Topological studies of the membrane component of the OleC ABC transporter involved in oleandomycin resistance in *Streptomyces antibioticus*

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Abstract

The OleC ABC transporter of *Streptomyces antibioticus* is constituted by an ATP-binding protein (OleC) and a hydrophobic protein (OleC5). Here we present experimental evidence demonstrating that the OleC5 protein is an integral membrane protein and we propose a topological model for its integration into the membrane. This model is based on the generation of hybrid proteins between different regions of OleC5 and an *Escherichia coli* β-lactamase (BlaM) and the determination of the minimal inhibitory concentrations to ampicillin in these constructions. Fusions were generated both by cloning specific fragments of oleC5 and by creating ExoIII nested deletions of the gene. In the topological model proposed there will be six α-helix transmembrane regions, two cytoplasmic and four periplasmic loops and a hydrophobic linker domain.

Keywords: Recombinant DNA; Macrolide; Polyketide; Secondary metabolism; Membrane protein

1. Introduction

ABC (ATP-binding cassette) transporters comprise a family of membrane-associated transport systems which are involved in the export and import of a great variety of molecules (for a review see [1]). In antibiotic-producing organisms, several genes have been described that confer resistance to the produced antibiotic and they encode ABC transporters [2–7]. Some of them [2,4,5,7] are constituted by two genes: one encoding a hydrophilic ATP-binding protein and a second encoding a hydrophobic membrane component. Others lack the membrane component or it has not yet been identified [3,6]. In *Streptomyces antibioticus*, an oleandomycin producer, we have identified three genes conferring resistance to oleandomycin [4]. Two of them, oleB and oleC, encode ABC transporters [4,6]. The oleC transporter is formed by two components: one protein (OleC) resembling a large family of ATP-binding proteins able to recognize and hydrolyse ATP and a second protein (OleC5) which shows the characteristic hydrophobic profile of membrane proteins. Here we present experimental evidence demonstrating that the OleC5 protein is a membrane protein and we report a topological study of this membrane protein through the generation of translational fusions to a β-lactamase gene (blaM).
2. Materials and methods

2.1. Microorganisms and vectors

Plasmids pOR8003 and pOR8007 (C. Olano, unpublished results) were used as a source of DNA for the OleC transporter of *Streptomyces antibioticus* ATCC 11891. pOR8003 is a pUC18 with a 1.3 kb *PstI-BamHI* fragment that contains the entire oleC and the 3'-end of the oleC5 gene. pOR8007 is a pUC18 with a 0.8 kb *BamHI* fragment that contains the 3'-end of oleC5. *Escherichia coli* TGI rec01504::Tn5 was used as host for subcloning. Phagemid pJBS633 [8] or its derivative pJBS633sm (this paper) were used as vectors for the construction of β-lactamase translational fusions in topological studies. pIJ2921 and pIJ2925 [9] were used as cloning vectors.

2.2. DNA manipulation and sequencing

DNA manipulations were according to standard procedures for *E. coli* [10] and *Streptomyces* species [11]. Sequencing was performed with the dideoxynucleotide chain-termination method [12] using [α-35S]dATP (1200 Ci/mmol; Amersham) and modified T7 DNA polymerase (Sequenase version 2.0; U.S. Biochemicals). For sequencing the gene fusions between oleC5 and blaM, a synthetic oligonucleotide (5'-dCTCGTGCACCCAACTGA-3') was used [8] that anneals to the 5'-end of blaM.

2.3. Determination of ampicillin resistance

The level of ampicillin resistance by single cells or cell patches was determined as described [8].

3. Results and discussion

3.1. Hydropathy profile of the OleC5 protein

Analysis of the deduced product of the oleC5 gene for hydrophobicity using the method of Kyte and
Fig. 2. Schematic representation showing the in-frame fusions created in pJBS633sm. A: Construction of plasmids pTM6 and pTM41. B: Sequence at the fusion between the tet and oleC genes (1) and between the oleC5 and blaM genes (2). B: BamHI; G, BglII; S, SalI; Sc, SacI; Sm, SmaI.

Doolittle [13] showed a hydropathic profile consistent with an integral membrane protein [4]. It contains six hydrophobic regions with the potential to form transmembrane helices, which could represent membrane-spanning domains.

3.2. Construction of translational fusions to a β-lactamase

In order to verify this model we used a method previously described [8,14] for the analysis of membrane topology of proteins. This method is based on the construction of translational fusions between portions of a membrane protein gene and a gene encoding a β-lactamase (BlaM) and the analysis of the different clones for ampicillin resistance. Since this BlaM lacks the leader peptide region it cannot be exported outside the cell unless it is fused to a region of the membrane protein which can translocate the β-lactamase through the membrane. Consequently, the determination of the susceptibility of the different constructions to ampicillin can give information about the membrane topology of the protein. So, if the BlaM is fused to a region of the protein that passes through the membrane it would be exported extracellularly and would confer a high level of resistance to ampicillin. In contrast, if it is fused
Fig. 3. Topological models for OleC5 protein. A: Theoretical model based on hydropathy profile analysis using the COMPARE/DOTPLOT program [17] and the predictions by Eisenberg et al. [18] and taking into consideration the positive-inside rule hypothesis [15]. B: Proposed topological model for the integration of OleC5 into the membrane assuming the theoretical considerations and the analysis of the results obtained with the OleC5-BlaM fusions. HI-HVI indicate the positions of potential transmembrane α-helices. CI-CII indicate the cytoplasmic loops. PI-PIV indicate the periplasmic loops. SI-SII indicate the hydrophobic linker domain. The arrows point to the amino acid positions in which the fusions to BlaM were made. Amino acids are shown in single letter code.
to a region of the protein located in the cytoplasm, it would only confer a low level of resistance as a consequence of the initial lysis of the cells and the release of the β-lactamase to the medium.

The vector used for these studies was pJBS633 [8]. Initially we generated pTM3 to allow the adequate expression of oleC5 when cloned into pJBS633 (Fig. 1). A Sall-BamHI fragment from pOR8003 was cloned into pIJ2921, rendering pTM1. This construction was linearized with BamHI to insert a BamHI fragment from pOR8007, generating pTM2. From this plasmid a Sall-EcoRI fragment (this last site from the polylinker) was obtained and cloned in pIJ2925, generating pTM3. Subcloning fragments from pTM3 using the Bglll site (site 1 in Fig. 1) and any other site from pTM3 into the BamHI site of pJBS633 (or pJBS633sm, see below) will inactivate the tetracycline resistance gene (ret) in pJBS633 generating a fusion protein between the N-terminus of the tetracycline gene product and the C-terminus of OleC (Fig. 2). Because tet-oleC and oleC5 are translationally coupled, this construction will allow its expression from the tetracycline promoter.

In order to confirm the correct expression of OleC5 and to verify that it was an integral membrane protein, two constructions (pTM4 and pTM5) were generated between two different regions of oleC5 and blaM. They were obtained by subcloning the Bglll-BamHI fragment (the BamHI site blunt ended with Klenow) (sites 1 and 2 of pTM3 in Fig. 1) and the Bglll-Styl fragment (the Styl site blunt ended with Klenow) (sites 1 and 3 in pTM3 in Fig. 1) into the BamHI and PvuII sites of pJBS633. In both cases in-frame fusions were created between specific regions of oleC5 and blaM. In pTM4 the hybrid protein generated contains the first 36 amino acids of OleC5 which corresponds to a hydrophilic region possibly located in the cytoplasmic face of the membrane. Therefore this protein is expected to confer a low level of resistance to ampicillin. pTM5 encodes a fusion protein containing the first 193 amino acids of OleC5 comprising a transmembrane hydrophobic region. This protein is expected to confer a high level of resistance to ampicillin if the OleC5 region is able to drive the leaderless β-lactamase through the membrane. As shown in Table 1, the determination of the minimum inhibitory concentration (MIC) of the corresponding clones confirmed the predicted results and consequently the fact that OleC5 was an integral membrane protein.

To determine an overall topological model for OleC5 protein, we generated other translational fusions at different positions of OleC5. To facilitate subcloning and because the oleC5 gene contains several PvuII sites, the PvuII site of pJBS633 located immediately before the β-lactamase gene was inactivated and replaced by a Smal site. This was done by inserting a 9-mer linker containing a Smal site, generating pJBS633sm. Then, the 1.2 kb Bglll fragment from this plasmid was subcloned into the BamHI site of pJBS633sm (Fig. 1). The resultant construction pTM6 was then linearized with EcoRI (an iso-schizomer of SacI) present in the polylinker and nested deletions generated by ExoIII digestion and further digestion with S1 nuclease. Then, after digesting with Smal and religation, the resultant constructions were transformed into E. coli competent cells and clones resistant to kanamycin (Km'; 50 ug/ml) selected. The different clones obtained were then tested for their susceptibility to ampicillin and the results are shown in Table 1. Some of the clones were highly resistant to ampicillin (MIC 200 ug/ml), others grew in relatively high concentrations of ampicillin (100 ug/ml) while others only grew in the presence of discrete ampicillin concentrations (between 10 and 50 ug/ml). The exact position in the OleC5 protein where the β-lactamase was fused was determined by sequencing the DNA fusion regions using a synthetic oligonucleotide designed to anneal to the β-lactamase coding region 40 bp downstream from the fusion point. The amino acids where the β-lactamase was fused in the different translational fusions are shown in Table 1.

3.3. A proposed topological model for OleC5

The hydropathy profile of OleC5 suggested that it was possibly an integral membrane protein with six hydrophobic regions that could span through the membrane [4]. From this analysis and considering (i) that a transmembrane segment should contain at least 20 hydrophobic (apolar) amino acids to form an α-helix spanning the lipid bilayer and (ii) the positive-inside rule hypothesis [15] that positively charged amino acids are mainly present in the cytoplasmic face of the membrane and negatively
charged amino acids at the extracellular face, a topological model for integration of OleC5 in the cell membrane can be predicted (Fig. 3A). To confirm this model we created and analyzed several fusion proteins between OleC5 and a β-lactamase. Some of the results obtained with these fusion proteins were in agreement with the predicted model. Thus the fusions made to the amino acids L6 and I36 conferred a very low level of resistance to ampicillin, confirming their position in the cytoplasmic side of the membrane. The same occurred with fusions in D223 and V243, which conferred a very high level of resistance to ampicillin, confirming their localization in the external side of the membrane. The levels of resistance obtained with the fusions to V59, M184, M135 and V130 are those expected if the fusions were made to amino acids localized at different positions along a membrane-spanning domain. However, some of the data were in completely disagreement with the theoretical topological model. Thus M193, V109, G108, S107, G98, Gl00 and D104, which were all, according to the theoretical model, presumably located in the cytoplasmic face of the membrane, showed very high levels of resistance to ampicillin. Based on these results, together with the theoretical considerations mentioned before, we propose a more plausible topological model for the integration of OleC5 in the membrane (Fig. 3B). In this model there are six α-helix transmembrane regions (HI–HVI) and a hydrophobic linker domain (SI–SII) that resides between HI and HII and that it is also inside the membrane. A similar model has been postulated and confirmed for the protein KpsM of Escherichia coli involved in the transport of capsule polysaccharides [16].

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References

