Interaction of the transmembrane domain of lysis protein E from bacteriophage ϕ X174 with bacterial translocase MraY and peptidyl-prolyl isomerase SlyD

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The molecular target for the bacteriolytic E protein from bacteriophage ϕ X174, responsible for host cell lysis, is known to be the enzyme phospho-MurNAc-pentapeptide translocase (MraY), an integral membrane protein involved in bacterial cell wall peptidoglycan biosynthesis, with an essential role being played by peptidyl-prolyl isomerase SlyD. A synthetic 37 aa peptide E_{pep} , containing the N-terminal transmembrane α -helix of E, was found to be bacteriolytic against *Bacillus licheniformis*, and inhibited membrane-bound MraY. The solution conformation of E_{pep} was found by circular dichroism (CD) spectroscopy to be 100 % α -helical. No change in the CD spectrum was observed upon addition of purified *Escherichia coli* SlyD, implying that SlyD does not catalyse prolyl isomerization upon E. However, E_{pep} was found to be a potent inhibitor of SlyD-catalysed peptidyl-prolyl isomerization (IC_{50} 0.15 μ M), implying a strong interaction between E and SlyD. E_{pep} was found to inhibit *E. coli* MraY activity when assayed in membranes (IC_{50} 0.8 μ M); however, no inhibition of solubilized MraY was observed, unlike nucleoside natural product inhibitor tunicamycin. These results imply that the interaction of E with MraY is not at the MraY active site, and suggest that a protein–protein interaction is formed between E and MraY at a site within the transmembrane region.

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INTRODUCTION

Bacteriophages use two strategies to achieve lysis of the bacterial host cell: dsDNA phages (such as phage λ) produce a muralytic enzyme and a holin permease protein; while ssRNA and ssDNA phages produce a single lytic protein, which does not act as a muralytic enzyme (Young, 1992; Young *et al.*, 2000). The latter strategy is used by bacteriophage ϕ X174, in which a single E gene mediates host-cell lysis. The encoded 91 aa E protein causes cell lysis at concentrations of 100–300 molecules per cell (Young & Young, 1982). Overexpression of the cloned E gene is sufficient to cause cell lysis. Analysis of the amino acid sequence reveals a hydrophobic transmembrane domain close to the N terminus (residues 9–32), followed by a positively charged soluble domain. A mutant E protein, in which the C-terminal region is replaced by the *lacZ* gene product, is still capable of cell lysis, indicating that the soluble domain is not of primary importance for lytic action (Blasi & Lubitz, 1985; Maratea *et al.*, 1985).

Genetic studies by Young and co-workers have implicated the SlyD and MraY gene products in the mechanism of action of the antibacterial E protein. Mutations in the *slyD* locus have been found to confer resistance to E (Roof *et al.*, 1994). Sequencing of the *slyD* gene has revealed that the encoded protein shares sequence similarity with the peptidyl-prolyl *cis-trans* isomerase family of enzymes, such as FK506-binding protein (FKBP) (Hottenrott *et al.*, 1997; Roof *et al.*, 1997). However, subsequent experiments imply that SlyD is not the lethal target for E (Bernhardt *et al.*, 2000). An *Epos* mutant of the E gene has been isolated, containing two point mutations R3H and L19F, which is bacteriolytic even in the absence of an active *slyD* gene, suggesting that SlyD acts as an accessory protein during cell lysis, rather than acting as the cellular target. Using the cloned *Epos* gene, searches have been made for further gene loci where mutations could confer resistance to *Epos*, and a mutation has been found at minute 2 of *Escherichia coli* which maps to the *mraY* gene (F288L point mutation) encoding phospho-MurNAc-pentapeptide translocase (MraY) (Bernhardt *et al.*, 2000).

MraY catalyses the first step of the intramembrane cycle of reactions involved in bacterial peptidoglycan biosynthesis,

Abbreviations: CD, circular dichroism; FKBP, FK506-binding protein; MraY, phospho-MurNAc-pentapeptide translocase.

Fig. 1. Lipid-linked steps of peptidoglycan biosynthesis, showing the MraY-catalysed reaction, and the site of action of E. The lipid carrier is undecaprenyl phosphate.

namely the reaction of the cytoplasmic precursor UDPMurNac-pentapeptide with the lipid carrier undecaprenyl phosphate, to give a lipid-linked intermediate undecaprenyl-P-P-MurNac-pentapeptide and UMP (Bugg, 1999), as shown in Fig. 1. This enzyme, an integral membrane protein, is known to be the site of action of three nucleoside natural product antibiotics: tunicamycin, mureidomycin A and liposidomycin B (Brandish *et al.*, 1996a, b). The inhibition kinetics for these inhibitors show that they compete for the UDPMurNac-pentapeptide and undecaprenyl phosphate binding sites at the MraY active site, and in the case of mureidomycin A, compete for the Mg^{2+} cofactor binding site (Howard & Bugg, 2003).

Expression of an epitope-tagged E_{myc} protein was found by Bernhardt *et al.* (2001) to lead to accumulation of the UDPMurNac-pentapeptide peptidoglycan precursor, and MraY activity in membranes containing expressed E_{myc} protein was found to be reduced by 75%, using a radiochemical exchange assay, consistent with inhibition of MraY by the E protein. However, it was not certain from these earlier results whether the interaction of E with MraY was at the MraY active site, or through a site in the transmembrane region; nor was the precise role of prolyl isomerase SlyD certain, although it was possible that the enzyme catalysed prolyl isomerization of E (Witte *et al.*, 1997).

In view of our previous studies on the inhibition of MraY, and its structure and mechanism (Lloyd *et al.*, 2004), we wished to investigate the molecular basis for the interaction of E with MraY. In this paper we report the interaction *in vitro* of purified *E. coli* SlyD and overexpressed *E. coli* MraY with a synthetic peptide containing the transmembrane domain of E.

METHODS

Materials. The 37mer peptide E_{pep} (H_2N -MVRWTLWDTL-AFLLLSLLPSLLIMFIPSTFKRPVS- CO_2H) was synthesized by solid-phase peptide synthesis by Severn Biotech to >99% purity. Data: m/z (MALDI-TOF) 4334.7 (MH^+), calculated M_r 4333.44. E_{pep} was dissolved in 0.5 M Tris buffer, pH 7.5, containing 1% SDS, to a concentration of 0.1 mg ml^{-1} . UDPMurNac-L-Ala- γ -D-Glu-m-DAP(N-dansyl)-D-Ala-D-Ala was prepared as previously described by Brandish *et al.* (1996a). Undecaprenyl phosphate was purchased from Larodan Fine Chemicals. Peptides phospholamman and ERB2 were a gift from Dr A. Beevers (University of Warwick, UK). Peptide substrates for SlyD and other biochemicals were purchased from Sigma-Aldrich.

Cloning of N- and C-terminal domains of E. DNA constructs containing the N- and C-terminal domains of E were amplified by PCR, using ϕ X174 DNA (Pharmacia) as a template, with the following primers: Ememb1 (containing the NdeI site), 5'-ACGTACCATATGGTACGTGGACTTT-3'; Ememb2 (containing the SapI site), 5-TGATCGGCTCTTCTGCAGAGACAGGCCGTTTGAA-TG-3'; Ecyt1 (containing the SapI site and N-terminal Cys), 5'-TATACCGCTCTTCTAACTGCTGGAAGGCGCTGAATT-3'; and Ecyt2 (containing a PstI site), 5'-TAATGCACGCCTTCC-TCACTGACGTCACATCA-3'.

The amplified DNA fragments were digested with SapI/NdeI (Ememb) or SapI/PstI (Ecyt), and cloned into the pTwin2 vector (New England Biolabs), according to the manufacturer's instructions. Transformation of the Ememb-intein 1-CBD construct into *E. coli* ER2566 gave no viable colonies, indicating high toxicity. Transformation of CBD-intein 2-Ecyt gave the desired construct, containing an N-terminal S38C mutation. Expression of this construct, followed by purification on a chitin affinity column, gave the expected 31 kDa band by SDS-PAGE.

Antibacterial activity of E peptide. Antibacterial activity was tested against *E. coli* K-12, *Bacillus licheniformis*, *Pseudomonas putida*, *Leuconostoc mesenteroides* and *Arthrobacter globiformis*. Activity on solid media was determined by agar diffusion assay.

Solutions of E_{pep} (0.1 mg ml⁻¹ stock in 0.5 M Tris buffer, pH 7.5, containing 1% SDS) were applied to a filter paper, which was placed onto a lawn of bacteria on an agar plate, and incubated for 24–48 h. Activity on liquid media was determined by addition of an aliquot of E_{pep} (0.1–2.6 µg ml⁻¹ final concentration) to a 100-fold dilution of an overnight culture in Luria Broth, followed by monitoring of OD₆₀₀ in a microtitre plate reader over 6–8 h, in duplicate. Control experiments were carried out using buffer containing 0.025% SDS, which showed <4% growth inhibition, as measured by OD₆₀₀.

Circular dichroism (CD) spectroscopy. CD spectra were measured over the range 200–275 nm using a Jasco spectropolarimeter model J-175. Measurements were made using a 1 cm cell length and a protein concentration of 0.1 mg ml⁻¹ in 50 mM Tris buffer, pH 7.5, at 20 °C. For the interaction of E_{pep} with SlyD, 1.0 ml of each was mixed, with a 1 s response time and a scan rate of 100 nm min⁻¹. Spectra were measured eight times and averaged. The data were analysed using K2D software (<http://www.embl-heidelberg.de/~andrade/k2d/>) to predict the percentage α -helix and β -sheet content.

Fluorescence measurements. Fluorescence analysis of E_{pep} and SlyD was carried out using a Perkin-Elmer LE5 fluorescence spectrophotometer, with fluorescence excitation at 280 nm, and recording fluorescence emission at 280–500 nm. Assays (total volume 500 µl) contained 50 mM Tris buffer, pH 7.5, and 0.1% SDS, and solutions of E_{pep} (0.1 mg ml⁻¹, 22 µM) and SlyD (0.1 mg ml⁻¹, 3.7 µM). Assays were carried out in triplicate.

Expression and purification of SlyD. An overexpression plasmid containing the *E. coli* *slyD* gene cloned into the pQE30 vector, as described by Hottenrott *et al.* (1997), was a gift of Professor G. Fischer (Max Planck Institute Halle). The plasmid was transformed into *E. coli* strain BL21. SlyD was expressed in 1 l Luria Broth medium, containing 100 µg ampicillin ml⁻¹, grown at 37 °C, and induced with 0.5 mM IPTG at OD₆₀₀ 0.6, followed by a further 3 h growth. Cell extract was purified on a Talon Metal Affinity Resins column (BD Biosciences), using a 0–200 mM imidazole gradient in 50 mM sodium phosphate, pH 7.0, containing 300 mM NaCl. Fractions containing SlyD protein were identified by SDS-PAGE, and were dialysed against 10 mM MOPS, pH 7.0, containing 1 mM DTT. Further purification was achieved by size-exclusion chromatography on an Ultrogel AcA44 column (Sigma) using the same buffer, to give homogeneous protein of *M_r* 27 000. Purified SlyD was then concentrated using an Amicon Ultra15 filter to a final concentration of 0.130 mg ml⁻¹.

Assays of SlyD activity. Assays of SlyD activity were based on the method of Hottenrott *et al.* (1997). Assays were carried out in 96-well microtitre plates using a Genios Tecan Plate Reader, with a total volume of 260 µl, monitoring at 400 nm over 5 min at 20 °C. Assays contained 50 mM Tris buffer, pH 7.5, 0.02 mg N-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide ml⁻¹ (32 µM; stock 10 mg ml⁻¹ in DMSO), 0.3 µM α -chymotrypsin and 0.1 mg SlyD ml⁻¹. Under these conditions, a specific activity of 0.12 µmol min⁻¹ (mg protein)⁻¹ was observed, using fresh SlyD. Inhibition assays using E_{pep} (0.1 mg ml⁻¹ stock in 50 mM Tris buffer, pH 7.5, containing 1% SDS) were carried out at 0–4 µM final concentration. Inhibition assays were also carried out using the following peptides: phospholamman (DYQSLQIGGLVIAGILFILGILVLSRR, 28mer, *M_r* 3040); ERB2 (RASPVTFLIAIVEGVLLFLILVVVGILIKRRR, 33mer, *M_r* 3672); insulin chain B, oxidized (FVNQHLCGSHLVEALYLVCGERGFFYTPKA, 30mer, *M_r* 3496, C represents cysteine acid); mast cell degranulating peptide HR1 (INLKAIAALVKKVL, 14mer, *M_r* 1493). Phospholamman and ERB2 were dissolved at 0.1 mg ml⁻¹ in 50 mM Tris buffer, pH 7.5, containing 1% SDS; and insulin chain

B and mast cell peptide HR1 were dissolved at 1.0 mg ml⁻¹ in 50 mM Tris buffer, pH 7.5. Control incubations using buffer containing 0.1% SDS showed no inhibition of SlyD.

Preparation of *E. coli* membrane extracts containing overexpressed MraY activity. A 500 ml culture of *E. coli* strain DH5 α /pJFY3c, containing the overexpressed *mraY* gene (Lloyd *et al.*, 2004), was grown in Luria Broth with 100 µg ampicillin ml⁻¹ at 37 °C, and overexpression was induced at OD₆₀₀ 0.6 by addition of 0.5 mM IPTG. Cultures were grown for a further 4 h, and cells were harvested by centrifugation at 4400 *g* for 10 min. Cells were stored overnight at –70 °C. Thawed cells were resuspended in buffer A (50 mM Tris, pH 7.5, 2 mM β -mercaptoethanol, 1 mM MgCl₂) at 2 ml buffer (g cells)⁻¹. Lysozyme (5 mg) was added, and the cells were gently stirred at room temperature for 30 min, followed by sonication on ice. Whole cells and debris were removed by centrifugation at 12 000 *g* for 30 min, and membranes were collected from the supernatant by centrifugation at 60 000 *g* for 1 h. Membrane pellets were resuspended in buffer A plus 1 M NaCl, and stirred gently for 15 min at 4 °C. Salt-stripped membranes were collected by centrifugation at 60 000 *g* for 1 h, and resuspended in 400 µl buffer A, to give a protein concentration of 1.5–2.0 mg ml⁻¹.

Solubilized MraY membranes. The membranes were resuspended in extraction buffer plus 1.5% CHAPS and 20% v/v, glycerol, and stirred for 1 h. Unsolubilized material was removed by centrifugation at 60 000 *g* for 1 h to give a protein concentration of 0.5 mg ml⁻¹, and a specific activity of 1–2 nmol min⁻¹ (mg protein)⁻¹ (Brandish *et al.*, 1996a).

MraY assays. The fluorescence enhancement assay described by Brandish *et al.* (1996a) was used, in a 96-well microtitre plate format (excitation at 340 nm, emission at 535 nm). Incubation was carried out at 20 °C in a total volume of 100 µl. Assays contained 50 mM Tris buffer, pH 8.0, 1 µl (12 µM) undecaprenyl phosphate (stock 1 mg ml⁻¹), 1 µl (8 µM) UDPMurNAc-L-Ala- γ -D-Glu-*m*-DAP(*N*-dansyl)-D-Ala-D-Ala (stock 1 mg ml⁻¹), and 10 µl MraY membranes (stock 2.0 mg protein ml⁻¹). Measurements were taken over a 10 min interval with a Genios Tecan Plate Reader. Changes of 2000 fluorescence units were measured over a 10 min assay, relative to controls lacking enzyme. Inhibition was measured by adding different concentrations of E_{pep} (0.1 mg ml⁻¹ stock in 0.5 M Tris buffer, pH 7.5, containing 1% SDS), to 1–9 µM final concentration, and tunicamycin at 1–50 µM final concentration. Control assays using buffer containing $\leq$ 0.4% SDS showed no inhibition of MraY activity.

RESULTS

Expression of N- and C-terminal domains of E

In order to carry out *in vitro* studies of the interaction of E with target proteins SlyD and MraY, a source of purified E was needed. The extremely high toxicity of E precluded the bacterial expression of native E protein (Young & Young, 1982). Attempts to express E with a 45 kDa N-terminal maltose-binding-protein fusion still showed very high toxicity in *E. coli*, and attempts to express E in yeast were also unsuccessful (data not shown). DNA constructs encoding the N- (residues 1–37) and C-terminal (residues 38–91) domains, containing Cys in place of Ser-38, were generated by PCR, and expressed as intein fusions, with a view to using intein-mediated protein ligation to prepare full-length protein (see Fig. 2). It was observed that the N-terminal domain construct was still highly toxic towards

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φX174 MVRWTLWDTLAFLLLSLLLPSSLIMFIPSTFKRPVSSWKALNLRKTLTLLMASSVRLKPLNCSRLPCVYAQETLTFLLTQKKTCVKNYVRKE
s13   .VRWTLWDTLAFLLLSLLLPSSLIMFIPSTFKRPVSSWKALNLRKTLTLLMASSVRLKPLNCSRLPCVYAQETLTFLLTQKKTCVKNYVQKE
phk   MERWTLAAILAFLLLSLLLPSSLIMFIPSTFRQHASLWKARSLAKTLTLLMASSARLTPLSSSRTPCVLRQDSKKL
a13   MERWTLDDILAFLLLSLLLPSSLIMFIPSMYQHASLWKARSLAKTLTLLMASSARLTPLSSSRTPCVLRQDSKKL
g4    MEHWTLGILAFLLLSLFLPSLLITFIPLTSKPPVSSWKVLSLPKTSMMVLNAPLPLNCSPLFLFAPETKILSVTLKQTSVNSYALK

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Fig. 2. Amino acid sequence alignment of ϕ X174 E protein with other bacteriophage E sequences, showing the position of the transmembrane domain (grey bar). The sequence of the 37 aa synthetic peptide E_{pep} is underlined.

E. coli, whereas the C-terminal domain construct could be transformed and expressed in *E. coli*. These observations imply that the toxicity of E lies in the N-terminal domain, consistent with the published observation that a fusion protein containing the N-terminal region of E fused to LacZ is bacteriolytic (Blasi & Lubitz, 1985). Therefore, a synthetic peptide (E_{pep}) containing residues 1–37 of E (M_r 4333) was prepared by solid-phase peptide synthesis.

Due to the hydrophobic character of E_{pep} , solubilization in buffer containing 1% SDS was needed. Preliminary experiments indicated that E_{pep} showed inhibition of membrane-bound MraY (see below); therefore, experiments were carried out to determine whether E_{pep} showed antibacterial activity. Since E is produced *in vivo* inside the bacterial host, and since it is large (4 kDa), it was not certain whether E_{pep} would be able to penetrate the bacterial cell from the exterior. E_{pep} showed no growth inhibition towards Gram-negative *E. coli* or *P. putida*, or Gram-positive *A. globiformis* or *L. mesenteroides*, but was found to inhibit the growth of Gram-positive *B. licheniformis* in liquid culture, assayed by an increase in OD_{600} over 6–8 h. A reduction of 98% in OD_{600} was observed in overnight cultures containing $2.6 \mu\text{g } E_{\text{pep}} \text{ ml}^{-1}$, and 15% growth inhibition at $1.0 \mu\text{g ml}^{-1}$, compared with cultures without E_{pep} . Incubation of *B. licheniformis* with 0.025% SDS showed only a 4% reduction in OD_{600} , indicating that the antibacterial effect was caused by E_{pep} . It is possible that the presence of detergent, required for solubilization, assisted the antibacterial activity of the peptide, as has been observed

in the case of antibacterial peptide P1 (Ohk & Kuramitsu, 2000). E_{pep} showed no growth inhibition of the above strains on agar plates by zone diffusion assay, suggesting that E_{pep} kills growing bacterial cells.

Interaction of E_{pep} with *E. coli* SlyD

Peptidyl-prolyl isomerase SlyD from *E. coli* was expressed and purified as described by Hottenrott *et al.* (1997), to give homogeneous protein of M_r 27 000, which was catalytically active for the *cis-trans* isomerization of *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide, detected by cleavage with α -chymotrypsin.

The sequence of E contains a proline residue close to the centre of the transmembrane α -helix, Pro-21, which has been shown to be essential for the activity of E (Witte *et al.*, 1997). Using the synthetic peptide E_{pep} , it was possible to examine whether treatment with SlyD resulted in any change in conformation of E_{pep} . A solution of $0.1 \text{ mg } E_{\text{pep}} \text{ ml}^{-1}$ in 1% SDS was examined using CD spectroscopy. A strong CD spectrum was observed, as shown in Fig. 3, showing a peak at 225 nm, diagnostic of an α -helical conformation. Analysis of the CD spectrum using K2D software predicted 100% α -helical conformation for E_{pep} . A 1:1 mixture of E_{pep} and SlyD gave a CD spectrum with a peak at 226 nm, showing no significant change from that of E_{pep} alone (see Fig. 3). These data imply that E_{pep} readily adopts an α -helical conformation, and that SlyD does not catalyse a conformational change upon E_{pep} under these conditions.

Fig. 3. CD spectra of 0.1 mg ml^{-1} solutions of E_{pep} , SlyD, and a 1:1 mixture of E_{pep} and SlyD, in 50 mM Tris buffer, pH 7.5, recorded as described in Methods.

Fig. 4. Fluorescence spectra of 0.1 mg ml^{-1} solutions of E_{peg} , SlyD, and a 1 : 1 mixture of E_{peg} and SlyD, in 50 mM Tris buffer, pH 7.5, containing 0.1 % SDS, recorded as described in Methods.

The interaction of E_{peg} and SlyD was also studied using fluorescence spectroscopy. The fluorescence spectrum of $0.1 \text{ mg } E_{\text{peg}} \text{ ml}^{-1}$ (Fig. 4) showed an emission maximum at 345 nm (excitation at 280 nm), due to the presence of tryptophan residues at positions 4 and 7. Upon addition of $0.1 \text{ mg SlyD ml}^{-1}$ in a 1:1 ratio, a 10% increase in fluorescence emission intensity at 345 nm was observed, compared to the fluorescence spectrum of E_{peg} , suggesting that there was an interaction between E_{peg} and SlyD. There were no time-dependent changes in fluorescence emission following mixing of E_{peg} and SlyD, over 5–10 min.

The interaction of E_{peg} with SlyD was studied further by measurement of the inhibition of SlyD-catalysed peptidyl-prolyl isomerization by E_{peg} . Addition of E_{peg} at concentrations $> 0.3 \text{ } \mu\text{M}$ to assays of SlyD caused strong inhibition of SlyD-catalysed prolyl isomerization, as shown in Fig. 5. From the data in Fig. 5, an IC_{50} value of $0.15 \text{ } \mu\text{M}$ for E_{peg} was estimated. Control assays in the presence of 0.1 % SDS showed no inhibition of SlyD activity, indicating that inhibition was due to binding of E_{peg} . Four other peptides were also assayed as inhibitors of SlyD: two soluble peptides, insulin chain B (30mer, M_r 3496) and mast cell degranulating peptide HR1 (14mer, M_r 1493); and two transmembrane peptides, phospholamman (28mer, M_r 3040) and ERB2 (33mer, M_r 3672). The soluble peptides showed no inhibition of SlyD, at concentrations up to $20 \text{ } \mu\text{M}$. As shown in Fig. 5, the two transmembrane peptides phospholamman and ERB2 showed inhibition of SlyD, with IC_{50} values of 1.1 and $0.8 \text{ } \mu\text{M}$, respectively, 5–8-fold higher than that of E_{peg} . The sequence of ERB2 (see Methods) contains one proline residue, close to the N terminus, whereas the sequence of phospholamman contains no proline residues.

Inhibition of *E. coli* MraY by E_{peg}

We have previously used a continuous fluorescence assay to study the kinetics of inhibition of *E. coli* MraY by nucleoside natural products mureidomycin A, liposidomycin B and tunicamycin (Brandish *et al.*, 1996a, b). *E. coli* MraY has been overexpressed 30–40-fold and can be solubilized in

Triton X-100 or CHAPS detergents in high yields, but it is refractory to further purification (Brandish *et al.*, 1996a; Lloyd *et al.*, 2004). Therefore, the overexpressed *E. coli* enzyme was used for inhibition studies, in particulate form (membranes suspended in 50 mM Tris, pH 8.0) and in detergent-solubilized form (solubilized in 1.5 % CHAPS). Assays were carried out using $8 \text{ } \mu\text{M}$ fluorescent substrate UDPMurNAc-L-Ala- γ -D-Glu-*m*-DAP(ϵ -dansyl)-D-Ala-D-Ala and $12 \text{ } \mu\text{M}$ undecaprenyl phosphate in 50 mM Tris buffer, pH 8.0.

Using MraY enzyme solubilized in 1.5 % CHAPS, addition of $1\text{--}10 \text{ } \mu\text{M}$ E_{peg} gave no MraY inhibition (see Fig. 6), with or without additional SlyD. In contrast, inhibition of detergent-solubilized MraY was observed using tunicamycin ($\text{IC}_{50} 2 \text{ } \mu\text{M}$), as reported previously (Brandish *et al.*, 1996b).

Fig. 5. Inhibition of SlyD-catalysed *cis-trans* isomerization of succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide by E_{peg} , phospholamman (PLM), peptide ERB2, insulin chain B (InsB) and mast cell degranulating peptide HR1 (MCP1). Assays were carried out in 50 mM Tris buffer, pH 7.5, with monitoring at 400 nm, as described in Methods.

Fig. 6. Inhibition of membrane-bound and detergent-solubilized MraY by (A) E_{pep} and (B) tunicamycin. Activity was measured by fluorescence enhancement assay, using UDPMurNAc-L-Ala- γ -D-Glu-*m*-DAP(*N*-dansyl)-D-Ala-D-Ala as substrate in 50 mM Tris buffer, pH 8.0, as described in Methods. Data are expressed as percentage fluorescence enhancement activity.

Using particulate MraY enzyme, inhibition was observed in the presence of either E_{pep} or tunicamycin. Treatment with 2–10 μM E_{pep} gave 80–90% inhibition of particulate MraY activity, as shown in Fig. 6(A). From these data, an IC_{50} value of 0.8 μM was estimated, although complete inhibition was not observed at higher concentrations of E_{pep} . Inhibition of membrane-bound MraY by tunicamycin, under the same conditions, gave an IC_{50} value of 30 μM , as shown in Fig. 6(B). Addition of SlyD to these assays had no additional effect; however, the *E. coli* membranes would have contained endogenous SlyD. Control MraY assays carried out in the presence of $\leq 0.4\%$ SDS showed no inhibition of MraY activity. E_{pep} was also solubilized in buffer containing 1% CHAPS, and assayed against membrane-bound MraY; similar (or slightly higher) levels of inhibition were observed, compared with E_{pep} solubilized in 1% SDS.

DISCUSSION

Genetic studies on the mechanism of action of the bacteriolytic E protein have clearly established that

translocase MraY is the primary target (Bernhardt *et al.*, 2000, 2001). However, it is unclear exactly how the E protein interacts with MraY, since the known MraY inhibitors are nucleoside natural products, whereas E is a 9 kDa transmembrane protein. The precise biochemical role of peptidyl-prolyl isomerase SlyD in this process is also unclear.

This present work has established that the N-terminal 37 aa transmembrane domain of E retains bacterial cytotoxicity upon expression in *E. coli*, and that a synthetic peptide E_{pep} retains the ability to inhibit membrane-bound MraY, and possesses some antibacterial activity. The conclusion that the N-terminal domain is responsible for the biological activity of E is consistent with the earlier observation that a mutated form of E, in which the cytoplasmic domain was replaced by LacZ, is active (Blasi & Lubitz, 1985).

These studies have demonstrated experimentally that there is an interaction between E_{pep} and peptidyl-prolyl isomerase SlyD, since E_{pep} is a potent inhibitor of SlyD-catalysed peptidyl-prolyl isomerization (IC_{50} 0.15 μM). However, there is no observable change in the secondary structure of E_{pep} upon addition of SlyD, as shown by CD spectroscopy; therefore, under these conditions, SlyD does not appear to catalyse a prolyl isomerization upon E_{pep} , as proposed by Witte *et al.* (1997). Bernhardt *et al.* (2002) have proposed that the role of SlyD is to protect E from proteolysis, which is consistent with our data. The results shown in Fig. 5 indicate that SlyD is also inhibited by two hydrophobic transmembrane peptides, but is not inhibited by two hydrophilic soluble peptides. These data appear to indicate that SlyD has an affinity for hydrophobic α -helical peptides, which provides an explanation for why SlyD binds the nascent E protein *in vivo*.

One of the hydrophobic peptides, phospholamman, contains no proline residues; therefore, the presence of a proline residue is not a pre-requisite for binding to SlyD. Nevertheless, E_{pep} does bind 5–8-fold more tightly to SlyD than to ERB2 and phospholamman, so there is some selectivity for the E_{pep} sequence. Scholz *et al.* (2006) have recently reported that SlyD exhibits high chaperone activity upon unfolded proteins, and is inhibited strongly (K_i 0.2–2 μM) by several polypeptides lacking proline residues, with a preference for unstructured peptides. It is conceivable that there is an interaction between SlyD and MraY which assists the formation of a protein–protein interaction with E. We note that SlyD has been reported to co-purify with the integral membrane protein adenylate cyclase (Mitterauer *et al.*, 1999). We have not detected any inhibition of MraY by SlyD alone; however, we were unable to exclude SlyD completely from the MraY inhibition experiments, since wild-type *E. coli* SlyD is present in membrane fractions. The precise cellular roles for the FKBP and cyclophilin families of peptidyl-prolyl isomerases are still uncertain (Ivery, 2000), but it is believed that they are able to stabilize certain peptide conformations found in partly folded proteins, and hence stabilize the formation of protein complexes (Schiene-Fischer & Yu, 2001).

Fig. 7. (A) Predicted secondary structure for translocase MraY (Lloyd *et al.*, 2004; Bouhss *et al.*, 1999), showing the position of active site Asp residues (Lloyd *et al.*, 2004) and the mutation giving rise to resistance to E (Bernhardt *et al.*, 2000). Bold type indicates completely conserved residues (from multiple sequence alignments), whereas residues in non-bold type are conserved in bacterial enzymes, but not conserved in the mammalian homologue. (B) Hypothesis for inhibition of MraY by E via a protein-protein interaction, disrupting the formation of a high-activity protein complex involving MraY.

The N-terminal synthetic peptide E_{pep} was found to inhibit membrane-bound MraY, with an IC_{50} value of $0.8 \mu\text{M}$, to the extent of 80–90 %, whereas no inhibition of detergent-solubilized MraY was observed. By comparison, using a radiochemical assay, Bernhardt *et al.* (2001) observed a 75 % reduction in MraY activity in cell membranes, after expression of E_{myc} . This behaviour is quite different to that shown by nucleoside inhibitors tunicamycin, muridomycin A and liposidomycin B, which inhibit

membrane-bound and solubilized MraY (Brandish *et al.*, 1996a, b). These observations demonstrate that the mechanism of inhibition by E_{pep} is quite different to that of small-molecule inhibitors, and imply that the site of inhibition by E_{pep} is not at the MraY active site.

The secondary structure of MraY consists of ten transmembrane α -helices, whose positions have been predicted by β -lactamase fusion analysis (Bouhss *et al.*, 1999) and by

bioinformatic analysis (Lloyd *et al.*, 2004). The active site of MraY is apparently formed by the five cytoplasmic loops, in particular loops 2 and 4, which bear catalytic residues Asp-115, Asp-116 and Asp-267 (Lloyd *et al.*, 2004), as shown in Fig. 7(A). Two separate considerations imply that the interaction of E_{pep} with MraY is at a site in the transmembrane region: (1) E_{pep} consists of a transmembrane α -helix alone, whose formation has been demonstrated herein using CD spectroscopy; and (2) the mutation in MraY known to cause resistance to E (Phe-288 to Leu) (Bernhardt *et al.*, 2000) lies in transmembrane helix 9, close to the periplasmic face of the protein (see Fig. 7A). We propose that the inhibition of MraY by E_{pep} is caused by a protein–protein interaction at an intramembrane site, and not by active site binding. Attempts to visualize by immunoprecipitation the binding of E_{pep} to a (His)₆–MraY fusion protein, prepared previously by Lloyd *et al.* (2004), were unsuccessful (data not shown), since the membrane-bound (His)₆–MraY could not be visualized by immunoblotting.

The observed inhibition of membrane-bound MraY, but not solubilized MraY, is unusual. An explanation of this behaviour that is consistent with the incomplete inhibition of membrane-bound MraY by E_{pep} (Fig. 6A) is that there is a high-activity form of MraY in cell membranes, formed upon interaction with other membrane-bound proteins, and that the binding of E to MraY via a protein–protein interaction blocks the formation of this high-activity form, leading to inhibition. Detergent-solubilized MraY activity is not inhibited by E_{pep}, presumably because the formation of a high-activity protein complex involving MraY is disrupted by detergent solubilization. There is some evidence for the formation of a multi-protein biosynthetic complex at cell division (Höltje, 1998), at which point a high turnover of cell wall synthesis is needed, and at which point the E protein causes cell lysis (Young, 1992). If MraY is involved in this complex, then it is quite feasible that the binding of E to MraY at a transmembrane site disrupts the formation of essential protein–protein interactions involving MraY (see Fig. 7B), leading to inhibition of high-activity turnover *in vivo*, and hence to cell lysis. It is therefore of interest to determine the precise site of interaction between E and MraY, as this site could be a novel antibacterial target. A recent precedent is the inhibition of the transmembrane enzyme γ -secretase by α -helical peptides at a transmembrane site removed from the active site (Das *et al.*, 2003). MraY from *Bacillus subtilis* has recently been purified on a small scale (Bouhss *et al.*, 2004); thus, it may be possible in future to carry out studies using homogeneous MraY protein. The availability of a biologically active synthetic peptide now allows more detailed studies of the E–MraY interaction.

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