions are indicated by dashed lines.

depletion strain CM 124 cells (Traxler & Murphy, 1996) were transformed with pMal-p2b6 (for cytochrome b_6 expression as a fusion protein with MBP) or pET25b6 (for cytochrome b_6 expression as a fusion protein with SSpelB) and grown aerobically at 37 °C in M63 minimal medium supplemented with 0.4% D-glucose or 0.2% L-arabinose and 100 μg/ml ampicillin. For all experiments, 2 ml of overnight culture was pelleted, washed once with M63 medium and diluted 1:20 into 20 ml per 125 ml flask to obtain mid-logarithmic phase growth. To deplete the cells of SecE, cells were grown in the absence of L-arabinose. The production of the fusion protein (MBP-apocytochrome b_6 or SSpelB-apocytochrome b_6) was induced by the addition of IPTG to a final concentration of 0.3 mM. After shaking for 1.5 h, cells were harvested by centrifugation (4000×*g*, 10 min, 4°C), resuspended in 15 ml of 30 mM Tris/HCl, pH 8.0 buffer containing 20% sucrose and 1 mM EDTA, then incubated for 10 min at room temp. with gentle shaking. The culture was centrifuged at 8000×*g* for 10 min at 4 °C. The resulting pellet was resuspended in 10 ml of ice-cold 5 mM $MgSO_4$, shaken for 10 min at 4 °C, and centrifuged as above. The obtained supernatant was the cold osmotic shock fluid. The resulting pellet of *E. coli* was frozen at –20 °C and used for further membrane and cytoplasmic protein preparation. For overexpression of functional SecE, L-arabinose was added 1.5 h after IPTG induction and the cultures were grown for the next 30 min, or L-arabinose was added together with IPTG (control expression).

Expression of recombinant apocytochrome b_6 derivatives in *E. coli*. TB1 cells (New England Biolabs) transformed with pMal-p2b6 or pMal-p2b6 derivatives, or 21(DE3) cells (Novagen) transformed with pET25b6 or pET25CD were grown aerobically at 37 °C in 1 liter of LB medium (10 g Bacto-tryptone, 5 g yeast extract, 5 g NaCl per liter) adjusted to pH 7.5 with NaOH, containing 100 μg/ml ampicillin and inoculated with 10 ml of overnight cultures. The production of the fusion protein (MBP-apocytochrome b_6 or MBP-apocytochrome b_6 derivatives and SSpelB-apocytochrome b_6 or SSpelB-CD) was induced by the addition of IPTG to the final concentration of 0.1 to 0.5 mM at an OD_{560} of 0.6. After shaking for 4 hours, cells were harvested by centrifugation (5000×*g*, 15 min, 4 °C), resuspended in 30 ml of TEN buffer (20 mM Tris/HCl, pH 7.8, 10 mM EDTA, 100 mM NaCl, 1 mM PMSF) and stored at –20 °C overnight.

Membrane preparation from *E. coli* cells. After thawing the frozen *E. coli* cells, the suspension (30 ml) was sonicated on ice (5×2 min; 70 W with 20 kHz homogeneous sound 3 mm diameter microtip sonifier cell disrupter, Sonopuls HD 2070, Bandelin) and the lysate was centrifuged at 27000×*g* for 20 min at 4°C. The supernatant was centrifuged again at 27000×*g* for 20 min at 4°C to pellet inclusion bodies debris, and the resulting supernatant was centrifuged for a further 2 h at 70000×*g* at 4°C. A suspension of the membrane sediment in TEN buffer was centrifuged again at 70000×*g* for 90 min at 4°C. The inclusion bodies and membrane fractions were examined by SDS/PAGE (Betton & Hofnung, 1996) and Western blotting.

Preparation of spheroplasts. *E. coli* carrying pMal-p2b6 or pMal-p2b6 derivative plasmids were grown in a standard LB medium containing 100 μg/ml of ampicillin. Induction was initiated at OD_{560} about 0.6 by the addition of IPTG to the final concentration of 0.5 mM. After 4 h of shaking at 37 °C, cells were carefully harvested by centrifugation (3000×*g*, 10 min, 4°C) and spheroplasts were prepared. Cell pellets were resuspended in buffer I (10 mM Tris/HCl, pH 7.5, 10 mM EDTA, 5 mM $MgSO_4$, 0.7 M sucrose, 1 mM PMSF) and centrifuged at 5000×*g* for 10 min at 4 °C. The pellets were resuspended in 10 ml of ice-cold 5 mM $MgSO_4$ and incubated for 10 min on ice, centrifuged as above, then resuspended once more in buffer

I containing 0.2 mg/ml of lysozyme and incubated at room temp. for 25 min. The sample was centrifuged at 5000 × *g* for 10 min at 4°C and the supernatant was removed. The pellet was resuspended in buffer I and washed twice. The fresh spheroplasts were used for agglutination tests.

Agglutination of spheroplasts. The spheroplasts were incubated with an antibody against MBP or against the COOH-terminal decapeptide (Ile 206 to Leu 215) of cytochrome b_6 (Szczepaniak & Cramer, 1990). The agglutination was analyzed by optical microscopy, immediately after the addition of the antibody.

Spectra measurements. Visible absorption spectra were recorded at room temp. in 1 cm optical-path length cuvettes (1 ml) using an M40 spectrophotometer (Carl Zeiss Jena) with a spectral bandwidth of 1 nm and a scan speed of 300 nm/min. Spectra were usually recorded in 50 mM Tris/HCl, pH 8.0 buffer containing 50 mM NaCl, 0.1% SDS and 10% glycerol. The samples were oxidized by adding 100 mM ferricyanide and reduced by the addition of 100 mM dithionite.

Measurements of protein concentration. MBP concentration was determined directly from the absorbance at 280 nm with an absorbance coefficient of $\varepsilon = 68750\ M^{-1}cm^{-1}$ (Betton & Hofnung, 1996). The concentration of fused protein was determined by using the BCA reagent (Sigma) according to the manufacturer's instructions with BSA as a standard.

Proteolysis of spheroplasts. Spheroplasts (50 μl, 5 mg protein/ml) were incubated at room temp. T with factor Xa for 45 min at a w/w ratio of 0.5% of the amount of fusion protein. Cleavage was carried out in 10 mM Tris/HCl, pH 8.0 buffer containing 10 mM NaCl, and 0.05% SDS. The reaction with factor Xa was terminated by the addition of PMSF to a final concentration of 5 mM. The spheroplasts were pelleted, washed twice with buffer I (see section: Preparation of spheroplasts), resuspended in the same buffer and used for agglutination assay.

Cleavage with factor Xa. Cleavage with factor Xa of bacterial membranes containing fusion protein was carried out at a w/w ratio of 0.4% of total protein. The reaction mixtures in 20 mM Tris/HCl, pH 8.0 buffer containing 100 mM NaCl, 2 mM $CaCl_2$ and 0.03% SDS were incubated for 10.5 hours at room temp. The reaction was blocked by the addition of PMSF to a final concentration of 5 mM. The samples containing bacterial membranes were ultracentrifuged at 70000 × *g* for 2 h at 4°C. The pellets were resuspended in 1 ml of 20 mM Tris/HCl, pH 8.0, then precipitated with TCA (using 36% TCA solution to final concentration of 6%) and subjected to SDS/PAGE followed by Western blotting. In the case of dissolved fusion proteins after cleavage with factor Xa the separation of protein from MBP was carried out as described by Kroliczewski & Szczepaniak (2002).

SDS/PAGE and Western blotting. Proteins were separated on 15% polyacrylamide gel containing 8 M urea. Denaturation of samples was achieved by mixing the protein sample or bacterial spheroplast pellets with solubilization buffer (300 mM Tris/HCl, pH 6.8, 60% glycerol, 12 mM EDTA, 12% SDS, 864 mM 2-mercaptoethanol, 0.05% bromophenol blue) in a v\v ratio of 5:1. For membrane proteins, 2-mercaptoethanol was replaced with 480 mM DTT in a solubilization buffer. Western blotting was carried out as described by Szczepaniak & Cramer (1990).

RESULTS

The role of SecE in apocytochrome b_6 insertion into bacterial membrane

In a previous paper (Kroliczewski *et al.*, 2005) we showed that the fusion of apocytochrome b_6 to MBP directed the apocytochrome to the Sec-dependent pathway. In additional experiments, we examined the dependence of apocytochrome b_6 and fusion apocytochrome b_6 insertion on the SecE component of the secretion apparatus (Sec) of *E. coli*. In this case, we used the *E. coli* CM124 strain in which SecE can be depleted efficiently. In this strain, expression of the *secE* gene is under the control of the arabinose-inducible *araBAD* promoter. In the presence of glucose and absence of arabinose, SecE is not expressed.

In our experiment CM124 cells carrying pMal-p2b6 or pET25b6 (Fig. 1) were grown in the absence or presence of arabinose, then the insertion of MBP-apocytochrome b_6 or SSpelB-apocytochrome b_6 fusions into the cytoplasmic bacterial membrane was investigated (Fig. 2). When SecE was depleted (arabinose was not present in the medium) both MBP-apocytochrome b_6 and SSpelB-apocytochrome b_6 fusions were found exclusively in the cytoplasmic fraction (Fig. 2AB, lane 3). On the other hand, in the presence of arabinose, expression of the *SecE* gene led to the insertion of the investigated fusions into the bacterial membrane (Fig. 2AB, lane 6).

The results presented here show that the insertion of the polytopic apocytochrome b_6 fused to MBP or SSpelB into the cytoplasmic membrane is SecE-dependent.

Prediction of signal peptides and signal anchor sequences in apocytochrome b_6

In a search for potential signal sequences and signal anchor sequences in the apocytochrome b_6 sequence, we decided to carry out a computational analysis. Most prediction programs predict "secreted proteins" by identifying an N-terminal signal sequence, a signal sequence cleavage site or a combination of both features in a target sequence. In our case, for the prediction of signal sequence (SS) and/or anchor signal sequence (STS) we chose the program SignalP ver. 3. SignalP uses two HMMs, one that models a signal peptide and the second

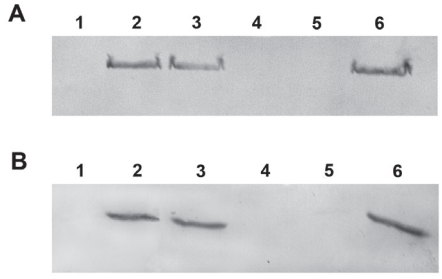

Figure 2. Effect of SecE depletion on MBP-apocytochrome b_6 and SSpelB-apocytochrome b_6 location in *E. coli*
(A) Western blot analysis of MBP-apocytochrome b_6 fusion protein expressed in CM124 strain. Lane 1, total protein fraction from *E. coli* cells before induction; lane 2, total protein fraction from *E. coli* cells after induction; lane 3, cytoplasmic fraction; lane 4, periplasmic fraction; lane 5, membrane fraction; lane 6, membrane fraction isolated after culturing with arabinose. **(B)** Western blot analysis of SSpelB-apocytochrome b_6 fusion protein expressed in CM124 strain. Lane description as in panel A. For immunodetection, polyclonal antibody against COOH-terminal part of apocytochrome b_6 was used. On each lane, 20 μg of protein was applied.

that models a signal anchor. N-terminal signal peptide is a short polypeptide (20–25 amino-acid residues) and has no strongly conserved sequence motifs but includes three distinct sequential regions: the n (N-terminal)-region, the h (hydrophobic)-region and the c (C-terminal)-region (von Heijne, 1985; 1986). The signal peptide model contains submodels that describe each of these three regions. The signal anchor model contains two submodels that characterize its n-region and h-region. In the signal peptide model the h-region is limited to between six and twenty residues, the n-region must have at least one residue (and start with a methionine), and the c-region must have at least three residues. The n-region and c-region contain self-cyclic states with exponentially decaying transitions. This type of transition state allows the model to fit signal peptides possessing n-regions and c-regions with variable lengths, while still constraining the system, preventing unusually long region lengths, and thereby encapsulating the known properties of these regions. In the signal anchor module, the architecture of the n-region is the same as in the signal sequence module, but the h-region also possesses a self-cyclic, exponentially decaying transition state (Nielsen *et al.*, 1997; Nielsen & Krogh, 1998; Klee & Ellis, 2005).

Signal anchors are also referred to as uncleaved signal peptides. However, they often have sites similar to signal peptide cleavage sites behind their hydrophobic (transmembrane) region. Therefore, a prediction method can easily be expected to mistake signal anchors for signal peptides. It is worth noting that most of eukaryotic signal anchor sequences (60%) have mean S-scores from 0.35 to 0.70 and, in the case of protein signal sequences, the mean S-scores are greater than 0.65, while non-signal sequences have mean S-scores from 0.0 to 0.25. The aforementioned data indicate that the mean S-score for signal anchors shows some overlap with the signal peptide distribution; however, signal anchors are generally significantly longer than signal peptides. Table 1 shows the results of the signal anchor prediction made by SignalP ver. 3 for apocytochrome b_6 deletion mutants (a maximum of the first 70 amino acids of each constructed mutant sequence were used for prediction).

For non-secretory proteins, all the S-scores represented in the SignalP ver. 3 output should ideally be very low. The HMM reports the probability of a signal anchor. The cleavage site is assigned by the probability score together with the scores for every amino acid in the n-region, h-region and c-region of the signal peptide, if one is found. Not all secretory proteins carry signal peptides or similar targeting signals. Some proteins enter the nonclassical secretory pathway without any currently known sequence motif (Bendtsen *et al.*, 2005).

Expression of apocytochrome b_6 mutants in *E. coli*

Figure 1 illustrates the derivatives of cytochrome b_6 constructed for this study. They were made from spinach *petB* gene encoding cytochrome b_6. Different regions of cytochrome b_6 were fused to the carboxyl terminus of periplasmic bacterial MBP or to the carboxyl terminus of SSpelB in an attempt to identify regions of cytochrome b_6 which function as SSs or STSs. The predicted topology and orientation of the expressed apocytochrome b_6 mutants in bacterial cytoplasmic membrane are shown in Fig. 3. Having established that apocytochrome b_6 overexpressed as a fusion protein to MBP can be incorporated into bacterial cytoplasmic membrane (Kroliczewski *et al.*, 2005), we tested whether the removal of a specific portion of mature cytochrome b_6 (related to helices A, B, C or D) would disrupt the proper incorporation.

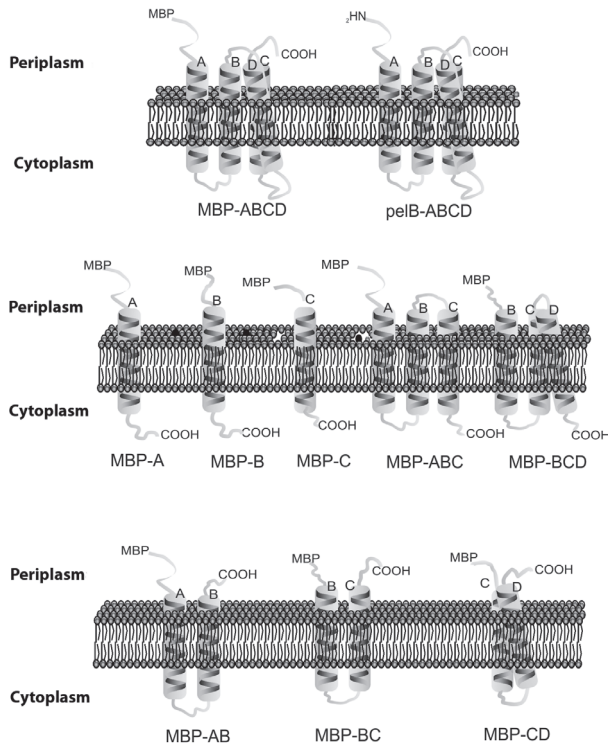

Figure 3. Expected topology and orientation of apocytochrome b_6 mutants in bacterial cytoplasmic membrane
Membrane helices are named according to apocytochrome b_6 terminology.

To determine the subcellular localization of the expressed proteins, periplasm, cytoplasm and membranes were isolated from the induced cells. The protein content of these fractions was investigated by Western blotting. The subcellular localization of the expressed deletion mutants, MBP-BCD and MBP-BC, revealed that both fusion proteins were exclusively found in the bacterial membrane (Fig. 4A, lane 4 and Fig. 4B, lane 1). A subcellular analysis of the mutants MBP-ABC, MBP-AB and MBP-A was carried out as well (not shown) and they were detected only in the membrane fractions (Fig. 5).

In contrast, the MBP-CD and MBP-C mutant fusion proteins were found exclusively in the periplasm (Fig. 6AB). To make sure that the transport of truncated apocytochrome b_6 containing only C and D transmem-

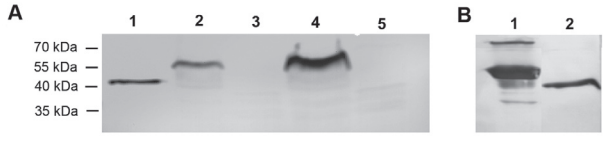

Figure 4. Western blot analysis of isolated cell subfractions from induced *E. coli* cells carrying pMal-p2BCD or pMal-p2BC plasmid
(A) Cells overexpressing MBP-BCD. Lane 1, MBP; lane 2, total protein fraction; lane 3, cytoplasmic fraction; lane 4, membrane fraction; lane 5, periplasmic fraction. **(B)** Cells overexpressing MBP-BC. Lane 1, membrane fraction; lane 2, MBP. For immunodetection, polyclonal antibody against MBP was used. On each lane, 20 mg of protein was applied.

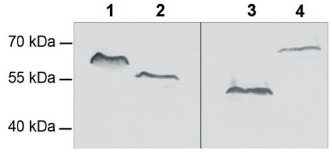

Figure 5. Western blot analysis of membrane fractions isolated from induced *E. coli* cells carrying pMal-p2ABC, pMal-p2AB, pMal-p2A or pMal-p2b6 plasmid
Lane 1, overexpressed MBP-ABC fusion protein; lane 2, overexpressed MBP-AB fusion protein; lane 3, overexpressed MBP-A fusion protein; lane 4, overexpressed MBP-apocytochrome b_6 fusion protein. For immunodetection, polyclonal antibody against MBP was used. Each lane contains 20 μg of protein of bacterial membranes.

brane helices to the periplasm is not caused by MBP, we constructed a new expression vector, pET25CD. In this plasmid, the truncated apocytochrome b_6 containing only C and D transmembrane helices was fused to pelB signal sequence (SSpelB). The fusion to SSpelB directs truncated apocytochrome b_6 to the Sec-dependent pathway — similar to MBP in expression vectors derived from pMal-p2. The results of the expression of pET25CD vector revealed that the mutant protein was also found exclusively in the periplasm (not shown).

Determination of apocytochrome b_6 mutant topology in the bacterial inner membrane

To determine the topology of all fusions, *E. coli* cells bearing the appropriate plasmid were induced and then converted to spheroplasts. Next, the obtained spheroplasts were incubated either with an antibody against MBP or with an antibody against COOH-terminal decapeptide of cytochrome b_6. As a control, we used uninduced or induced cells carrying pMal-p2b6 or pMal-c2b6, respectively (negative controls), and induced cells carrying Malp2b6 plasmid (positive control). As an additional (negative/positive) control, we used induced cells carrying pMal-p2b6 converted to spheroplasts followed by factor Xa treatment. The results are presented in Table 2. As it is shown, after the induction of pMal-p2A, pMal-p2B, pMal-p2AB, pMal-p2BC or pMal-p2BCD vectors and incubation of spheroplasts with an antibody against MBP, a strong or medium agglutination of the spheroplasts was observed. Such results clearly demonstrate the periplasmic location of MBP and anchoring of the studied apocytochrome b_6 deletion mutants into the bacterial cytoplasmic membrane.

Only spheroplasts derived from cells bearing the pMal-p2b6 plasmid have the possibility of showing agglutination after incubation with antibody against COOH-terminal decapeptide of cytochrome b_6. The observed lack of this type of agglutination implies that the COOH terminus of apocytochrome b_6 mutants is located on the cytoplasmic side of the membrane. Additional experiments on spheroplasts bearing MBP-BCD fusion and treated with factor Xa show a lack of agglutination with an antibody against MBP. Such results clearly demonstrate that the NH_2 terminus of the studied apocytochrome b_6 deletion mutant is on the periplasmic side of the bacterial membrane, as predicted (Fig. 3).

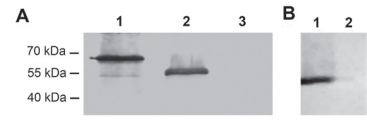

Figure 6. Western blot analysis of isolated cell subfractions from induced *E. coli* cells carrying pMal-p2CD or pMal-p2C plasmid
(A) Cells overexpressing MBP-CD. Lane 1, MBP–apocytochrome b_6; lane 2, periplasmic fraction; lane 3, membrane fraction. **(B)** Cells overexpressing MBP-C. Lane 1, periplasmic fraction; lane 2, membrane fraction. For immunodetection polyclonal antibody against the COOH-terminus part of apocytochrome b_6 was used. On each lane, 20 μg of protein was applied.

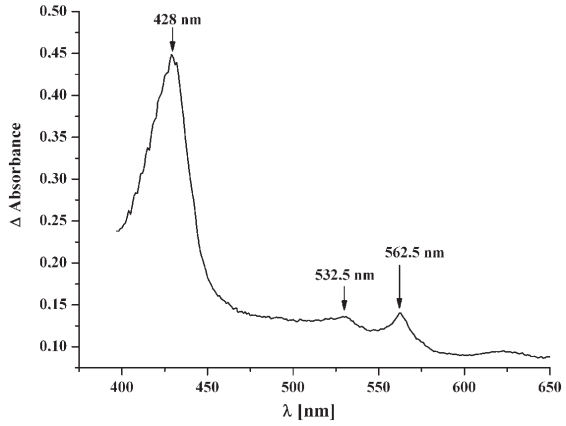

Figure 7. Redox difference spectrum of reconsituted BCD mutant of cytochrome b_6
Ferricyanide and dithionite were used as oxidant and reductant, respectively. For details, see the text.

Heme incorporation

It has been shown previously that in the *E. coli* cytoplasmic membrane apocytochrome b_6 and exogenic heme added to the culture medium spontaneously form a complex with similar spectroscopic properties to native cytochrome b_6 (Kroliczewski *et al.*, 2005). Among the apocytochrome b_6 mutants only MBP-BCD has the possibility to bind hemes because of possessing the B and D helices bearing the axial ligands to heme. Indeed, when heme was present in the culture media, during the protein expression from the pMal-p2BCD vector, the dithionite-reduced spectra of isolated cytoplasmic membrane demonstrated the presence of b-type heme. After cutting off and isolating MBP, the absorption spectra of the reduced form revealed the characteristic Soret (γ-band) at 428 nm and prominent β and α bands at 532.5 nm and 562.5 nm, respectively (Fig. 7), and were similar to those of isolated cytochrome b_6 (Stuart & Wasserman, 1973; Boronowsky *et al.*, 2001). These results demonstrate the presence of properly reconstituted hemes in the protein structure of the BCD deletion mutant and also suggest proper topology of the BCD mutant, with the NH_2-terminus on the periplasmic and the COOH-terminus on the cytoplasmic side of the membrane, as predicted (Fig. 3).

DISCUSSION

It appears that in *E. coli* transport across or into the bacterial membrane is carried out by at least four independent precursor-specific transport pathways: the SRP-dependent, TAT/ΔpH-dependent, spontaneous and Sec-dependent pathways (Froderberg *et al.*, 2003; du Plessis *et al.*, 2006). The fusion of polytopic cytochrome b_6 to

Table 1. Signal anchor sequence (STS) and signal sequence (SS) predictions made by SignalP ver. 3 for apocytochrome b_6 deletion mutants

Mutant*	Signal sequence probability	Signal anchor probability	Mean S-score
ABC AB A	0.000	0.420	0.163
BCD BC	0.024	0.864	0.628
B	0.020	0.258	0.625
CD C	0.658	0.333	0.689

*A, B, C, D stand for appropriate helices

MBP directs the cytochrome to the Sec-dependent pathway, which also requires SecA.

The endosymbiont hypothesis has spurred parallels between the translocation or insertion of proteins through or into *E. coli* cytoplasmic membrane and the translocation or insertion of chloroplast proteins through or into thylakoid membranes (Schatz & Dobberstein, 1996; Dalbey & Kuhn, 2004). This encouraged us to use *E. coli* for studies of cytochrome b_6 incorporation into the biological membrane. In a previous paper (Kroliczewski *et al.*, 2005), we showed that the topogenic signals in the amino-acid sequence of the nascent chain of the chloroplast cytochrome b_6 protein were recognized by the *E. coli* SecYEG translocon, leading to the integration of this protein into the bacterial inner membrane. Cytochrome b_6 has a native structure in the bacterial cytoplasmic membrane but has an opposite orientation compared to that in the thylakoid (Szczepaniak & Cramer, 1990; Kurisu *et al.*, 2003). Recently it has been reported that polytopic cytochrome b_6 (which naturally contains four transmembrane helices: A,B,C,D) can spontaneously insert into bacterial cytoplasmic membrane (Dreher *et al.*, 2007). Such a conclusion is in opposition to our earlier findings. That is why we decided to perform additional experiments to verify the inconsistent data. We took up the study with *E. coli* CM124 cells in which SecE (integral subunit of SecYE translocon) could be depleted. The obtained results show that apocytochrome b_6 expressed in cells with depleted SecE is found only in the cytoplasm (Fig. 2AB, lane 3), with no signal deriving from apocytochrome b_6 detected in the membrane fraction (Fig. 2AB, lane 5). However, expression in the presence of arabinose leads to incorporation of apocytochrome into the bacterial membrane (Fig. 2AB, lane 6). These results clearly demonstrate that the insertion of apocytochrome b_6 into the membrane is strongly dependent on SecE and no spontaneous incorporation was observed. So, the insertion of polytopic cytochrome b_6 into the cytoplasmic bacterial membrane is absolutely dependent on the presence of an N-terminal presequence artificially added, and its N-terminus is always transported to the periplasmic side of the bacterial membrane (Kroliczewski *et al.*, 2005). Here arises the question: where does from the mentioned divergence of the results obtained by Dreher *et al.* (2007) and our group come? In the cited paper the presence of cytochrome b_6 in the membrane was indicated only by measurements of visible spectra of membranes isolated from *E. coli*. From the literature it is known that exogenic heme can interact with the membrane itself or *via* other proteins in a non-specific manner (Cassoly, 1978; Marden *et al.*, 1989; Solar *et al.*, 1989; Solar & Shaklai, 1989; Leclerc *et al.*, 1993). Such unspecific interaction of heme with bacterial membrane was also observed by us in a previous study (Kroliczewski *et al.*, 2005). In this context, the results presented by Dreher *et al.* (2007) should be verified for example in washing experiments of isolated bacterial membrane with chaotropic agents (in such conditions integral proteins are not washed out from the membrane) or in experiments with spheroplasts (prepared from induced bacterial cells) using proper antibodies/proteases which could allow the study of the transmembrane topology of expressed cytochrome. Such experiments were not done in the research in question. Moreover, the visible absorption spectra of reduced cytochrome b_6 reveal specific bands characterized as α, β and γ, named the Soret band. In the discussed paper, visible spectra of reduced membranes isolated from bacteria do not reveal a characteristic well-marked Soret band. Additionally, to confirm that visible spectra are characteristic for cytochrome b_6 it is recommended to analyze the width of the α-band at the half-height; such analysis was not carried out either. In light of the presented facts, in our opinion the authors (Dreher *et al.*, 2007) could observe signals deriving from hemes interacting with the bacterial membrane in a non-specific manner.

Table 2. Agglutination of spheroplasts isolated from induced and uninduced *E. coli* cells carrying indicated plasmids

Plasmid		anti-MBP	anti-apocytochrome b_6	without antibody
pMal-c2b6[a,b]	induced	- - -	- - -	- - -
pMal-p2b6[a,b]	uninduced	- - -	- - -	- - -
pMal-p2b6[a,b]	induced	+ + +	+ + +	- - -
pMal-p2b6[a]	induced and incubated with factor Xa	- - -	+ + +	- - -
pMal-p2A	induced	+ + +	ns	- - -
pMal-p2B	induced	+ + +	ns	- - -
pMal-p2AB	induced	+ +	ns	- - -
pMal-p2BC	induced	+ + +	ns	- - -
pMal-p2CD	induced	- - -	- - -	- - -
pMal-p2BCD	induced	+ + +	- - -	- - -
pMal-p2BCD	induced and incubated with factor Xa	- - -	- - -	- - -

(- - -) lack of agglutination, (+) weak agglutination, (++) medium agglutination, (+++) strong agglutination, [a]b6 stands for ABCD helices, [b]control of spheroplast preparation, (ns) not studied

Returning to the translocation/insertion of proteins across/into the membrane, it has been proposed that when the elongating sequence passes through the SecY complex, the translocon has to "decide" whether the segment should be secreted or shunted into the membrane as a TM helix. Their integration into the membrane utilizes a mechanism that requires an upstream cleavable SS or noncleavable TM segment. The subsequent appearance of the signal anchor sequence (STS) within the

translocation apparatus aborts the SS/TM-initiated translocation of the polypeptide chain across the membrane. STSs occur as hydrophobic segments, flanked by charge residues, which are sufficiently long to form TM α-helix and provide a permanent anchorage (Saaf *et al.*, 1998). Other investigations concerning the insertion of proteins into the membrane have shown that the coexpression of two or three complementary polypeptide fragments of rhodopsin allows the formation of noncovalently linked rhodopsin, showing that the covalent linkage of the polypeptide chain is not required for the *in vivo* insertion into the membrane, folding and assembly of rhodopsin (Ridge *et al.*, 1995). It has also been shown that many rhodopsin fragments with discontinuities in the intradiscal, transmembrane and cytoplasmic regions fold and insert into the membrane (Ridge *et al.*, 1996).

During our study on cytochrome b_6 assembly into the membrane, the following question arose: which part/parts of cytochrome b_6 apoprotein is/are responsible for the protein insertion into the membrane? In the case of polypeptide fragments of cytochrome b_6 fused to MBP expressed in *E. coli* only TM helices A and B and constructs containing both these helices were folded and inserted into the membrane (Fig. 5). Unexpectedly, constructed mutants containing TM helix C or helices CD were transported through the cytoplasmic membrane and were found exclusively in the bacterial periplasm (Fig. 6AB), implying that helices C and D do not include an STS. Computational analysis has shown a lack of STS in fragments containing helix C or D and simultaneously revealed the presence of STS in helices A and B (Table 1); these predictions are in agreement with our experimental results. In contrast to rhodopsin (Ridge *et al.*, 1995; Saaf *et al.*, 1998), for proper folding and correct insertion into the membrane (with the possibility of heme insertion into the protein structure) helices C and D of cytochrome b_6 require covalent linkage to the preceding TM helix B, and the presence of helix A is not necessary for the anchoring process (Fig. 4 and Fig. 7).

Other important results obtained in our study are that the spectral and redox properties of the BCD mutant of cytochrome b_6 mimic those of the native protein and are similar to those of cytochrome b_6 spectra obtained from isolated protein or cytochrome b_6-*f* complex (Stuart & Wasserman, 1973; Boronowsky *et al.*, 2001). The spectral and redox properties of the BCD mutant are typical for bis-histidine liganded b-type cytochromes. This very strongly suggests that the structural features responsible for the significant redox differences between the two hemes reside in the BCD part of cytochrome b_6 itself, rather than arising from a higher order conformation induced by the other subunits or transmembrane helix A. It is also clear that membrane-incorporated BCD helices reveal a proper structure and an appropriate distance between histidine axial ligands that allow proper heme incorporation.

CONCLUDING REMARKS

In the present study we have shown that the fused signal sequence (SS) is responsible for the orientation of apocytochrome b_6 into bacterial cytoplasmic membrane and the fused part is located on the periplasmic side. Helices A and B of apocytochrome b_6 are responsible for the anchoring of cytochrome b_6 and B (with or without A) ensures insertion and proper folding of helices C and D, while helices C and D do not possess a functional anchor signal sequence (STS). The results presented here also show that proper incorporation of hemes into a truncated form of apocytochrome b_6 is possible, as long as helices B and D (parts bearing the axial ligands to heme) are present.

Acknowledgements

This work was supported by a grant from the State Committee for Scientific Research (grant No.: 6 P04 C 00420 to A.S.).

The authors are grateful to Professor J. W. de Gier for providing *E. coli* CM 124 strain.

REFERENCES

Aldridge C, Cain P, Robinson C (2009) Protein transport in organelles: Protein transport into and across the tylakoid membrane. *FEBS J* **276:** 1177–1186.

Bendtsen JD, Kiemer L, Fausboll A, Brunak S (2005) Non-classical protein secretion in bacteria. *BMC Microbiol* **5:** 58.

van der Berg B, Clemons WM, Collinson I, Modis Y, Hartman E, Harrison SC, Rapoport TA (2004) X-ray structure of a protein-conducting channel. *Nature* **427:** 36–44.

Betton JM, Hofnung M (1996) Folding of a mutant maltose-binding protein of *Escherichia coli* which forms inclusion bodies. *J Biol Chem* **271:** 8046–8052.

Boronowsky U, Wenk S, Schneider D, Jager C, Rogner M (2001) 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*(6)*f* complex. *Biochim Biophys Acta* **1506:** 55–66.

Cassoly R (1978) Evidence against the binding of native hemoglobin to spectrin of human erythrocytes. *FEBS Lett* **85:** 357–360.

Choquet Y, Stern DB, Wostrikoff K, Kuras R, Girard-Bascou J, Wollman FA (1998) Translation of cytochrome *f* is autoregulated through the 5' untranslated region of *petA* mRNA in *Chlamydomonas* chloroplasts. *Proc Natl Acad Sci USA* **95:** 4380–4385.

Cline K, Ettinger WF, Theg SM (1992) Protein-specific energy requirements for protein transport across or into thylakoid membranes. Two lumenal proteins are transported in the absence of ATP. *J Biol Chem* **267:** 2688–2696.

Cline K, Dabney-Smith C (2008) Plastid protein import and sorting: different paths to the same compartments. *Curr Opin Plant Biol* **11:** 585–592.

Dalbey RE, Kuhn A (2004) YidC family members are involved in the membrane insertion, lateral integration, folding, and assembly of membrane proteins. *J Cell Biol* **166:** 769–774.

Dreher C, Prodohl A, Weber M, Schneider D (2007) Heme binding properties of heterologously expressed spinach cytochrome *b*(6): implications for transmembrane *b*22 type cytochrome formation. *FEBS Lett* **581:** 2647–2651.

Emanuelsson O, von Heijne G (2001) Prediction of organellar targeting signals. *Biochim Biophy Acta* **1541:** 114–119.

Froderberg L, Houben E, Samuelson JC, Chen M, Park SK, Phillips GJ, Dalbey R, Luirink J, De Gier JW (2003) Versatility of inner membrane protein biogenesis in *Escherichia coli*. *Mol Microbiol* **47:** 1015–1027.

von Heijne G (1985) Signal sequences. The limits of variation. *J Mol Biol* **184:** 99–105.

von Heijne G (1986) A new method for predicting signal sequence cleavage sites. *Nucleic Acids Res* **14:** 4683–4690.

Heinemeyer W, Alt J, Herrmann RG (1984) Ncleotide sequence of the clustered genes for apocytochrome b_6 and subunit 4 of the cytochrome b_6f complex in the spinach plastid chromosome. *Curr Genet* **8:** 543–549.

Herrmann RG (1982) The preparation of circular DNA from plastids. In *Methods in Chloroplast Molecular Biology*. Edelman M, Hallick RB, Chua N-H eds, pp 259–279. Elsevier, Amsterdam.

Hynds PJ, Robinson D, Robinson C (1998) The Sec-independent twin-arginine translocation system can transport both tightly folded and malfolded proteins across the tylakoid membrane. *J Biol Chem* **273:** 34868–34874.

Klee EW, Ellis LB (2005) Evaluating eukaryotic secreted protein prediction. *BMC Bioinformatics* **6:** 256.

Kroliczewski J, Szczepaniak A (2002) *In vitro* reconstitution of the spinach chloroplast cytochrome b_6 protein from a fusion protein expressed in *Escherichia coli*. *Biochim Biophys Acta* **1598:** 177–184.

Kroliczewski J, Hombek-Urban K, Szczepaniak A (2005) Integration of the thylakoid membrane protein cytochrome *b*6 in the cytoplasmic membrane of *Escherichia coli*. *Biochemistry* **44:** 7570–7576.

Kurisu G, Zhang H, Smith JL, Cramer WA (2003) Structure of the cytochrome b_6f complex of oxygenic photosynthesis: tuning the cavity. *Science* **302:** 1009–1014.

Leclerc E, Leclerc L, Cassoly R, der Terrosian E, Wojcman H, Poyart C, Marden MC (1993) Heme binding to calmodulin, troponin C, and parvalbumin, as a probe of calcium-dependent conformational changes. *Arch Biochem Biophys* **306:** 163–168.

Marden MC, Hazard ES, Leclerc L, Gibson QH (1989) Flash photolysis of the serum albumin-heme-carbon monoxide complex. *Biochemistry* **28:** 4422–4426.

Marques JP, Schattat MH, Hause G, Dudeck I, Klosgen RB (2004) *In vitro* transport of folded EGFP by the DeltapH/TAT-dependent pathway in chloroplasts of *Arabidopsis thaliana*. *J Exp Bot* **55:** 1697–1706.

Nielsen H, Krogh A (1998) Prediction of signal peptides and signal anchors by a hidden Markov model. *Proc Int Conf Intell Syst Mol Biol* **6:** 122–130.

Nielsen H, Brunak S, von Heijne G (1999) Machine learning approaches for the prediction of signal peptides and other protein sorting signals. *Protein Eng* **12:** 3–9.

du Plessis DJ, Nouwen N, Driessen AJ (2006) Subunit a of cytochrome *o* oxidase requires both YidC and SecYEG for membrane insertion. *J Biol Chem* **281:** 12248–12252.

Rapoport TA, Jungnickel B, Kutay U (1996) Protein transport across the eukaryotic endoplasmic reticulum and bacterial inner membranes. *Annu Rev Biochem* **65:** 271–303.

Ridge KD, Lee SSJ, Yao LL (1995) *In vivo* assembly of rhodopsin from expressed polypeptide fragments. *Proc Natl Acad Sci USA* **92:** 3204–3208.

Ridge KD, Lee SSJ, Abdulaev NG (1996) Examining rhodopsin folding and assembly through expression of polypeptide fragments. *J Biol Chem* **271:** 7860–7867.

Rochaix JD (1996) Post-transcriptional regulation of chloroplast gene expression in *Chlamydomonas reinhardtii*. *Plant Mol Biol* **32:** 327–341.

Robinson C, Thompson SJ, Woolhead C (2001) Multiple pathways used for the targeting of thylakoid proteins in chloroplasts. *Traffic* **2:** 245–251.

Rothstein R, Gatenby AA, Willey DL, Gray JC (1985) Binding of pea cytochrome *f* to inner membrane of *Escherichia coli* requires the bacterial secA gene product. *Proc Natl Acad Sci USA* **82:** 7955–7959.

Roy LM, Barkan A (1998) A SecY homologue is required for the elaboration of the chloroplast thylakoid membrane and for normal chloroplast gene expression. *J Cell Biol* **141:** 385–395.

Saaf A, Wallin E, von Heijne G (1998) Stop-transfer function of pseudo-random amino acid segments during translocation across prokaryotic and eukaryotic membranes. *Eur J Biochem* **251:** 821–829.

Schatz G, Dobberstein B (1996) Common principles of protein translocation across membranes. *Science* **271:** 1519–1526.

Schuenemann D (2007) Mechanisms of protein import into tylakoids of chloroplasts. *Biol Chem* **388:** 907–915.

Schuenemann D, Amin P, Hartman E, Hoffman NE (1999) Chloroplast SecY is complexed to SecE and involved in the translocation of the 33-kDa but not the 23-kDa subunit of the oxygene-evolving complex. *J Biol Chem* **274:** 12177–12182.

Solar I, Muller-Eberhard U, Shaklai N (1989) Serum proteins as mediators of hemin efflux from red cell membranes: specificity of homeopexin. *FEBS Lett* **256:** 225–229.

Solar I, Shaklai N (1989) Association of hemin with protein 4.1 as compared to spectrin and actin. *Biochim Biophys Acta* **983:** 199–204.

Stuart AL, Wasserman AR (1973) Purification of cytochrome b_6 a tightly bound protein in chloroplast membranes. *Biochim Biophys Acta* **314:** 284–297.

Szczepaniak A, Cramer WA (1990) Thylakoid membrane protein topography-location of the termini of the chloroplast cytochrome b_6 on the stromal side of the membrane. *J Biol Chem* **265:** 17720–17726.

Traxler B, Murphy C (1996) Insertion of the polytopic membrane protein MalF is dependent on the bacterial secterion machinery. *J Biol Chem* **271:** 12394–12400.

Wallin E, von Heijne G (1998) Genome-wide analysis of integral membrane proteins from eubacterial, archaean, and eukaryotic organisms. *Protein Sci* **7:** 1029–1038.

Woolhead CA, Mant A, Kim SJ, Robinson C, Rodger A (2001) Conformation of a purified "spontaneously" inserting thylakoid membrane protein precursor in aqueous solvent and detergent micelles. *J Biol Chem* **276:** 14607–14613.

Yi L, Dalbey RE (2005) Oxa1/Alb3/YidC system for insertion of membrane proteins in mitochondria, chloroplasts and bacteria (review). *Mol Membr Biol* **22:** 101–111.

Zhang L, Paakkarinen V, Suorsa M, Aro EM (2001) A SecY homologue is involved in chloroplast-encoded D1 protein biogenesis. *Biol Chem* **276:** 37809–37814.

Yuan J, Henry R, McCaffery M, Cline K (1994) SecA homolog in protein transport within chloroplasts: evidence for endosymbiont-derived sorting. *Science* **266:** 796–798.