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The transmembrane topology of the α subunit from the ATPase in *Escherichia coli* analyzed by PhoA protein fusions

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The *atpB* gene encodes the α subunit of the H^+-ATPase of *E. coli*. The topology of this membrane protein has been analyzed by PhoA fusions. The results support an eight transmembrane segment model that is consistent with the hydropathic profile.

F_0-ATPase; Subunit α topology; PhoA protein-fusion

1. INTRODUCTION

The proton-translocating ATPase from *Escherichia coli* is responsible for synthesis of ATP (for review see [1,2]). The enzyme complex consists of two distinct parts: the cytoplasmic F_1 that houses the catalytic sites, and the membrane bound F_0 which forms a proton channel through the membrane.

The three subunits α, β and γ is a stoichiometry of 1:2:6-10 [3] constitutes F_0 and all three are essential for normal ATPase function. The secondary structure of subunits β and γ have been determined [4] whereas the structure of subunit α has not been elucidated. The hydropathic profile of subunit α exhibits alternating hydrophobic and hydrophilic segments and indicates 6-7 transmembrane segments [4]. This has led to proposals of two-dimensional models [4-8] wih five, six or seven transmembrane segments. Eisenberg et al. [9] have on the basis of an algorithm, which also considered requirements for cooperative folding of protein segments into the membrane, found tht residues 40-60, 70-90, 101-121, 122-142, 146-166, 182-202, 217-237 and 242-262 of subunit α might form transmembrane segments, thus giving rise to a conformation with eight transmembrane segments. A few experiments give information about the orientation of the α subunit in the membrane: (i) Digestion experiments with proteases suggest that the N-terminal end is located at the cytoplasmic side of the membrane [4], and (ii) cross-linking between subunit α and β with dithiobissuccimidylpropionate indicates that the α subunit has lysine residues exposed to the cytoplasm [10].

We have studied the conformation of the α subunit in the membrane with *atpB:phoA* fusions. From the results we can conclude that the C-terminal end of the α subunit is located at the cytoplasmic side of the inner membrane, and that the only model that is consistent with all data, is a model with eight transmembrane segments.

2. MATERIALS AND METHODS

2.1. *Bactereia*

Bacteria were grown in LB medium [11]. Rabbit antibodies against alkaline phosphatase was a gift from H. Fridén, University of Lund.

Strains: The *E. coli* strains used were MC1000 [12] and CC118 [13].

2.2. *Construction of atpB:phoA gene fusions*

Tn*phoA* transposition: The Tn*phoA* was transposed into the *atpB* gene on plasmids pOMC11-1 [14] (pOMC121 and pOMC122) and pFHC167 [15] (pOMC120) as described [13].

Construction of *atpB:phoA* fusions (pCB plasmids): In plasmid pOMC122 (this study) the *atpB:phoA* fusion site is located 11 bases downstream of the second *Bam*HI site in the *atpB* gene. From this plasmid the *phoA* and the Km resistance genes can be isolated on a *Bam*HI fragment. Plasmid pCMC1070 [16], which carries the λP_R promotor in front of the *atpBEF'* genes, was opened at the unique *Hpa*I site in the intergenic region between *atpE* and *F*. The DNA was digested with the exonuclease *Bal*31 and ligated with *Bgl*II linkers. The resulting plasmids were treated with restriction endonuclease *Bgl*II and religated in presence of the above described *Bam*HI fragment.

Strain MC1000 was transformed with the ligation mixture and plated on LB-agar plates supplemented with Amp (100 μg/ml), Km (50 μg/ml) and XP (50 μg/ml) and incubated at 30°C.

Plsamids were prepared from blue colonies and the positions of fusions were determined by restriction enzyme analysis. The *in frame* insertion of the *Bam*HI-fragment in a *Bgl*II linker-site, results in hybrid proteins which contains only four extra amino acids (K-I-L-N) at the fusion, when compared with a Tn*phoA* transposition. Sequencing

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Abbreviations: X-P, 5-bromo-4-chloro-3-indolylphosphate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; F_1, cytoplasmic ATPase part of the H^+-ATPase complex; F_0, membrane part of the ATPase complex; PhoA, alkaline phosphatase enzyme; *bla*, β-lactamase gene; Km, kanamycin; Amp, ampicillin.

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was done by the chain-termination method [17]. Preparation of DNA, plasmids, cleavage by restriction enzymes, ligation and transformation were done according to standard methods [18].

2.3. *Immunoblotting of SDS-PAGE*

Strains containing the pCB fusion plasmids were grown at 30°C and synthesis of the *atpB:phoA* fusion protein was induced by raising the temperature to 39°C. The cells were harvested and total cell proteins were subjected to SDS-PAGE and then transferred to nitrocellulose sheets. These were then reacted with the rabbit antibody specific to alkaline phosphatase from *E. coli*. A peroxidase-conjugated horse anti-rabbit immunoglobuline preparation was used as second antibody [unpublished method by J.K. Andersen].

2.4. *Enzyme assays and normalization of alkaline phosphatase activities*

Alkaline phosphatase and β-lactamase were assayed as described [19,20]. The PhoA activities were determined at 30°C, where the λP_R promotor carried by the pCB plasmids is completely repressed, and were corrected for differences in relative copy numbers, based on the β-lactamase activities expressed from the plasmids (table 1). The *bla* gene on pCB plasmids was in addition to the transcription from the *bla* promotor also transcribed from the λP_{RM} promotor present on the plasmids, while the *bla* gene on the pOMC plasmids only was transcribed from the *bla* promotor. We therefore supposed that the copy numbers of pCMC1070 and pFHC167 were equal, as both have the normal pBR322 origin of replication.

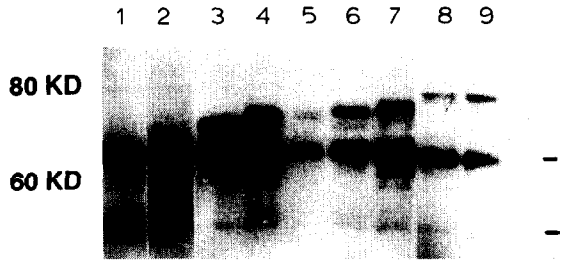

Fig.1. Immunoblot analysis of α-PhoA fusion proteins. Plasmids in strain MC1000: Lane 1: pCB51; lane 2; pCB135; lane 3: pCB201; lane 4: pCB234; lane 5: pCB236; lane 6: pCB241; lane 7: pCB246; lane 8: pCB263 and lane 9: pCB265. The antiserum also contained other *E. coli* protein specific antibodies (marked with -).

3. RESULTS

In order to analyze the topology of the α subunit of the ATPase from *E. coli*, we used the genetic approach of isolating *atpB:phoA* fusions. The rationale behind this approach is that alkaline phosphatase only becomes active when it has been transported through the cytoplasmic membrane. Fusion proteins will then only have enzymatic activity if the transmembrane segment

Table 1

Characteristics of *atpB':phoA* gene fusions

Strain/ plasmid[a]	Fusion position[b]	PhoA activity[c]	Relative PhoA activity	Lethal when overproduced[e]	Expected cellular localisation[f]	Predicted molecular weigth of fusions (kDa)
Strain MC1000						
pCB51	51	5	M	–	M	53
pCB135	135[s]	< 1	L	+	C	63
pCB139	139	< 1	L	+	C	63
pCB142	142	< 1	L	+	C	63
pCB148	148	< 1	L	+	C	64
pCB201	201[s]	< 1	L	+	C	70
pCB234	234	27	H	+	P	74
pCB236	236	30	H	+	P	74
pCB241	241[s]	30	H	+	P	75
pCB246	246[s]	6	M	+	M	75
pCB263	263	6	M	+	M	77
pCB265	265	11	M	+	M	77
Strain CC118						
pOMC120	116	46	H	nd	P	60
pOMC121	229[s]	59	H	nd	P	73
pOMC122	238[s]	48	H	nd	P	74

[a] pCB plasmids are constructed by in vitro techniques (see section 2.1.)
[b] Number of amino acids from the α subunit present in the hybrid
[c] Alkaline phosphatase activities (arbitrary PhoA units)
[d] Relative alkaline phosphatase activities, corrected for plasmid copy number differences. H: 55–100% of maximum, M: 15–30% of maximum and L: <2% of maximum
[e] + for lethal effect when overproduced after induction of the λP_R-promoter (CI_{857})
[f] Expected localization of fusion position from PhoA activities: M, membrane; C, cytoplasmic; P, periplasmic
[s] Sequenced fusion-positions
nd not determined

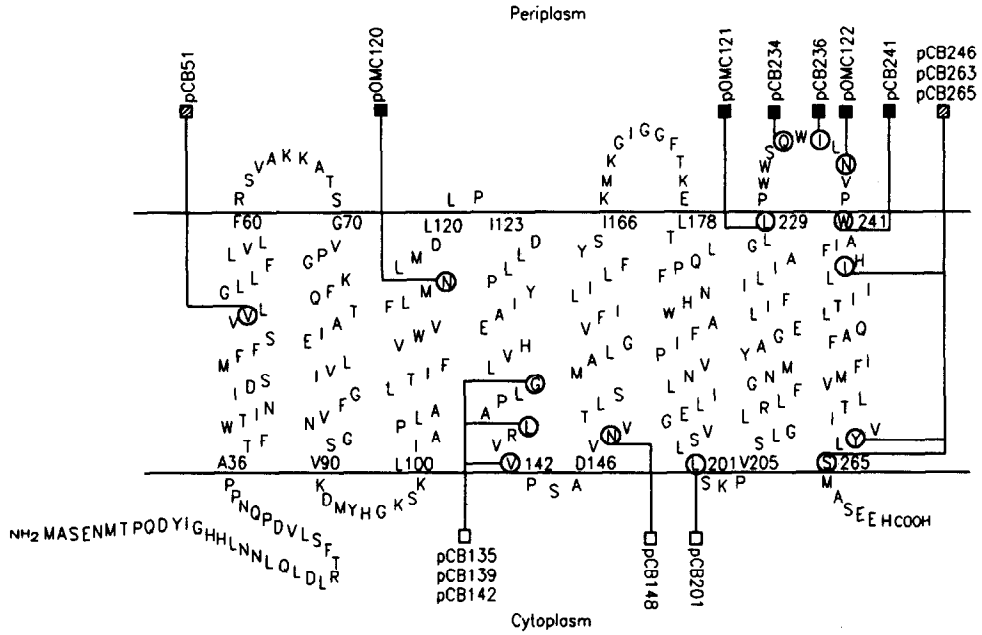

Fig.2. Suggested arrangement of the α subunit polypeptide across the membrane. Positions of α-PhoA fusions are encircled and the relative alkaline phosphatase enzyme activities of the corresponding hybrid proteins are indicated: filled square, 55–100% of maximum; hatched square, 15–30% of maximum and open square, <2% of maximum.

in front of the alkaline phosphatase moiety brings this part of the hybrid protein to the periplasmic space [21].

Fifteen different fusions were constructed. Three of these were obtained after transposition of Tn*phoA*, and the rest were constructed using in vitro techniques.

Fusion plasmids yielding high and low PhoA activities were found and easily distinguished on media containing the PhoA indicator, X-P. Strains with fusions exhibiting the low activities only became pale blue after 3 days incubation at 30°C. The *atpB:phoA* fusions were expressed from the *atpB2p* promotor carried by all plasmids. The positions of the fusions were determined by restriction enzyme analysis. In selected plasmids the precise point was determined by DNA sequencing (table 1). The sizes of the hybrid proteins were determined by immunoblotting (fig.1) and corresponded well to the sizes predicted from the analysis of the plasmid DNA, assuming 47 kDa as the molecular mass of alkaline phosphatase and an average weight of 115 for amino acid residues in subunit α.

All fusion proteins tested, except that expressed from pCB51 (α (51):PhoA), had a lethal effect when overproduced after induction of the upstream λP_R-promotor (table 1). This indicates that the α subunit part of these proteins is integrated and folded normally in the cytoplasmic membrane. For the wild type α a subunit this effect has been shown previously [16,22], and is probably caused by a collapse of the membrane potential.

4. DISCUSSION

Fusions with alkaline phosphatase might fall into three categories: (i) those with high activities, which have the alkaline phosphatase moiety exposed to the periplasm, (ii) those with low activities, which have the phosphatase moiety exposed to the cytoplasm, and (iii) those with intermediate activities, which either have reduced enzymatic activity because the phosphatase moiety is partly buried in the membrane (e.g. pCB51), or only some of the molecules have the phosphatase moiety positioned in the periplasm (e.g. pCB246, pCB263 and pCB265) [21].

We obtained a cluster of fusions with high activity around I236, showing that this region must be exposed to the periplasm. The C-terminal end of the *a* subunit must be located at the cytoplasmic side of the inner membrane, since the fusions around S265 have lower activities than those around I236. The fusions with low activity fall into two clusters, one in the region around V142 and one around L201. These two regions must therefore be exposed to the cytoplasm, either separated by two transmembrane segments as shown in fig. 2, or the whole region could be situated in the cytoplasm. The high hydrophobicity of the region, V142–L201, strongly indicates that this region forms two transmembrane segments as shown in fig.2.

These conclusions are based on 10 different subunit *a*-PhoA fusions, which have enzymatic activities that indicate either cytoplasmic or periplasmic localization and 3 fusions with activities, that indicate ambiguity in the localization ambiguity in the localization of the phosphatase moiety. The activity of the fusion encoded by plasmid pOMC120 indicates that N116 is located close to the periplasm and suggests a transmembrane segment between N116 and V142. The activity of the fu-

sion encoded by plasmid pCB51 indicates that V51 is located in the membrane and suggests that the first transmembrane segment has the orientation as shown in fig.2.

The conformation of the N-terminal end of the α subunit cannot be assigned unambiguously from this study, but the experiments with protease digestion indicate that the N-terminal end of the α subunit is located in the cytoplasm [4]. This, taken together with our localization of the C-terminus to the cytoplasm, rules out all models [5,8] with unequal numbers of transmembrane segments. Furthermore in the model with 5 transmembrane segments [5,23] alkaline phosphatase moieties with both high and low activities would be placed at the cytoplasmic side of the membrane. A model with 6 transmembrane segments would imply that the region, R61–P122, should be exposed to the periplasm. This seems very unlikely, because the highly hydrophobic region, L100–L121, which in all models [5,7–9] is located in the membrane, together with the hydrophobic region, G70–V90, which in two models [8,9] is located in the membrane, then would be in the periplasm. The model shown in fig.2 also takes into account that the cross-linking experiments with dithiobissuccimidylpropionate suggest that subunit α has lysine residues exposed to the cytoplasm. A model with 6 transmembrane segments would only expose K203 to the cytoplasm, while the model suggested here exposes K91, K97, K99 and K203 to the cytoplasm.

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