Volume 247, number 2, 396-400 FEB 07070 April 1989

Topological analysis of the amino-terminal region of lactose permease using the *Escherichia coli* outer membrane protein, OmpA, as a marker

Sheila MacIntyre, Marie-Luise Eschbach, Heinz Schwarz and Ruth Ehring*

Max-Planck-Institut für Biologie, Correnstrasse 38, D-7400 Tübingen and **Institut für Genetik der Universität zu Köln, D-5000 Köln 41, FRG*

Received 3 March 1989

*Lac*Y-*omp*A fusions, encoding the N-terminal 50, 71 or 143 residues of lactose permease, were constructed. The observed orientation of the OmpA part of each hybrid protein with respect to the plasma membrane supports current models of the N-terminus of Lac permease. Hybrids possessing the entire mature OmpA were very stable; those with only a part thereof were much less stable. Due to their in vivo stability and accessibility to antibody it is proposed that such hybrids may represent potential models to investigate the assembly pathway of lactose permease.

Lactose permease; Topology; Signal sequence; Protein, OmpA; Membrane assembly

1. INTRODUCTION

The proton:galactoside symporter, lactose permease (LacY), is a polytopic integral protein of the inner membrane of *Escherichia coli*. Current structural models predict that it is organised in the membrane in a series of between 10 and 14 membrane-spanning helices [1-3]. Biochemical analysis using antibodies, proteases and surface iodination have localised both the N- and C-termini as well as a small segment around residue 135 to the inner side of the plasma membrane (reviews [1-4]). However, much of the polypeptide, especially in the N-terminal domain, is apparently inaccessible at either surface of the membrane [5], making topological analysis of the protein more difficult. With the exception of leader peptidase I and M13 procoat [6], there is a general dearth of information on the assembly process of bacterial inner membrane proteins. Regarding LacY, recent studies on truncated LacY fragments in minicells have suggested that the N-terminal region is capable of independently associating with the membrane [7,8]. Here, by constructing several LacY-OmpA hybrids, we have used the *E. coli* outer membrane protein, OmpA, as a marker firstly to test the model of the topological arrangement of the N-terminus of LacY and secondly with the aim that in the future these hybrids can be used to investigate the pathway of membrane assembly of the permease.

Correspondence address: S. MacIntyre, Max-Planck-Institut f. Biologie, Corrensstraße 38, 7400 Tübingen, FRG

2. EXPERIMENTAL

2.1. *Bacterial strains and growth conditions*

Strain UH203 is *Δlac supF* ompA *rec*A *pro*A or *B rps*L F'*lacI*^{Q} *lacZ* M15 *pro*AB^{+} [9]. The chromosomally *omp*A^{+} strain parent of UH203 and has otherwise the same characteristics. Cells were grown at 37°C in L-broth containing glucose (0.6%) or IPTG (1 mM) plus ampicillin (50 μg/ml) or tetracycline (12.5 μg/ml).

2.2. *Plasmids and construction of fusion genes*

Plasmids pY50 and pBI [8] both carry the *lac*Y gene under lac control. In pY50, a 24-mer linker encoding a suppressible amber codon prior to a unique *Bgl*II site follows codon 50 of *lac*Y (fig.1). pBI carries unique sites for *Ava*I and *Bss*HII. The sources of the *omp*A gene were (i) pTU500 [9], (ii) a derivative of this plasmid in which the *Bam*HI site at position 228 had been

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

converted to an *Sma*I site by insertion of an *Sma*I linker (dCCCCGGGG) and (ii) pINOmpA-RD which possesses a unique *Eco*RI site corresponding to amino acids −1/+1, i.e. the signal sequence cleavage site, of OmpA. pINOmpA-RD will be described elsewhere.

To construct pY50/228-325, pY50/85-325 and pY50/1-325, pY50 was linearized with *Bgl*II, filled in and religated independently with pTU500(*Bam*228-*Sma*), pTU500, and pINOmpA-RD, respectively, each of which had been digested as shown in fig.1A. Tetracycline-resistant transformants derived from each ligation mixture were screened for plasmids and gene products of the correct size. pUCY50/A228-325, pUCY50/A85-325 and pUCY50/A1-325 carry, respectively, the 2.77, 3.17 and 3.4 kb *Eco*RI fragments, derived from pY50/228-325, pY50/85-325 and pY50/1-325, subcloned in pUC19 in the opposite direction to the pUC19 *lac* promoter. pUC71/A228-325 and pUC143/A1-325 were constructed in a similar way using the *Ava*I and *Bss*HII sites of pBI (fig.1B,C). Due to the recreation of an *Eco*RI site at the fusion joint within Y143/A1-325, transfer of this gene from the pBI vector to pUC19 was achieved by deleting a *Bgl*II fragment from pUCY71/A228-325 and inserting the equivalent fragment from pY143/A1-325. Fusion joints of mutant genes were confirmed by DNA sequencing using synthetic oligonucleotide primers which were complementary to appropriate regions within the *ompA* gene.

2.3. *Trypsin digestion in plasmolysed cells, alkali extraction and immunoprecipitation*

Following 10 min induction with IPTG, exponentially growing cells (strain UH203 chromosomally *ompA*$^+$) were labelled with [^{35}S]methionine (50 μCi/ml, >800 Ci/mmol; New England Nuclear) for 5 min at 37°C. For trypsin digestion, cells were plasmolysed with sucrose/EDTA and treated with trypsin for 1 h at 4°C [10]. For alkali extraction, cells were extracted with 0.1 N NaOH. Soluble and insoluble proteins were recovered by centrifugation, precipitation with trichloroacetic acid and immunoprecipitation [11]. All immunoprecipitations were performed using rabbit anti-OmpA serum.

2.4. *Electron microscopy*

Cells, grown in the presence of IPTG for 2.5 h, were plasmolysed with 25% sucrose, fixed with 2% formaldehyde and 0.05% glutaraldehyde and embedded in Lowicryl K4M [9]. Ultrathin sections were labelled with rabbit anti-OmpA serum and protein A-gold complexes.

3. RESULTS

3.1. *Construction of fusion genes and properties of encoded polypeptides*

Five *lacY-ompA* fusions, encoding three different N-terminal segments of lactose permease and either the entire mature part of OmpA, i.e. minus the signal sequence, or shorter C-terminal parts thereof, were constructed as described in fig.1 and section 2. All constructs remained under *lac* transcriptional control and contained the *ompA* stop codon and terminator sequence.

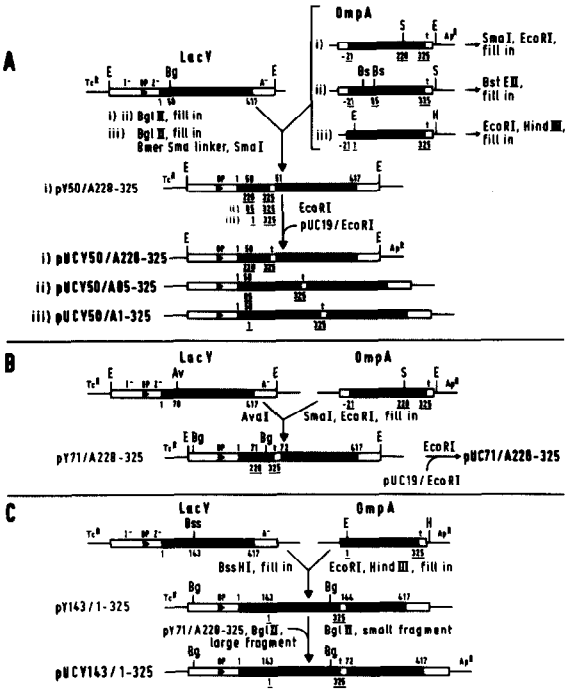

Fig.1. Construction of fusion genes. (A) (i) pUCY50/A228-325, (ii) pUCY50/A85-325, (iii) pUCY50/A1-325; (B) pUCY71/A228-325; (C) pUCY143/A1-325. Boxed in region, structural *lacY* gene; I$^-$, partial *lacI* gene; OP, *lac* promoter region; Z$^-$, partial *lacZ* gene; A$^-$, partial *lacA* gene; cross-hatched region, structural *ompA* gene; t, *ompA* terminator; numbers, codons for respective amino acids of LacY or OmpA (underlined). Only relevant restriction enzyme sites are shown: Bg, *Bgl*II, Bs, *Bst*EII; Bss, *Bss*HII; E, *Eco*RI; H, *Hin*dIII; S, *Sma*I. Additional amino acids encoded at the fusion joints were (A) (i) (Y)LGR + SG, (ii) (Y)LGR + SPR$^+$, (iii) (Y)LGR$^+$ SPNS; (B) G, and (C) NS. (Y) represents tyrosine encoded by the amber stop codon in the presence of the *sup*F suppressor. See section 2 for details.

Following induction of each of the genes with IPTG, polypeptides of about the expected M_r (15 980, 31 320, 41 250, 18 250 and 50 760, for Y50/A228-325, Y50/A85-325, Y50/A1-325, Y71/A228-325 and Y143/A1-325, respectively) could be detected in whole-cell preparations by immunoblotting with anti-OmpA serum and by radiolabelling (see fig.2). However, the quantity of each of these proteins varied dramatically. Those constructs containing the entire mature part of OmpA were very stable. Y143/A1-325 was the major protein present in membrane preparations from cells expressing this gene (fig.2A). Y50/A1-325 could

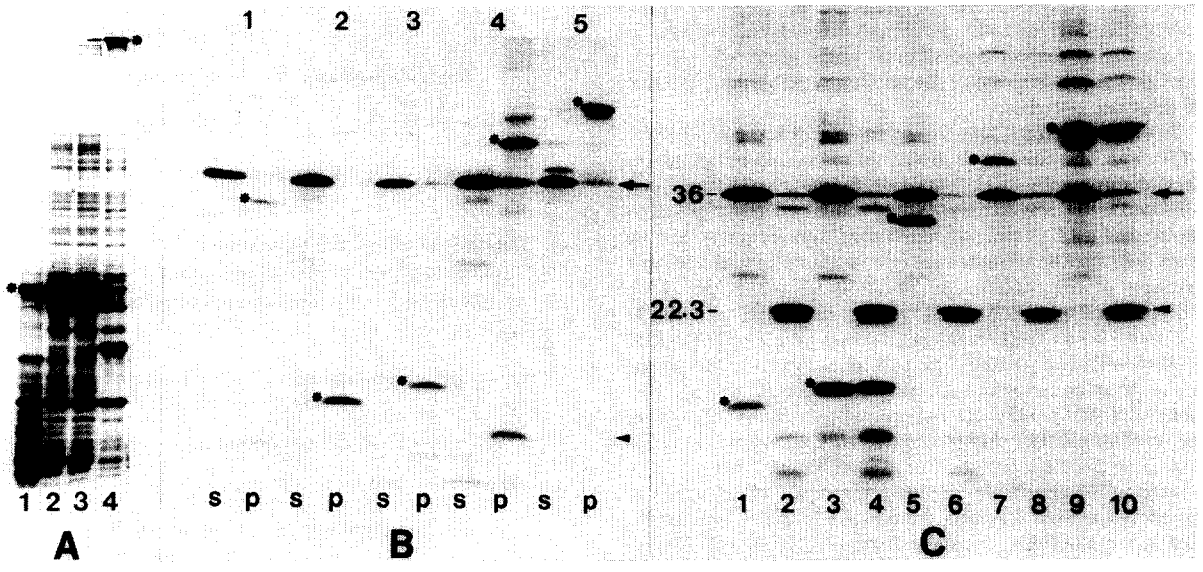

Fig.2. (A) SDS-polyacrylamide (10%) gel of membrane preparations, solubilised at (1) 25, (2) 37, (3) 50, (4) 100°C, from strain UH203 expressing Y143/A1-325. (B) Alkali extraction of cells expressing fusion proteins. (1) Y50/A85-325, (2) Y50/A228-325, (3) Y71/A228-325, (4) Y50/A1-325, (5) Y143/A1-325. s, supernatant fraction; p, pellet fraction. (C) Trypsin digestion of fusion proteins in plasmolysed cells. (1,2) Y50/A228-325, (3,4) Y71/A228-325, (5,6) Y50/85-325, (7,8) Y50/A1-325, (9,10) Y143/A1-325. Odd numbers, controls incubated in the absence of trypsin; even numbers, trypsin-treated. Asterisk, gene products; arrow, chromosomally encoded OmpA; arrowhead, (A) breakdown product from Y50/A1-325, (B) wild-type OmpA tryptic fragment(s). 36 and 22.3, apparent molecular masses of OmpA and OmpA tryptic fragment (in kDa). (B,C) Autoradiograms of SDS-polyacrylamide (14%) gels of immunoprecipitates are shown.

also be visualised in whole-cell or membrane preparations by staining with Coomassie blue although it was present in lower quantities due to the presence of the suppressible stop codon (see section 2 and fig.1). In contrast, hybrids containing only the small C-terminal fragment of OmpA (Y50/A228-325 and Y71/A228-325) were extremely unstable (half-life about 1-2 min) and were only barely visible by immunoblotting. Y50/A85-325 was also degraded but at a much slower rate. The stability of these fusion proteins was clearly more closely related to the presence of the complete OmpA protein than to their final cellular location, i.e. at either the periplasmic or the cytoplasmic surface of the plasma membrane (see section 3.2). It was also noted that like wild-type lactose permease [12], the Y143/A1-325 polypeptide, but none of the other constructs, aggregated when heated at 100°C in SDS, whereas, following solubilisation at 50°C, it migrated on SDS-polyacrylamide gels with close to the expected M_r (fig.2A).

3.2. *Location of the fusion proteins*

Insolubility in alkali is a well-known property of integral membrane proteins including those anchored by a single hydrophobic domain [13,14]. As wild-type OmpA is one notable exception [13], we analyzed the solubility of the LacY/OmpA hybrids in alkali. In every case, the hybrid was recovered in the pellet (fig.2B). According to this criterion, each of these fusion proteins would appear to be inserted into the membrane.

To determine the location of the OmpA portion of the fusion proteins with respect to the plasma membrane, plasmolysed cells were treated with trypsin and any OmpA-related fragments immunoprecipitated. In such cells, proteins exposed at the trans(periplasmic) surface of the plasma membrane would be accessible to the protease whereas those on the cis(cytosolic) face would remain inaccessible. The results are shown in fig.2C. The first 50 residues of lactose permease functioned as a signal sequence to mediate transloca-

tion of the complete or partial OmpA portion of the hybrid polypeptides across the plasma membrane; in all three cases the OmpA part was degraded by trypsin. In contrast, the OmpA segments of Y71/A228-325 and Y143/A1-325 evidently remained cytosolic; neither was affected by trypsin although both could be degraded in lysed cells. In this experiment removal of the periplasmic tail, to leave the membrane-protected tryptic fragment [15] of the chromosomally encoded OmpA, demonstrated that the protease had penetrated the outer membrane in all cases. As no such tryptic fragment could be detected following digestion of Y50/A1-325, in an *ompA*$^-$ background (not shown), the OmpA portion of Y50/A1-325 was clearly not assembled into the outer membrane. This is consistent with the absence of two other characteristic properties of outer membrane assembled OmpA, viz. heat-modifiable migration on SDS-polyacrylamide gels and sensitivity of cells expressing the protein to OmpA-specific phage [15].

Association of the polypeptide, Y143/A1-325, with the plasma membrane was directly demonstrated by electron microscopic examination of immunogold-labelled thin sections of cells expressing the protein (fig.3A). The label was relatively evenly distributed around the plasma membrane although there was some indication of a low level of aggregation of the protein at the membrane after prolonged induction times. Unfortunately, due to the presence of the suppressible stop codon in Y50/A1-325 not enough of this protein was synthesized to be visualized by electron microscopy.

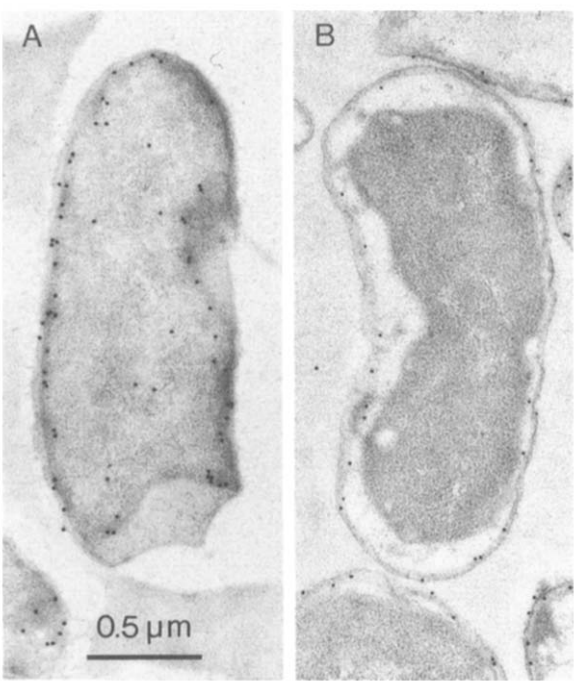

Fig.3. Localization of Y143/A1-325 by immunoelectron microscopy. Strain UH203 carrying (A) pY143/A1-325 or (B) pTU500 (wild-type OmpA).

4. DISCUSSION

The above evidence then provides biochemical support for the model of the N-terminal region of lactose permease as depicted by Vogel et al. [1] (see fig.4) and similarly by Kaback [2]. The LacY/OmpA hybrids were each inserted into the membrane in the orientation which would have been predicted from this model, in which residues 71 and 143 are within cytosolic segments and residue 50 is at the beginning of the second transmembrane strand.

The first 50 residues of LacY functioned efficiently not only as an export signal for OmpA but also as a membrane anchor. This is in agreement with our previous observation that, in the absence of an N-terminal signal, an artificial lipophilic sequence inserted within the OmpA protein acted as a signal-anchor domain [11]. It might also be expected when one considers the extreme hydrophobicity of this first transmembrane strand of LacY [1] together with the fact that no specific processing equivalent to that of signal peptidase I cleavage was ever observed with the LacY50 hybrids (the alkali-insoluble fragment, about 12 kDa, immunoprecipitated from cells producing Y50/A1-325 was probably due to partial degradation of this hybrid). The inner membrane bound OmpA (Y50/A1-325) was unable to fold into the conformation characteristic of outer membrane associated OmpA. This may have been simply due to the inability of the protein to reach and interact properly with the outer membrane. We have noted that the presence of several different additional sequences at the N-terminus of OmpA does not inhibit its membrane assembly (MacIntyre, unpublished). Thus, it may be interesting to determine whether insertion of a 'spacer' permits the hybrid to span the periplasm

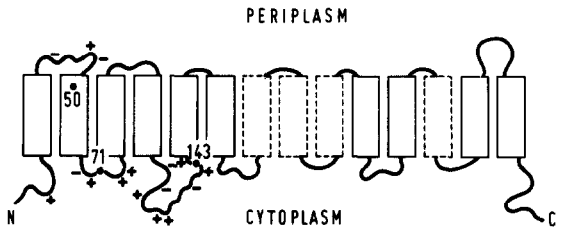

Fig.4. Diagrammatic representation of the structure of lactose as proposed by Vogel et al. [1]. Boxed in regions, hydrophobic membrane spanning α-helices; dashed lines, more hydrophilic α-helices. The proposed positions of residues 50, 71 and 143 and charged residues within the N-terminus are shown. Loss of the 2 basic residues in the first cytosolic loop in Y71/A228-325 is replaced by a sequence ($GD^-PK^+D^-PK^+D^-G$) also containing 2 basic residues although it has an overall negative charge.

and the OmpA domain to assemble into the outer membrane.

The assembly pathway of the LacY50/OmpA hybrids (probably typical type I membrane proteins [16]) is likely to have properties in common with that of wild-type OmpA as both involve export of OmpA across the cytoplasmic membrane. In contrast, the LacY71 and LacY143/OmpA hybrids (complex membrane proteins with a cytosolic OmpA) may represent good model systems for the assembly of the N-terminal domain of lactose permease into the membrane. Earlier studies in minicells involving selective detergent and solvent extraction, and proteolytic digestion have indicated that membrane-associated fragments consisting of only the first 50, 71 or 143 residues of LacY behave similarly to the intact permease [8]. The orientation of the LacY/OmpA hybrids in the inner membrane together with the aggregation phenomenon of Y143/A1-325 then further support the idea that the N-terminus can assemble independently of the rest of the permease. Moreover, these hybrids have several properties which are likely to be advantageous in studying this assembly process, e.g. the involvement of additional protein factors. These include synthesis in high levels, stability and the ability to be followed in vivo by immunoprecipitation with antibody directed against OmpA.

Acknowledgements: We are very grateful to Ulf Henning and Fritz Jähnig for many helpful discussions and for critically reading the manuscript.

REFERENCES

[1] Vogel, H., Wright, J.K. and Jähnig, F. (1985) EMBO J. 4, 3625-3631.
[2] Kaback, H.R. (1988) Annu. Rev. Physiol. 50, 243-256.
[3] Bieseler, B., Prinz, H. and Beyreuther, K. (1985) Ann. NY Acad. Sci. 456, 309-325.
[4] Wright, J.K., Seckler, R. and Overath, P. (1986) Annu. Rev. Biochem. 55, 225-248.
[5] Page, M.G.P. and Rosenbusch, J.P. (1988) J. Biol. Chem. 263, 15906-15914.
[6] Wickner, W. (1988) Biochemistry 27, 1081-1086.
[7] Stochaj, U. and Ehring, R. (1987) Eur. J. Biochem. 163, 653-658.
[8] Stochaj, U., Fritz, H.-J., Heibach, C., Markgraf, M., Von Schaewen, A., Sonnewald, U. and Ehring, R. (1988) J. Bacteriol. 170, 2639-2645.
[9] Freudl, R., Schwarz, H., Klose, M., Movva, N.R. and Henning, U. (1985) EMBO J. 4, 3593-3598.
[10] MacIntyre, S., Freudl, R., Degen, M., Hindennach, I. and Henning, U. (1987) J. Biol. Chem. 262, 8416-8422.
[11] MacIntyre, S., Freudl, R., Eschbach, M.-L. and Henning, U. (1988) J. Biol. Chem. 263, 19053-19059.
[12] Ehring, R., Beyreuther, K., Wright, J.K. and Overath, P. (1980) Nature 283, 537-540.
[13] Russel, M. and Model, P. (1982) Cell 28, 177-184.
[14] Davis, N.G. and Model, P. (1985) Cell 41, 607-614.
[15] Schweizer, M., Hindennach, I., Garten, W. and Henning, U. (1978) Eur. J. Biochem. 82, 211-217.
[16] Wickner, W. and Lodish, H.F. (1985) Science 230, 400-407.