

INSERTION OF CYTOCHROME b_6 DERIVATIVES INTO MEMBRANE BY THE BACTERIAL SEC-DEPENDENT PROTEIN TRANSPORT SYSTEM

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Chloroplast contains at least four distinct pathways for targeting proteins into the thylakoid membrane [1]. All of these, the Sec-dependent, SRP-dependent, Δ pH-dependent and spontaneous pathways have direct cognates in bacteria [2]. Translocation of precursor proteins across the cytoplasmic membrane in bacteria is mediated by a multi-subunit protein complex termed translocase, which consists of the integral membrane heterotrimer SecYEG and the peripheral homodimeric ATPase SecA. Preproteins are bound by the cytosolic molecular chaperone SecB and targeted in a complex with SecA to the translocation site at the cytoplasmic membrane. This interaction with SecYEG allows the SecA/preprotein complex to insert into the membrane by binding of ATP to the high affinity nucleotide binding site of SecA. At that stage, presumably recognition and proofreading of the signal sequence occurs [3].

Cytochrome b_6 (215 aa) like many integral membrane proteins operate with uncleaved signal for insertion into membrane but these signal are not identified. The only exception between chloroplast encoded proteins is cytochrome f made in chloroplast as a precursor with typical signal peptide that operate for membrane insertion *in vivo* [4] and in *Escherichia coli* [5]. The SEC-dependent pathway for integration of cytochrome b_6 into thylakoid membrane has been suggested [6]. The explanation for integration of cytochrome b_6 into the bacterial membrane by SEC-dependent pathway come from results suggesting that in chloroplast, at least SRP- and SEC- dependent pathways overlap [7]. We have assumed that also chloroplast uncleaved signal for insertion of cytochrome b_6 would operate in *E.coli*.

An overexpression system for spinach apocytochrome b_6 as a fusion protein to a maltose binding protein (MBP) in *E.coli* was established using the expression vector pMal-p2. Fusion of the cytochrome b_6 to the MBP directs the cytochrome on the SEC-dependent pathway. Using this approach and molecular biology methods we designed few mutants of fusion protein which differed of cytochrome b_6 sequence. The mutants contain: only first, second or third α -helix (pMalp2-A, pMalp2-B, pMalp2-C), first and second α -helix (pMal-p2AB), second and third α -helix (pMalp2-BC), third and fourth α -helix (pMalp2-CD) and the last mutant contain α -helices B,C and D. Upon expression transfer of this artificial transport substrates across the cytoplasmic membrane of *E.coli* have been examined by western blot analysis of protein from different compartments also *E.coli* cells bearing the appropriate plasmid after induction

were converted to spheroplast, and either incubated with an antibody against the MBP or COOH-terminal decapeptide of cytochrome b_6 . These experiments were used for determination of MBP-apocytochrome b_6 mutant topology in the bacterial inner membrane. As expected all mutant were transported by SEC-dependent pathway to periplasm. Western blot analysis of isolated protein and analysis of precipitated *E.coli* spheroplast of above overexpressed plasmids show that helices A, B anchored fusion protein in membrane. In similar experiments with mutant containing helices AB, BC, CD or BCD we observed that only fusion with helices AB, BC and BCD of cytochrome were anchored in the membrane. Mutant of cytochrome b_6 with helices CD was translocated by translocase and detected in periplasm. For a number of reasons we conclude that the signals responsible for insertion and anchoring of apocytochrome b_6 in membrane there are in the first 115 aa contain helices A and B but new investigation are necessary for finding stop transfer signal.

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