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Integration of the Thylakoid Membrane Protein Cytochrome b_6 in the Cytoplasmic Membrane of *Escherichia coli*†

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ABSTRACT: An overexpression system for spinach apocytochrome b_6 as a fusion protein to a maltose-binding protein in *Escherichia coli* was established using the expression vector pMalp2. The fusion of the cytochrome b_6 to the periplasmic maltose-binding protein directs the cytochrome on the Sec-dependent pathway. 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 but has the opposite orientation compared to that in thylakoid. Our data also show that in the *E. coli* cytoplasmic membrane, apocytochrome b_6 and exogenic hemes added into a culture media spontaneously form a complex with similar spectroscopic properties to native cytochrome b_6. Reconstituted membrane-bound cytochrome b_6 contain two *b* hemes (α band, 563 nm; average $E_{m,7} = -61 \pm 0.84$ and -171 ± 1.27 mV).

Chloroplast contains at least four distinct pathways for targeting proteins to the thylakoid membrane (*1*, *2*). The Sec-dependent, SRP-dependent, pH-dependent, and spontaneous pathways all have direct cognates in bacteria (*3*, *4*). The observed similarities of these mechanisms are consistent with the evolution of the chloroplast from a prokaryotic ancestor. Several thylakoid signal peptides can target a protein for secretion across the plasma membrane of bacteria (*5*, *6*). Usually after overexpression in the *Escherichia coli* membrane, proteins accumulate in bacteria as inclusion bodies (*7*, *8*); however, the integration of some thylakoid membrane proteins (LHCP and cytochrome *f*) into the *E. coli* cytoplasmic membrane has also been demonstrated (*9*, *10*, *11*).

Little is known about the targeting of chloroplast-encoded proteins; however, it is likely that they share many of the same translocation components as nuclear-encoded proteins. Because membrane proteins contain domains that must be translocated across the bilayer, it is no surprise that the enzymatic machinery responsible for translocating proteins across the membrane also plays a role during the assembly of integral membrane proteins. An important notion is that the insertion machinery may not only insert the membrane protein but also determine its topology. Only when the insertion machinery is able to understand the "language" used by the polypeptide chain will the polypeptide have a native structure in the membrane.

Chloroplast-encoded cytochrome b_6, like many other integral proteins, operates with an uncleaved signal for insertion into the thylakoid membrane. A Sec-dependent pathway for the integration of cytochrome b_6 into the thylakoid membrane has been suggested (*12*). To determine whether cytochrome b_6 may be inserted into a bacterial membrane, cytochrome b_6 was fused to a periplasmic maltose-binding protein (MBP)[1] carrying a signal sequence expressed in *E. coli*. This directs the cytochrome b_6 on the Sec-dependent pathway.

The cytochrome expressed in bacteria, which is incorporated into the cytoplasmic membrane, has to adopt the native structure but has an opposite orientation compared to that in thylakoids. The integration of the thylakoid membrane protein in the cytoplasmic membrane of *E. coli* offers new possibilities for future studies of the structural properties and the mechanism of incorporation into biological membranes. Part of this work was presented at the 11th International Congress on Photosynthesis (*13*).

MATERIALS AND METHODS

Construction of the Expression Plasmid pMalp2b6. The recombinant plasmid pMalc2b6 containing a gene for spinach apocytochrome b_6 (*8*) was cleaved by *Nde*I and *Bgl*II (Promega). Restriction products (4458 kb) were purified from gel electrophoresis and cloned into *Bgl*II and *Nde*I sites of the expression vector pMalp2 (New England Biolabs) by a standard T4 DNA ligase procedure, resulting in the expression of the target gene as a fusion protein to MBP containing a signal sequence for the transport of the fusion protein to periplasm. The recombinant plasmid, designed pMalp2b6,

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[1] Abbreviations: BCA, bicinchoninic acid; b_h, high-potential haem; b_l, low-potential haem; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; E_m, midpoint potential; IPTG, isopropyl thio-β-D-galactoside; MBP, maltose-binding protein; MOPS, 3-(*N*-morpholino)propanesulfonic acid; OD, optical density; ORP, oxidation−reduction potential; PMSF, phenylmethylsulphonyl fluoride; RT, room temperature; SDS, sodium dodecyl sulfate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; spMBP, maltose-binding protein with signal peptide; TMBZ, 3,3′,5,5′-tetramethylbenzidine.

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was checked by restriction analysis and verified by DNA sequencing.

Construction of the Expression Plasmid pET16b6. The gene-encoding apocytochrome b_6 was isolated by restriction with *Nde*I and *Bam*HI enzymes from the plasmid pBluescript-II-SKpetb carrying genes *psbA* and *petB* (unpublished data). The restriction product was inserted into the vector pET16b (Novagen) cleaved with the same enzymes by a standard T4 ligase procedure. Both fragments were separated by electrophoresis on a 1% agarose gel. This construct (pET16b6) was sequenced to confirm any unwanted mutation and was used to express apocytochrome b_6 fused to the His-tag domain at the N terminus.

Construction of the Expression Plasmid pET25b6. Plasmid pET16b6 was digested with restriction endonucleases *Nco*I and *Bam*HI. A corresponding digestion was applied to plasmid pET25b (Novagen). Both fragments were run on 1% agarose gel and isolated using a gel-extraction kit (Macherey−Nagel). The fragments were then ligated by standard T4 ligase procedure to obtain plasmid pET25b6, resulting in the expression of the target gene as a fusion protein to the pelB signal sequence. After the transformation of the DH5α cells, a plasmid preparation with a subsequent *Nco*I and *Bam*HI digest showed the correct fragment lengths (not shown).

Expression of Recombinant Apocytochrome b_6 in E. coli. TB1 cells transformed with pMalp2b6 or BL21(DE3) transformed with pET16b6 or pET25b6 were grown aerobically at 37 °C in 1 L of Luria−Bertani medium containing 100 μg/mL ampicillin and inoculated with 10 mL of overnight cultures. The production of fusion protein (MBP−apocytochrome b_6, His−apocytochrome b_6, or pelB−apocytochrome b_6) was induced by the addition of 0.5 mM IPTG at an OD_{550} of 0.6. After shaking for 4 h, cells were harvested by centrifugation (5000*g* for 15 min), resuspended in 30 mL of TEN buffer (20 mM Tris-HCl at pH 7.8, 10 mM EDTA, 1 mM PMSF, and 100 mM NaCl), and then frozen overnight.

Membrane Preparation from E. coli Cells. After the frozen *E. coli* cells were thawed, the solution was sonicated (6 × 15 s, 65 W, microtip sonifier cell disrupter, Barnsted) 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 to pellet inclusion bodies, and the resulting supernatant was centrifuged for a further 2 h at 70000*g* at 4 °C. A suspension of the membranous 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 (*14*) and Western blot.

Preparation of Spheroplasts. E. coli carrying plasmid pMalp2b6 were grown in a standard LB medium containing 100 μg/mL of ampicillin. Induction was initiated at OD_{560} 0.6 by the addition of the 0.5 mM IPTG. After 4 h of shaking at 37 °C, cells were carefully harvested by centrifugation (3000*g* for 10 min at 4 °C) and spheroplasts were prepared. The cell pellets were resuspended in buffer I (10 mM Tris-HCl, 10 mM EDTA, 5 mM $MgSO_4$, 0.7 M sucrose, and 1 mM PMSF at pH 7.5) and centrifuged again at 5000*g*, for 10 min at 4 °C. The bacterial pellets were resuspended again in 10 mL of ice-cold 5 mM $MgSO_4$ and incubated for 10 min in ice. The resuspended cells were again pelleted under the same conditions, resuspended in buffer I containing 0.2 mg/mL lysozyme, and incubated at room temperature for 25 min. The sample was then centrifuged at 5000*g* for 10 min at 4 °C, and the supernatant was removed. The pellets were resuspended in buffer I and washed twice. The fresh spheroplasts were agglutinated using antibodies against MBP and cytochrome b_6.

Agglutination of Spheroplasts. The spheroplasts were incubated with an antibody against the MBP or against the COOH-terminal decapeptide (Ile206 to Leu215) of cytochrome b_6. The agglutination was analyzed by optical microscopy. The agglutination of bacterial spheroplasts was observed immediately after the addition of the antibody.

Isolation of Cytochrome b_6 from Synechocystis PCC 6803. Cytochrome b_6 was prepared from the cyanobacterial membrane according to the method of Boronowsky et al. (*15*).

Spectra Measurements. Visible absorption spectra were recorded at RT in 1 cm optical-path length cuvettes (1 mL) using a Cary 1 spectrophotometer with a spectral bandwidth of 1 nm and a scan speed of 600 nm/min. Spectra were usually recorded in 50 mM Tris-HCl at pH 8.0, 50 mM NaCl, 0.3% SDS, and 10% glycerol. The samples were oxidized by ferricyanide and reduced by the addition of a concentrated dithionite solution.

Low-Temperature Spectra Measurements. Spectra over liquid nitrogen at fixed redox potentials were measured using 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 quickly frozen in liquid nitrogen before low-temperature difference spectra were recorded.

Electrochemical Redox Titrations. Membranes were prepared from *E. coli* as described above (*2*, *3*) to allow titration of the α band of the reconstituted cytochrome b_6. The membrane pellet was resuspended in 1 volume of deoxygenated by nitrogen 50 mM MOPS buffer at pH 7.0 containing 50 mM KCl and the following redox mediators at the concentration of 100 μM each: *p*-benzoquinone (+280 mV), 2,5-(methyl)-2-benzoquinone (+180 mV), 1,2-naphthoquinone (+145 mV), phenazine methyl sulfate (+80 mV), 1,4-naphthoquinone (+60 mV), menadione (0 mV), 2,5-(OH)-2-benzoquinone (−60 mV), antraquinone (−100 mV), 2-(OH)-1,4-naphthoquinone (−145 mV), antraquinone 1,5-disulfonate (−170 mV), antraquinone 2-sulfonate (−225 mV), and benzyl viologen (−359 mV). The midpoint potential of mediators is given for pH 7.0. All redox potentiometric measurements were made with a micro ORP combination electrode, with an Ag/AgCl electrode as a reference, (Microelectrodes, Inc.) at 25 °C. The potential was adjusted by the additions of small quantities of a concentrated solution of potassium ferricyanide (oxidative titrations) and sodium hydrosulfite (reductive titrations). Titrations in steps ∼10 mV were performed in the oxidative and the reductive directions in the potential range from +200 to −350 mV. The equilibration time was between 3 and 5 min after each change in ambient redox potential before spectra were scanned. Spectra were recorded between 500 and 600 nm with a M40 spectrophotometer (Carl Zeiss Jena) with modification. At the end of titration, the pH was checked to ensure that no drift had occurred. Spectral changes recorded from −300 to +100 mV were analyzed by taking the peak wavelengths at 563 nm and fitting the absorbance values to Nernst curves (*16*). Component spectra were obtained by

redox cut analysis (*16*). The spectrum of cytochrome b_h was obtained by subtracting from the average of five spectra taken at -62 ± 8 mV and an average of three spectra recorded at 0 ± 5 mV, and the spectrum of cytochrome b_l was obtained by subtracting from the average of four spectra taken at -250 ± 10 mV and an average of four spectra recorded at -125 ± 5 mV. The midpoint potential of the hemes and their corresponding spectra were calculated either by Origin 7.0 (OriginLab Corporation) in a global fit procedure (Boltzman equation, general least-squares algorithm) or by fitting the absorbance values at a peak wavelength of 563 nm to two species to Nernst curves each with $n = 1$.

Measurements of the Protein Concentration. The MBP concentration was determined directly from the absorbance at 280 nm with an excitation coefficient of $\epsilon = 68.750\ M^{-1}\ cm^{-1}$ (*17*). The concentration of fused protein was determined by using the BCA reagent (Sigma) according to the instructions of the manufacturer with BSA as a standard.

Proteolysis. Spheroplasts (50 μL, 5 mg of protein/mL) were incubated at room temperature (about 25 °C) with chymotrypsin-free trypsin or factor Xa for 30 min. The trypsin and factor Xa reaction were terminated by the addition of 5 mM PMSF. The spheroplasts were pelleted, washed twice with buffer I, resuspended in the same buffer, and used for agglutination.

Cleavage with Factor Xa. Cleavage was carried out at a w/w ratio of 0.5% of the amount of fusion protein in 10 mM Tris-HCl at pH 8.0, 10 mM NaCl, and 0.05% SDS buffer.

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 (*18*). For heme staining, samples were dissolved immediately before electrophoresis in 3% SDS, 50 mM Tris-HCl, and a 10% glycerol at 37 °C for 15 min. The staining of heme proteins was performed according to ref *19*.

RESULTS AND DISCUSSION

Expression of Apocytochrome b_6 in E. coli. To direct cytochrome b_6 to the bacterial inner membrane, the plasmids pMalp2b6 and pET25b6 were constructed. In plasmid pMalp2b6, the coding region for the mature cytochrome b_6 was fused to the gene encoding bacterial protein, MBP with the signal peptide. We determined whether the spMBP−apocytochrome b_6 fusion protein could become an integral membrane protein. Proteins that were fractionated from *E. coli* cells strain TB1 bearing the plasmid pMalp2b6 induced with IPTG are shown in Figure 1A. A polyclonal antibody against the COOH-terminal decapeptide of cytochrome b_6 detects a protein in whole-cell lysates that is ~47 kDa larger than mature cytochrome b_6, indicating that the fusion protein is correctly expressed (lane 2).

Cells expressing spMBP−apocytochrome b_6 were sonicated and centrifuged at 27000*g*, producing a pellet containing unlysed cells, cell walls, and inclusion bodies (P1, Figure 1A) and a supernatant of membranes and cytoplasm (*20*). This latter supernatant was centrifuged again at 27000*g* to clear residual cell debris and inclusion bodies. A total of 30% of the spMBP−apocytochrome b_6 pellets was detected at 27000*g*, but 70% is also detected in a 27000*g* supernatant (not shown). This supernatant was centrifuged twice at

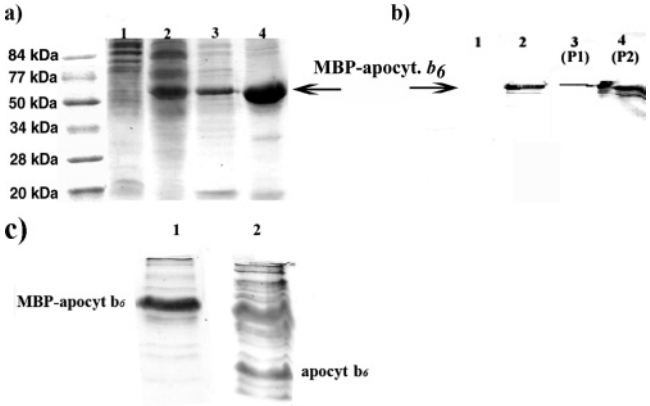

FIGURE 1: (a) SDS−PAGE of the purification of the expressed MBP−apocytochrome b_6 fusion protein. Lane S, standard; lane 1, total protein of *E. coli* cells carrying the plasmid pMalp2b6 before induction; lane 2, total protein after induction with IPTG; lane 3, isolated inclusion bodies (P1); and lane 4, membranes (P2). (b) Western blot analysis of the expressed MBP apocytochrome b_6 fusion protein. Lane 1, total protein of *E. coli* cells carrying the plasmid pMalp2b6 before induction; lane 2, total protein after induction with IPTG; lane 3, inclusion bodies (P1); and lane 4, membranes (P2). (c) SDS−PAGE of the membrane fraction of the expressed MBP−apocytochrome b_6 fusion protein (lane 1) and apocytochrome b_6 overexpressed in *E. coli* cells carrying the plasmid pET25b6. The sample of bacteria was taken at the same OD, and each lane contains an identical amount of bacterial membrane.

70000*g* to pellet the cytoplasmic membranes shown in Figure 1A (P2). MBP−apocytochrome b_6 was detected only in pelleting material.

In plasmid pET25b6, the apocytochrome b_6 was fused to the pelB signal sequence. The fusion of the cytochrome b_6 to the pelB signal sequence directs the apocytochrome b_6 on the SEC-dependent pathway, similar to the MBP fusion in pMalp2b6. Gene *petB* in the plasmid pET16b6 was cloned without any additional signal sequence. Both "pET" plasmids were expressed in *E. coli* strain BL21(DE3). The expression product apocytochrome b_6 fused to the C terminus of the pelB signal sequence was incorporated into the membrane, but in comparison to MBP−apocytochrome b_6, the level of the expressed apocytochrome b_6 determined by densitometric analysis was 10 times lower (Figure 1C). Plasmid pET16b6 was induced by IPTG in 37 and 25 °C. After overexpression in 37 °C, we found apocytochrome b_6 protein only in a fraction of the inclusion bodies, but in 25 °C, we also found a weak signal in the inner membrane of *E. coli*. Moreover, expressed apocytochrome b_6 in the membrane was completely degraded during the 2 h of expression. This experiment suggests that apocytochrome b_6 overexpressed in plasmid pET16b6 has the wrong structure, which is not resistant to proteolysis degradation and rather associated with the membrane than inserted into it (data not shown).

Determination of MBP−Apocytochrome b_6 Topology in the Bacterial Inner Membrane. E. coli cells bearing the plasmid pMalp2b6 after induction were converted to spheroplast and either incubated with an antibody against the MBP or an antibody against the COOH-terminal decapeptide (Ile206−Leu215) of cytochrome b_6. In both cases, a strong agglutination of the spheroplasts was observed, demonstrating the periplasmic location of the MBP and COOH terminus of cytochrome b_6. Uninduced cells were used as a control (Table 1).

Table 1: Agglutination of Spheroplast Isolated from Induced and Uninduced *E. coli* TB1 Cells Carrying the Indicated Plasmids[a]

plasmid		anti-MBP	anti-apocytochrome b_6	without antibody
pMalp2b6	uninduced	− − −	− − −	− − −
pMalp2b6	induced	+ + +	+ + +	− − −
pMalp2b6	induced and incubated with factor Xa	− − −	+ + +	− − −
pMalp2b6	induced and incubated with trypsin	− − −	− − −	− − −
pMalc2b6	induced	− − −	− − −	− − −

[a] (− − −) Lack of agglutination, (+) weak agglutination, and (+ + +) strong agglutination

The MBP−apocytochrome b_6 contains one cleavage site for factor Xa protease, located between the COOH terminus of MBP and NH_2 terimnus of cytochrome b_6. Externally added factor Xa to spheroplast isolated from induced cells cut off MBP from the fusion protein as shown by the lack of agglutination of spheroplast with the antibody against MBP and the presence of agglutination with the antibody against the COOH terminus of cytochrome b_6 (Table 1). The liberation of MBP was confirmed by Western blot analysis of the supernatant after pelleting spheroplasts (not shown). The data in this experiment indicate that the NH_2 terminus of the apocytochrome b_6 is on the periplasmic side of the membrane. The treatment of the spheroplast with trypsin resulted in a lack of agglutination with both antibodies (Table 1). The sensitivity of the COOH terminus of cytochrome b_6 in the thylakoid membrane was shown early (*18*). These results imply that both the NH_2 and COOH termini of apocytochrome b_6 are on the periplasmic side of the bacterial membrane.

Heme Incorporation. The measurement of the dithionite-reduced spectra of the isolated cytoplasmic membranes from induced *E. coli* cells bearing the plasmid pMalp2b6, pET25b6, or pET16b6 did not show the presence of any cytochrome b_6 heme (not shown), implying that the cytochrome b_6 moiety of the fusion protein was in the apocytochrome form. During the protein expression from pMalp2b6, heme was present in the culture medium; the dithionite-reduced spectra of isolated cytoplasmic membranes demonstrate the presence of *b*-type heme (Figure 2A). The spectra of the oxidized form have the characteristic Soret (γ band) at 413 and a broad α, β band at 535 nm (Figure 2A). The reduction of the ferrous form yields the characteristic red shift of the γ band to 421 nm and prominent β and α bands in the 535 and 563 regions, respectively (Figure 2A). The width of the α band at the half-height is ~11 nm.

The spectra profiles of α and β bands in MBP−cytochrome b_6 in bacterial membrane are similar to those of isolated cytochrome b_6 (*15*, *21*). Literature on the position of the Soret band of cytochrome b_6 are inconsistent at 434 nm (*21*) and 425 nm (*15*). In our case, this band is additionally overlapped by the absorption band of free hemes in the bacterial membrane, which are not removable by dialysis (Figure 2A). For this reason, the Soret band was broadened and shifted to a shorter wavelength. The reconstituted MBP−cytochrome b_6 in the membrane was cleaved by factor Xa, and MBP was removed from the cleavage reaction by washing the membranes twice with buffer containing 10 mM Tris-HCl at pH 8.0, 10 mM NaCl, and 0.05% SDS (*8*). After cutting off and isolating, the absorption γ peak (Soret band) is shifted to 416.5 and 429 nm, which are more similar to those of isolated cytochrome b_6 (Figure 2B) (*15*, *21*). In the experiment with plasmids pET25b6 and pET16b6 after expression in the presence of hemin only,

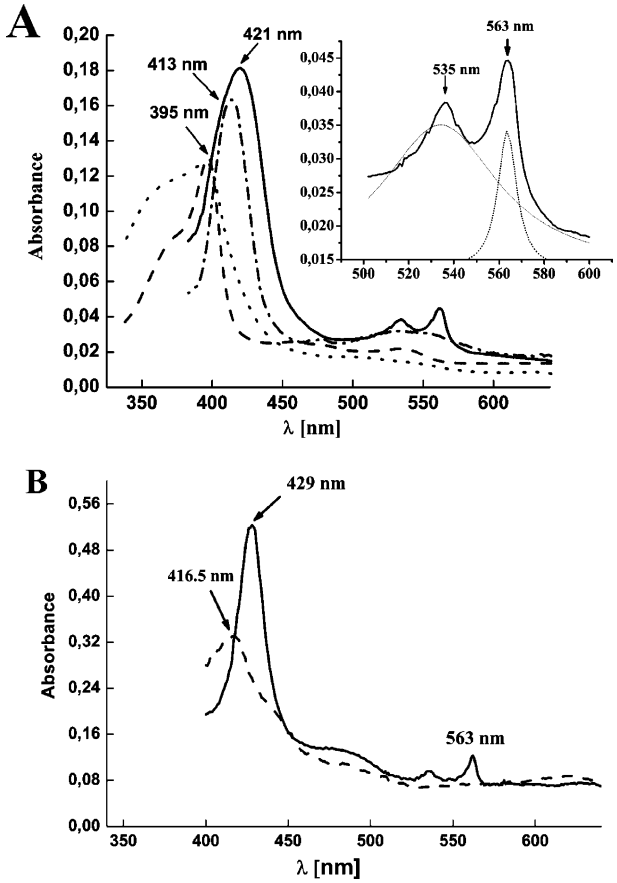

FIGURE 2: (A) Absorption spectra of oxidized (-·-) and reduced (—) hemes inserted into MBP−apocytochrome b_6 fusion protein expressed in *E. coli* carrying pMalp2b6 plasmid obtained as described under the Materials and Methods. Spectrums of free protoporphyrin IX (···) in R1 buffer and free hemes incorporated into membranes (- - -) are shown for comparison. Ferricyanide and dithionite were used respectively as oxidants and reductants. The inset in A shows absorption spectra of reduced MBP−cytochrome b_6 measured in the range from 500 to 600 nm. Gaussian distribution of α and β band was shown as a thin line. (B) Absorption spectra of reduced (—) and oxidized hemes (- - -) inserted into cytochrome b_6 after cutting off by factor Xa and isolating from the fusion protein obtained as described under the Materials and Methods. Ferricyanide and dithionite were used respectively as oxidants and reductants.

cytochrome b_6 fused to the pelB signal sequence gave maximum absorbance of that Soret band near 430 nm (reduced by dithionite), suggesting proper insertion of cytochrome b_6 into the bacterial membrane and also the presence of reconstituted hemes. Because of the low expression of protein, the reconstitution level of cytochrome b_6 was also low, which is why we chose *E. coli* harboring plasmid pMalp2b6 for our next experiments.

Low-Temperature Spectra. The cytochrome b_6, either within the cytochrome b_6f complex or in its isolated form, can be separated into two distinct forms, as derived from

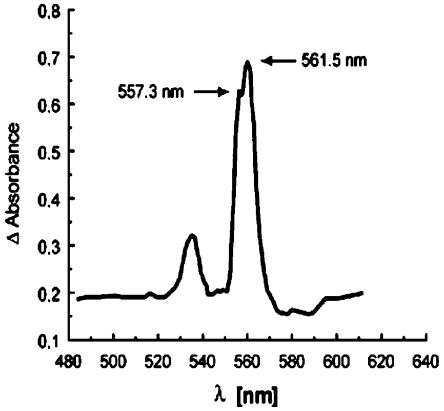

FIGURE 3: Low-temperature absorption spectra of a reconstituted cytochrome b_6 (—). Difference spectra of reconstituted cytochrome b_6 obtained as described under the Materials and Methods. Ferricyanide and dithionite were used respectively as oxidants and reductants.

the higher resolution low-temperature difference spectra (*21*, *22*). The membrane-incorporated cytochrome b_6 has an asymmetrical α band at 561 nm in the low-temperature difference spectrum (Figure 3). Gaussian deconvolution of this α band shows two spectral species with peaks at 557.3 and 561.6 nm, which are similar to the maxima of the native cytochrome b_6.

Electrochemical Redox Titrations. Equilibrium redox titration of isolated cytochrome b_6 from *Synechocystis* 6803 and the *E. coli* cytoplasmic membrane containing reconstituted MBP−cytochrome b_6 are shown in Figure 4A. Visible spectra were recorded over the redox potential range from −350 to 200 mV. They were analyzed by fitting the absorbance values at a peak wavelength of 563 nm to Nernst curves with $n = 1$. The data could be fitted to curves corresponding to two species with an E_{m7} of -73 ± 1.05 and -168 ± 0.75 mV for *Synechocystis* cytochrome b_6. Also, the data for MBP−cytochrome b_6 can be fitted in a fashion similar to a two-component curve with an E_{m7} of -61 ± 0.84 and -171 ± 1.27 mV.

These values are similar to the published data (*23*, *24*). The small changes in redox potential between the *Synechocystis* cytochrome b_6 and reconstituted MBP−cytochrome b_6 can be explained in terms of changes in the environment or from the difference in the amino acid sequence. The isolated cytochrome b_6 in contrast to the reconstituted cytochrome b_6 has bound chlorophyll, carotene, and detergents. The reconstituted MBP−cytochrome b_6 is surrounded by bacterial membrane lipids. The redox titration experiments show evidence that two hemes b_h and b_l are incorporated into the reconstituted MBP−cytochrome b_6 (Figure 4A) and the ratio of b_h to b_l is close to 1 (Figure 4B).

Heme Stain. Because cytochrome b_6 can be stained after electrophoresis while the related cytochrome *b* cannot (*25*, *26*), the peroxidase activity of the overexpressed protein was tested using isolated cytoplasmic membranes from the expression strain. Samples of the bacterial membrane were dissolved in 2% SDS and analyzed for heme content after SDS−PAGE (Figure 5). The membrane from uninduced *E. coli* cells and cytochrome *c* was used as a control. The MPB−cytochrome b_6 was heavily stained with TMBZ under

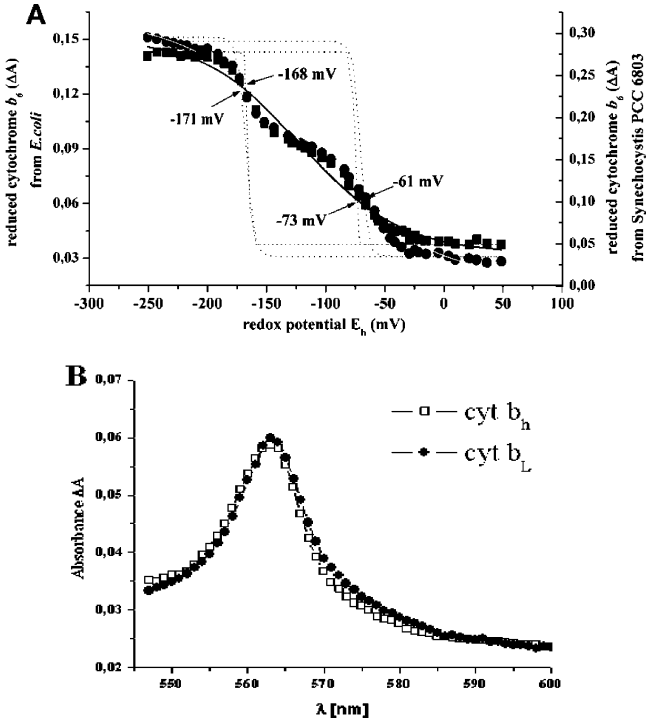

FIGURE 4: Redox titration of cytochrome b_6. Oxidation−reduction potential measurements were performed as described under the Materials and Methods. (A) Circles represent titration of *Synechocystis* PCC 6803 cytochrome b_6, and squares represent titration of *E. coli* membranes with reconstituted cytochrome b_6 fused to MBP. The continius line corresponds to the best fit to the Nernst equation ($n = 1$), with $E_{m7} = -171$ and -61 mV (*E. coli* reconstituted cytochrome b_6), $E_{m7} = -168$ and -73 mV (*Synechocystis* cytochrome b_6). The dotted line corresponds to the Boltzmann equation. (B) Absorbance spectra of cytochrome b_l and b_h in the α band region were obtained by redox cut analysis, see the Materials and Methods. The ratio of cytochrome b_h to cytochrome b_l is close to 1.

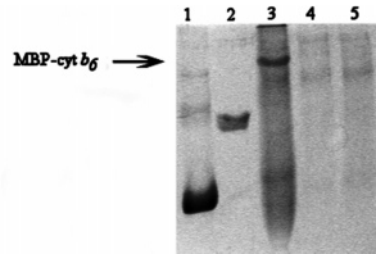

FIGURE 5: Heme staining of reconstituted cytochrome b_6 in the *E. coli* membrane. TMBZ staining was after urea SDS−PAGE. Lane 1, control (cytochrome *c*); lane 2, control (cytochrome *f*); lane 3, cytoplasmic membranes containing MBP−cytochrome b_6; lane 4, cytoplasmic bacterial membranes of *E. coli* strain TB1 without pMalp2b6 plasmid; lane 5, cytoplasmic membranes containing MBP−cytochrome b_6 after heating at 100 °C.

denaturating conditions, which suggest that the heme is strongly associated with the polypeptide.

Recently, the presence of an additional heme bound to cytochrome b_6 has been shown. The heme *x* (heme c_i) is covalently bound by one thioether linkage and has no axial amino acid ligand (*27*, *28*). It explains the peroxidase activity of cytochrome b_6 in SDS−PAGE, even after heating in the presence of 2% SDS at 100 °C, which is not observed with cytochrome bc_1. It is also consistent with the involvement of four nuclear genes in heme binding to *Chlamydomonas reinhardtii* cytochrome b_6 (*25*). We do not have the heme *x*

in our cytochrome b_6 inserted in the *E. coli* cytoplasmic membrane. The peroxidase activity of cytochrome b_6 expressed in *E. coli* in SDS−PAGE after heating is not observed (Figure 5). The heme *x* is located at the interface of the cytochrome b_6 and the subunit IV, and it is suggested that the CCB nuclear factors define the biogenesis pathway required for the binding of heme *x* to cytochrome b_6 (*25*). The subunit IV and these factors are not present in *E. coli*; this is probably the reason we do not have the heme *x* in cytochrome b_6 expressed in *E. coli*, as in the case of *in vitro* reconstituted spinach cytochrome b_6 (*8*).

The results presented in this paper show that the topogenic signals (signal anchor and stop transfer) in the amino acid sequence of the nascent chain of the chloroplast protein cytochrome b_6 are recognized by the *E. coli* SecYEG translocon, leading to the integration of this protein into the bacterial inner membrane. The fusion of the cytochrome b_6 to the periplasmic MBP directs the cytochrome on the Sec-dependent pathway. The cytochrome b_6 has a native structure in the bacterial cytoplasmic membrane with both NH_2 and COOH termini on the same, periplasmic (positive) side of the membrane but has an opposite orientation compared to that in the thylakoid. The chloroplast Sec-dependent pathway is responsible for the translocation of nuclear-encoded proteins, such as the 33-kDa polypeptide of the oxygen-evolving complex, plastocyanin, the F subunit of photosystem I (*1*), and at least one chloroplast-encoded protein, cytochrome *f* (*30*, *31*). The translocation or insertion of these proteins across or into the thylakoid membrane is absolutely dependent on the presence of N-terminal presequences, and in this case, their N termini are always transported to the other side (lumenal, negative) of the membrane.

The Sec-dependent pathway for the integration of cytochrome b_6 into the thylakoid membrane has been suggested (*12*). However, the lack of the N-terminal presequences in this cytochrome b_6 and the opposite orientation in the cytoplasmic membrane after expression in *E. coli* imply other pathways for integration, and additional experiments are needed for a final determination on which method is used by cytochrome b_6 for incorporation into the thylakoid membrane.

Plastid-encoded membrane proteins are more likely to be inserted by a cotranslational or post-translational SRP-dependent pathway. The explanation for the integration of cytochrome b_6 into the bacterial membrane by the Sec-dependent pathway come from the results showing that the SRP- and Sec-dependent pathways both use the Sec YEG translocase and that discrimination is done on the SRP/Sec A (Sec B) level (ref *4* and citation within refs *32* and *33*). It is worth stressing that the protein expressed in *E. coli* apocytochrome b_6 is not incorporated into the membrane. These results imply that the cytochrome b_6 apoprotein is not recognized by *E. coli* either SRP or Sec A (Sec B) proteins and also that more experiments are needed for a final determination on which method is used by cytochrome b_6 for incorporation into the bacterial membrane.

At least four nuclear gene products designed CCB1, CCB2, CCB3, and CCB4 have been shown to be specifically involved in heme attachment to cytochrome b_6 (*25*). These observations indicated that heme binding to cytochrome b_6 is catalyzed *in vivo* by the specific enzymatic machinery. Our data shows that *E. coli* cytoplasmic membrane apocytochrome b_6 and exogenic hemes added into culture media spontaneously form a complex. It is important that the heme is added during the expression of the fusion protein. Heme added after the expressions were not incorporated into apocytochrome b_6 (not shown). It is interesting that apocytochrome b_6 has a native topology in the bacterial cytoplasmic membrane and that heme is not needed for its incorporation into the membrane. The native topology is suggested by the presence of both NH_2 and COOH termini of apocytochrome b_6 on the same periplasmic side of the membrane (Table 1) (*18*).

The most important results are that spectral and redox properties of cytochrome b_6 mimic those of the native protein. This very strongly suggest that the structural features responsible for the significant redox differences of two hemes reside in cytochrome b_6 itself rather than arising from a higher order conformation induced by the other subunits.

The experimental procedure presented in this work offers new possibilities for future studies of cytochrome b_6 incorporation into biological membranes. Using this approach and molecular biology methods should allow a more detailed look at the characterization of the stop transfer and signal anchor sequences.

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