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Membrane Topology of MntB, the Transmembrane Protein Component of an ABC Transporter System for Manganese in the Cyanobacterium *Synechocystis* sp. Strain PCC 6803

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The structure of the membrane protein MntB, a component of a manganese transporter system in *Synechocystis* sp. strain PCC 6803, was examined with a series of fusions to the reporter proteins alkaline phosphatase and β-galactosidase. The results support a topological model for MntB consisting of nine transmembrane segments, with the amino terminus of the protein being in the periplasm and the carboxyl terminus being in the cytoplasm.

The first high-affinity transporter system for the transition metal manganese was identified in the cyanobacterium *Synechocystis* sp. strain PCC 6803 (1, 2). This system is a member of the ABC transporter (10) or traffic ATPase (5) superfamily of transporters that mediate movements of numerous diverse substrates across various biological membranes from microbes to humans (17, 19). In gram-negative bacteria, a typical uptake ABC transporter consists of five components: a substrate-binding protein located in the periplasm, two transmembrane proteins that form the pathway for the substrate, and two ATP-binding proteins peripherally located at the cytoplasmic face of the inner membrane. Except with the ATP-binding domains in the last-named polypeptides, sequence similarities between the different ABC transporters are usually not high. However, the overall structures of all ABC transporters are believed to be similar. The two hydrophobic transmembrane domains of the majority of the ABC transporters were originally thought to span the membrane 12 times (six transmembrane segments per domain) (10), a prediction that has since been experimentally confirmed in a number of cases. An exception was found for the MalF protein of the maltose transporter, which was predicted, and then experimentally shown, to have eight transmembrane segments (7).

In *Synechocystis* sp. strain PCC 6803, the ABC transporter system for manganese is encoded by an operon of three closely linked genes: *mntC*, which encodes the substrate-binding protein; *mntA*, which encodes the ATP-binding protein; and *mntB*, which encodes the transmembrane protein (1). The MntB protein is predicted to be extremely hydrophobic, with a molecular mass of 33.4 kDa. Hydropathy analysis with the Kyte and Doolittle algorithm in the TopPred II program (4) suggested the presence of eight putative transmembrane segments in the MntB protein (1). However, using the same program but a different algorithm developed by Engelman and coworkers (6), we found that the MntB protein may have up to 10 membrane-spanning segments (data not shown). To determine the actual number of transmembrane spans in this protein, we used a reporter gene fusion approach to examine the membrane topology of MntB. This method is based on the observation that the enzyme activities of certain reporter proteins translationally fused to a membrane protein can indicate the subcellular locations of the fusion sites in the hybrid protein (9, 20). In the present study, we selected two such reporters that have been widely used to study topologies of many bacterial proteins expressed in *Escherichia coli* cells. The first of these, alkaline phosphatase, encoded by the *phoA* gene, is enzymatically active in the periplasm but not in the cytoplasm (11, 14). In contrast, the second reporter protein β-galactosidase, encoded by the *lacZ* gene, is active in the cytoplasm but not in the periplasm (7).

Construction of MntB fusion proteins. We used synthetic oligonucleotides to generate various fusion proteins. First, *Sal*I restriction sites were introduced throughout the *mntB* gene by using a site-directed mutagenesis procedure (15). For this purpose, DNA sequences from the *mntB* gene were amplified by PCR with one universal forward primer, 5′-TTTTGGTGAGT TGCGAAGGAGCGTTTTCCT-3′, and several reverse mutagenic primers (Table 1). All of these PCR products were sequenced to ensure that no undesired mutation was introduced. Next, for the MntB-PhoA fusions, the *mntB* gene present in an *Eco*RI-*Sal*I fragment of *Synechocystis* sp. strain PCC 6803 DNA (containing *mntC*, *mntA*, and various 5′-fragments of *mntB*) as well as the *phoA* gene present in a *Sal*I-*Not*I DNA fragment (containing the promoterless *phoA* gene from the pPHO7 plasmid [8]) was cloned in the pUK21 vector (21) (Fig. 1A). Alternatively, for the MntB-LacZ fusions, the same *Eco*RI-*Sal*I fragments of *Synechocystis* sp. strain PCC 6803 DNA were cloned into the polylinker region of the pLKC480 vector, which carries the *lacZ* gene (18) (Fig. 1B). The reporter fusions were selectively created at a number of sites present in various predicted cytoplasmic and periplasmic loops of the MntB protein.

Expression of MntB-PhoA fusions. The fusion proteins were expressed in *E. coli* CC118, which lacks the *phoA* and *lacZ* genes (13). The *mntCAB* operon is expressed in *E. coli* cells from its own promoter, as was detected by immunoblot analysis with antibodies raised against the MntC protein (data not shown).

Alkaline phosphatase activities of cells expressing MntB-PhoA fusions were determined by measuring the rates of hydrolysis of the substrate *p*-nitrophenyl phosphate (13). The PhoA activities (in Miller units per minute per milligram of total cellular protein) corresponding to various fusion sites are

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TABLE 1. Oligonucleotides used for the construction of *Sal*I sites in the *mntB* gene of *Synechocystis* sp. strain PCC 6803

Oligonucleotide	Sequence (5′ to 3′)[a]	Site of fusion in MntB protein
B1	CAAAGGCTCC**GTCGAC**CAATGCCAAAA	13W
B2	AGGCGCTCACCCAA**GTCGAC**CGGATTAA	25R
B3	CCATTAA**GTCGAC**CAACCTTTGAGA	49W
B4	GAATA**GTCGAC**GCATAGGCCAGCACTA	69A
B5	TGCCAATGACTGCA**GTCGAC**TTTAACCG	99K
B6	TGGAATAG**GTCGAC**TTACTAGGAA	124S
B7	CAAAACAAGAGCAG**GTCGAC**TTGCG	163K
B8	TGCGTA**GTCGAC**GCCTTGGCATGGTTT	177A
B9	TTGTAAA**GTCGAC**ACAATGGTGAGG	198V
B10	AAGCATA**GTCGAC**TCAAAGCGATCGCT	227F
B11	CCG**GTCGAC**ACATCAAAGTGATAGC	254V
B12	CCACA**GTCGAC**TTTTCATCGTCTTCC	297E

[a] *Sal*I sites are underlined. Boldface nucleotides indicate introduced mutations.

shown in Fig. 2. The activities were relatively high for fusions at amino acid positions 69, 124, 198, and 254 of the MntB protein, indicating that these sites are located in the periplasm. In comparison, PhoA fusions at amino acid positions 49, 99, 163, 177, 227, and 297 of MntB demonstrated significantly lower activities, suggesting that these residues are located in the cytoplasm. It is noteworthy that despite the low activities of the fusions at positions 13 and 25, we have reasoned that they belong to a periplasmic domain, because during TopPred II analysis (4), all algorithms strongly predicted the first transmembrane segment to be between residues 26 and 46. Reporter genes fused to periplasmic N termini of proteins that lack signal peptides display a cytoplasmic phenotype, since without a transmembrane segment following them, the reporters are not translocated through the membrane (3, 12).

To demonstrate that differences in the enzymatic activities of various fusion proteins are not a result of different levels of protein expression, we performed immunoblot analysis. Whole-cell extracts of exponentially growing *E. coli* cultures were

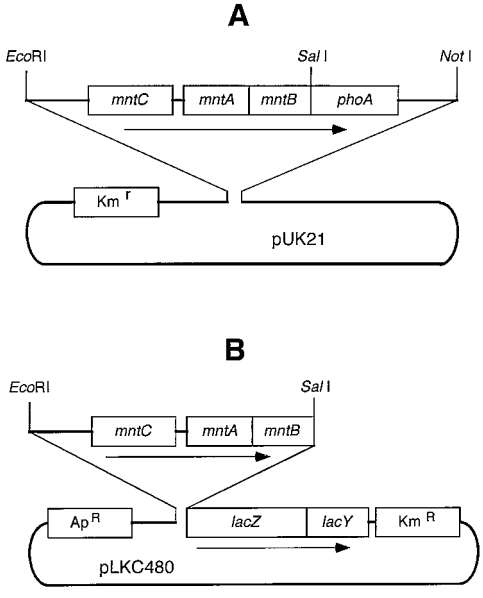

FIG. 1. Schematic illustrations of the plasmid constructions used for the expression of MntB-PhoA (A) and MntB-LacZ (B) fusion proteins in *E. coli* CC118.

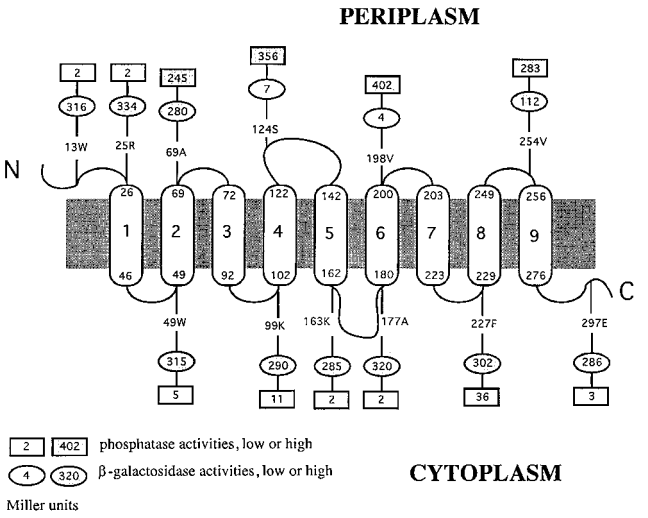

FIG. 2. Experimentally derived model for the structure of the MntB protein with nine transmembrane domains (numbered capsules) and locations of alkaline phosphatase and β-galactosidase fusions (rectangles and ovals, respectively). The small numbers at the tops and bottoms of the capsules correspond to positions of amino acid residues in MntB. Sites of fusions are indicated by similar numbers followed by the one-letter designations of the corresponding amino acids.

fractionated on a denaturing sodium dodecyl sulfate–12% polyacrylamide gel, transferred to nitrocellulose filters, and immunostained with a rabbit anti-PhoA immunoglobulin G preparation (5 Prime → 3 Prime, Inc.). The filters were presoaked with cellular extracts of untransformed *E. coli* CC118 to decrease nonspecific hybridization. As shown in Fig. 3, for the first six fusions, there was no significant difference in the levels of expression of the hybrid proteins irrespective of whether they exhibited low or high alkaline phosphatase activities. However, we could not detect the last six hybrid proteins, even for fusions with high activities (data not shown). It is possible that the larger fusion proteins were relatively unstable. Similar effects have been observed in other studies of different membrane proteins (reviewed in reference 13). Indeed, in all of the fusion-bearing strains, we observed a band at 47 kDa (Fig. 3), which corresponds to the size of the normal PhoA protein and which was not present in the control sample (extracts from *E. coli* CC118 without a plasmid [Fig. 3, lane 1]). We suggest that in all of these samples, the presence of the native PhoA protein results from the degradation of the hybrid proteins in these cells.

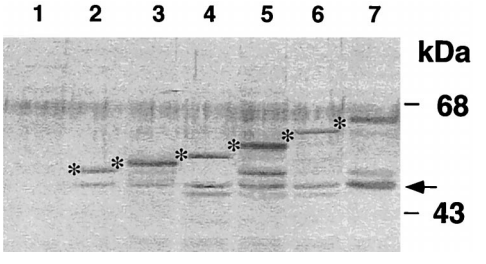

FIG. 3. Western blot analysis of MntC-PhoA fusion proteins expressed in *E. coli* CC118. The bands corresponding to the predicted molecular masses of various fusion proteins are marked by asterisks. The arrow denotes a 47-kDa band corresponding to the size of the normal PhoA protein. Lane 1, no plasmid; lanes 2 to 7, fusion constructions created with primers B1 to B6 (Table 1), respectively.

Expression of MntB-LacZ fusions. To examine the topology of MntB by a second method, we used an alternative reporter protein, β-galactosidase. β-Galactosidase activities of cells expressing MntB-LacZ fusions were analyzed by measuring the rates of hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside according to the method described in reference 16. As explained above, at any given fusion site, the β-galactosidase fusion is expected to display levels of activity opposite to those of the PhoA fusion. This was found to be true for 10 of the 12 MntB-LacZ fusions (Fig. 2). However, two fusions at amino acid positions 69 and 254 had higher than the expected levels of β-galactosidase activity, based on the analysis of the corresponding MntB-PhoA fusions. It is known that β-galactosidase is a less reliable reporter than PhoA (9). It has been suggested that fused to periplasmic domains, β-galactosidase sometimes exhibits high activity because of disruption of the membrane integration of the fusion protein by this reporter (9). In contrast, high activity of a PhoA fusion requires active translocation of the reporter enzyme moiety through the cytoplasmic membrane. Therefore, in a case where both reporters at the same fusion site displayed high activities, we concluded that the site was periplasmic.

Membrane topology of the MntB protein. Considering together the predictions of theoretical analysis and the experimental results with reporter gene fusions, we have derived a model of the topology of the MntB protein in the cytoplasmic membranes of bacterial cells (Fig. 2). According to this model, MntB has nine membrane-spanning segments and has its amino terminus in the periplasm and its carboxyl terminus in the cytoplasm. The extent of each transmembrane segment shown in Fig. 2 was as predicted by the TopPred II program (4) with the algorithm of Engelman et al. (6). The major difference between this experimentally derived nine-span model and the previous theoretically predicted eight-span model (1) of this protein is the identification of the domain between residues 49 and 69 as the second membrane span. We have previously reported that MntB has a high degree of sequence similarity with the membrane protein components of a number of putative ABC transporters (1). More recent analysis has revealed that the MntABC transporter is the representative member of a newly identified subfamily of ABC-type metal transporters present in a number of bacterial species (17, 19). Since the hydrophobicity profiles of the membrane protein components of all members if this subfamily are highly similar to that of MntB, we suggest that these proteins also have nine transmembrane segments.

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