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Energetically distinct early and late stages of HlyB/HlyD-dependent secretion across both *Escherichia coli* membranes

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The alternative secretion pathway which exports hemolysin across both *Escherichia coli* membranes into the surrounding medium is directed by an uncleaved C-terminal targeting signal and the membrane translocator proteins HlyD and HlyB. In order to identify stages and intermediates in this unconventional secretion process we have examined the effect of inhibition of the total proton motive force (ΔP) and its components during the *in vivo* HlyB/HlyD-dependent export of a 22.4 kDa secretion competent HlyA C-terminal peptide (Actp). Secretion of Actp was severely inhibited by the proton ionophore carbonylcyanide *m*-chlorophenylhydrazone (CCCP), which collapses simultaneously membrane potential $\Delta\psi$ and the proton gradient ΔpH, and also by valinomycin/K^+, a potassium ionophore which disrupts $\Delta\psi$. The inhibition of secretion by valinomycin/K^+ was ameliorated by imposition of a pH gradient, the second component of the ΔP, and selective depletion of ΔpH by nigericin also blocked secretion. This indicates that, as in the secretion of β-lactamase to the periplasm, HlyB/D-directed secretion requires ΔP itself and not specifically one of its components. However, inhibition of HlyB/D-dependent secretion was only marked when CCCP, valinomycin/K^+ or nigericin were present during the early stage of Actp secretion; at a later stage the secretion was not significantly inhibited. HlyB/D-dependent secretion appears therefore to share with conventional secretion across the cytoplasmic membrane an early requirement for ΔP, but comprises in addition a late stage which does not require ΔP, $\Delta\psi$ or ΔpH. The translocation intermediate identified in the ΔP-independent late stage of secretion was associated with the membrane fraction. Analysis of the protease accessibility of this intermediate in whole cells and spheroplasts showed that it was not in the periplasm, nor was it exposed on the cell surface or on the periplasmic faces of either the inner or outer membranes. This may reflect its close association with the inner membrane or a membrane translocation complex.

Key words: hemolysin/membrane translocation/protein secretion/secretion intermediate

Introduction

Export of proteins across the bacterial cytoplasmic membrane is characteristically directed by an N-terminal leader signal sequence and several cellular Sec proteins (Michaelis and Beckwith, 1982; Wickner and Lodish, 1985). Secretion across the outer membrane of Gram-negative bacteria is relatively rare and is generally achieved in distinct translocation steps involving periplasmic intermediates, e.g. IgA protease or cholera toxin (Pohlner *et al.*, 1987; Hirst and Holmgren, 1987). Secretion of the 110 kDa hemolysin protein (HlyA) by *Escherichia coli* is an exception in that HlyA has no N-terminal signal and is secreted across both cytoplasmic and outer membranes into the surrounding medium, apparently without a periplasmic intermediate (Haertlein *et al.*, 1983; Felmlee *et al.*, 1985; Mackman *et al.*, 1987; Koronakis *et al.*, 1989).

This alternative secretion pathway is dependent upon two specific secretion proteins, HlyD and HlyB (Wagner *et al.*, 1983), the latter being a putative ATP binding analogue of many prokaryotic and eukaryotic membrane transporters including the human multidrug resistance P-glycoprotein, the cystic fibrosis protein and the MHC-linked peptide tansporter protein (Gerlach *et al.*, 1986; Rommens *et al.*, 1989; Hyde *et al.*, 1990; Monaco *et al.*, 1990). The HlyB/D-dependent process directs secretion of related cytolytic bacterial toxins (Koronakis *et al.*, 1987; Strathdee and Lo, 1987; Glaser *et al.*, 1988; Gygi *et al.*, 1990) and also the 30 kDa NodO plant nodulation protein of *Rhizobium* (Economou *et al.*, 1990), and the 53 kDa and 50 kDa metalloproteases of *Erwinia* and *Serratia* (Letoffe *et al.*, 1990; Nakahama *et al.*, 1986). All these HlyB/D-secreted proteins have a C-terminal secretion signal sequence and in the well characterized *E.coli* HlyA it comprises the C-terminal 48-60 residues (Koronakis *et al.*, 1989; Hess *et al.*, 1990; Stanley *et al.*, 1991). HlyA proteins carrying wild-type or defective secretion signals appear only in the cytoplasm or membrane fractions, not in the periplasm (Koronakis *et al.*, 1989), supporting the possibility that HlyA may be exported directly into the medium via a HlyB/HlyD membrane contact site or channel. No intermediates and no steps in the secretion process have been described. In this paper we attempt to identify both by interrupting the transmembrane electrochemical potential during secretion.

The conventional secretion of proteins across the bacterial cytoplasmic membrane requires not only ATP-derived energy but also an energized envelope, i.e. the transmembrane energy potential or total proton motive force ΔP. The ΔP was first shown to be necessary *in vivo* by inhibiting with energy uncouplers the insertion of phage M13 coat protein into the cytoplasmic membrane (Date *et al.*, 1980). A second cytoplasmic membrane protein, the leader peptidase, requires proton motive force for assembly as a transmembrane protein (Wolfe and Wickner, 1984). Uncouplers also prevent cytoplasmic membrane translocation of precursor periplasmic proteins such as maltose binding protein, leucine-specific binding protein, alkaline phosphatase and β-lactamase, and of the outer membrane proteins OmpA, OmpF and LamB (Daniels *et al.*, 1981; Enequist *et al.*, 1981; Pages and Lazdunski, 1982;

Zimmermann and Wickner, 1983). The requirement for ΔP during β-lactamase secretion across the *E.coli* cytoplasmic membrane can be fulfilled by either of the two components of ΔP, membrane potential $\Delta\psi$ or the pH gradient ΔpH (Bakker and Randall, 1984). In conventional protein secretion the ΔP has a direct role in secretion independently of ATP levels (Yamada *et al.*, 1989; Geller, 1990) and appears to be required for the dissociation and translocation of inner membrane bound pre-protein intermediates following release from the SecA translocase (Schiebel *et al.*, 1991). Mitochondrial protein import occurs, like the HlyB/D-dependent secretion, across two membranes, and mitochondrial membrane contact sites have been identified by microscopy (Schleyer and Neupert, 1985). Import of the ADP/ATP carrier protein, which is not processed, is inhibited at an early stage by the presence of uncouplers, both *in vivo* and in cell-free systems (Gasser *et al.*, 1982; Schleyer *et al.*, 1982). However, in contrast to *E.coli* β-lactamase secretion, the import and insertion into the mitochondrial inner membrane of the ADP/ATP carrier requires specifically the single component of membrane potential and there is no contribution by ΔpH (Pfanner and Neupert, 1985). The apparent lack of a periplasmic intermediate in the secretion of hemolysin provokes the question of whether one or more components of ΔP are required, particularly as there may be a surprising role for ΔP in protein translocation across the outer membrane (Wong and Buckley, 1989).

The present study examines the effect of transmembrane energy depletion on the HlyB/D-directed secretion of an inducible secretion-competent 22.4 kDa C-terminal peptide of HlyA, termed Actp. In addition to demonstrating similarity in the requirement for ΔP between the early stage of HlyB/HlyD-dependent secretion and conventional cellular protein secretion, we show that a pool of translocating Actp, representing a subsequent stage of the process, is secreted independently of membrane electrochemical potential. Protease-accessibility studies indicate that this pool, or intermediate, is associated with the inner membrane or transmembrane complex.

Results

HlyB/HlyD-dependent secretion of HlyA C-terminal peptide (Actp)

Production of active cell-free hemolysin by *E.coli* is governed by four *hly* genes which dictate its synthesis (*hlyC* and *hlyA*) and export (*hlyB* and *hlyD*). Our previous data (Koronakis *et al.*, 1989) have shown that the information critical to the secretion of the hemolysin protein HlyA is carried in the C-terminal ~50 amino acids and that a HlyA C-terminal peptide (Actp) containing the signal is exported readily into the medium when complemented with the proteins HlyB and HlyD. The Actp comprises the HlyA C-terminal 194 amino acids fused with the 11 N-terminal residues of the T7 gene 10 product (Figure 10A). The Actp sequence N-terminal of the targeting signal contains >30% charged residues and 15% glycine residues, and the complete Actp contains four methionine residues for radiolabelling. In cell lysates Actp is less susceptible to proteolytic breakdown than the intact HlyA protein.

Synthesis of Actp followed IPTG induction of chromosomal p*lac*-directed T7 *pol* expression in mid-logarithmic phase cultures of *E.coli* BL21(DE3)(pT7Actp) carrying the 3′ *hlyA* sequence cloned downstream of the T7 gene 10 promoter (see Materials and methods and Figure 10). The *hlyB* and *hlyD* genes were expressed constitutively *in trans* by the compatible pACYC184-based plasmid pEK110. This system was used to examine the effect of depleting the transmembrane electrochemical potential during HlyB/D-dependent secretion, in particular by using two assays, termed co-translational and post-translational with respect

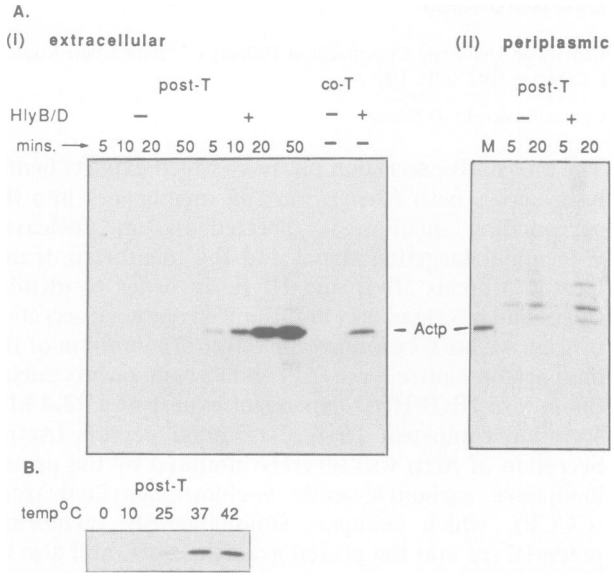

Fig. 1. HlyB/D-dependent secretion of the Hly A C-terminal peptide (Actp) into the extracellular medium. (**A**) (i) Radiolabelled Actp was assayed in the cell-free medium after 5, 10, 20 and 50 min secretion in the post-translational assay, or directly after 5 min pulse-labelling (co-translational assay), in both cases after induction of synthesis for 20 min in the presence and absence of HlyB/HlyD. (ii) Cells from the post-translational assay (± HlyB/HlyD), after 5 and 20 min secretion was allowed, were fractionated and periplasmic fractions analysed for the presence of Actp. The M track shows marker extracellular Actp. (**B**) Post-translational assay of HlyB/HlyD-dependent secretion of Actp into the extracellular medium at different temperatures (the induction step was in each case performed at 37°C).

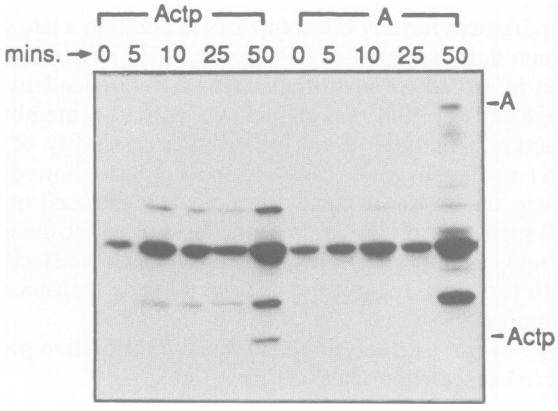

Fig. 2. The rate of HlyB/HlyD-dependent secretion of Actp and HlyA proteins by *E.coli* BL21(DE3) carrying, respectively, pT7Actp or pT7HlyA. Protein synthesis was induced for 5 min. Extracellular Actp was assayed at the times indicated following a 20 s pulse of labelling with [^{35}S]methionine and a chase with excess unlabelled methionine. The other dominant 34 kDa protein is flagellin.

to the time of uncoupling. Secretion of Actp in these two assays without uncoupling is depicted in Figure 1A. In the co-translational assay, induction of Actp expression was allowed to proceed for 20 min, before radioactively labelling proteins with [^{35}S]methionine for 5 min. Radioactivity was chased with excess unlabelled methionine and translation halted by addition of spectinomycin before pelleting of the cells and direct assay of secreted Actp in the cell-free medium. Actp was evident in the medium only when the specific secretion proteins HlyB and HlyD were present in the cell (Figure 1A). When cells induced and labelled as above were pelleted, washed and resuspended in gently shaking minimal medium prewarmed to 37°C, secretion of Actp was measured in the post-translational assay. In this case extracellular Actp was evident after 5 min and levels of Actp in the medium increased up to 50 min, but again only when HlyB and HlyD were present in the cell. For all further post-translational assays a 20 min secretion incubation was employed. Fractionation of cells after 5 and 20 min of the post-translational assay revealed that, as in the case of the complete HlyA protein (Koronakis *et al.*, 1989), no Actp could be isolated from the periplasmic fraction (Figure 1A, panel II).

The HlyB/HlyD-dependent secretion of Actp was strongly influenced by temperature (Figure 1B). Following induction of Actp synthesis at 37°C, cells were pelleted and resuspended in fresh media pre-cooled to 0°C. After 5 min aliquots were shifted to temperatures between 0°C and 42°C (i.e. temperature changes were imposed post-translationally) and extracellular Actp was measured after 20 min incubation. Secretion was prominent and comparable at 37°C and 42°C, but was reduced to $<10\%$ at 25°C. No secretion was detected at 10°C and 0°C. This temperature dependence has also been observed in the post-translational secretion of the entire 1024 residue HlyA protein (Springer and Goebel, 1980). Further identity with the secretion of HlyA was depicted by parallel assessment of the secretion rates of Actp and HlyA both synthesized separately in *E.coli* BL21(DE3) from T7 promoter-directed expression plasmids and secreted by HlyB and HlyD provided *in trans* by pEK110 (Figure 2). Following a 5 min induction proteins were labelled in a short pulse of 20 s (i.e. much shorter than the labelling employed in our other experiments) during induction and this was followed by an extensive chase with excess cold methionine, to a final concentration of 10 mg/ml. Both HlyA and Actp proteins began to appear in the extracellular minimal medium at 25 min and were both strongly evident at 50 min. This system is therefore a valid and amenable model for HlyB/HlyD-dependent secretion.

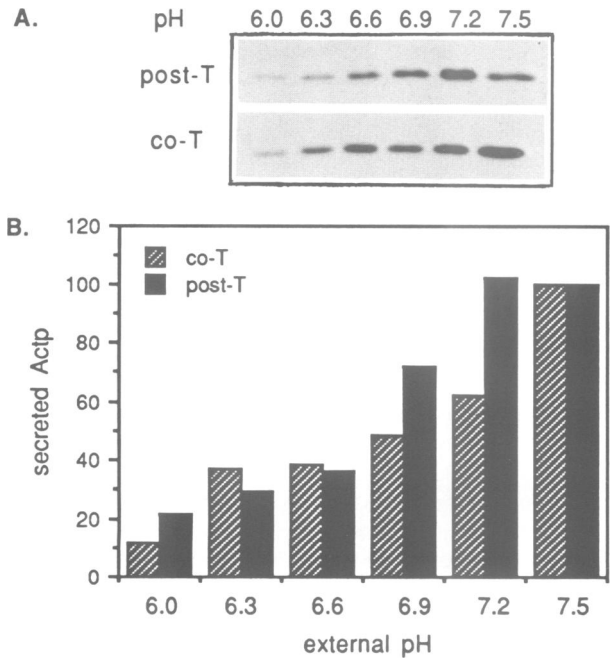

Fig. 3. Effect on Actp secretion of external pH imposed co-translationally (co-T) or post-translationally (post-T). (**A**) Secretion of radiolabelled Actp in a single representative experiment is shown with (**B**) mean values obtained from three separate assays, shown as a percentage of the pH 7.5 secretion levels.

That co-translational and post-translational assays share central functions of secretion is supported by the identical innate pH dependence of secretion in the two cases. Experiments in which external pH was imposed before or after Actp synthesis gave the same result. Secretion fell significantly with decreasing external pH; at pH 6.0 it was 13% (co-T) and 21% (post-T) of the values observed at pH 7.5 (Figure 3).

Differentiating early and late stages in the HlyB/D-directed secretion process

Assuming that following induced synthesis the Actp will as a first step bind and insert into the cytoplasmic membrane, differences observed in the secretion of Actp synthesized before or after imposing changes in the electrochemical potential would identify a specific requirement for an energized membrane early or late in the secretion process, i.e. the co-translational assay would reflect the requirement of the entire process but the post-translational assay would measure the requirement of only the late stage. The kinetics of both HlyA and Actp secretion in this system indicate that the secretion process is not occurring too rapidly to uncouple such stages.

The protonophore CCCP blocks Actp secretion at an early but not late stage

To establish whether the secretion of Actp by HlyB and HlyD requires an energized membrane, the electrochemical gradient across the *E.coli* envelope was depleted by addition of the protonophore carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), which collapses the electrochemical potential (total proton motive force, ΔP). Cells were treated with Tris-buffered EDTA, washed in K0.6 medium and resuspended in K0.6 medium containing increasing levels of CCCP (0.5−120 μM) either prior to or after induction of radiolabelled Actp synthesis (co-translational or post-translational treatment, respectively). Protein synthesis was not affected by the presence of 2 μM CCCP but was reduced to ~30% by 10 μM (data not shown). The results (Figure 4) show that Actp secretion was extremely sensitive to the co-translational addition of CCCP even at low levels previously shown not to depress dramatically the *in vivo* intracellular levels of ATP (Bakker and Randall, 1984; Muren and Randall, 1985; Geller, 1990); there was a 90% reduction in secretion at 0.5 μM CCCP and 99% loss at 2 μM.

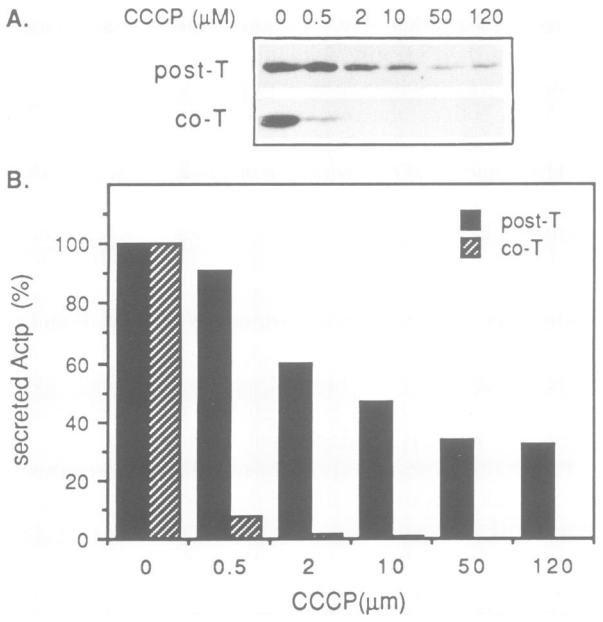

Fig. 4. Actp secretion in the presence of CCCP added either co-translationally or post-translationally, (**A**) the levels of secreted Actp in the cell-free medium assayed in a single experiment, (**B**) the mean of three independent experiments, presented as percentages of the levels achieved when no CCCP was added (co-T, co-translational assay; hatched columns) or (post-T, post-translational assay; filled columns).

In contrast to the above findings, secretion was essentially unaffected when Actp was synthesized prior to the addition of CCCP (post-translational treatment), with no reduction at 0.5 μM CCCP, 40% reduction at 2 μM, and ~35% of the control secretion level persisting in the presence of 120 μM uncoupler. These results give a first indication that the requirement for total proton motive force or one of its components is imposed at an early stage of the secretion process.

The potassium ionophore valinomycin blocks Actp secretion at an early but not late stage

The total proton motive force (electrochemical potential) comprises two components: the membrane potential $\Delta\psi$ and the proton gradient ΔpH. Dissipation of one component allows the other to increase and maintain ΔP essentially constant. To confirm first of all the requirement for ΔP and secondly to examine the components of this requirement, we assayed the effect on Actp secretion of co-translational or post-translational addition of valinomycin, an electrogenic potassium ionophore which specifically collapses membrane potential.

Secretion of Actp in the co-translational presence of a range of valinomycin concentrations, with and without KCl, was first assayed using as before Tris/EDTA-treated cells. The results (Figure 5A) demonstrated that, as found in β-lactamase secretion across the cytoplasmic membrane (Bakker and Randall, 1984), the presence of valinomycin alone had only a marginal inhibitory effect, but when present in combination with 10 mM KCl the inhibition was marked, with a substantial reduction in the appearance of extracellular Actp observed at 50 μg/ml (45 μM) valinomycin. When sodium was substituted for potassium no inhibitory effect

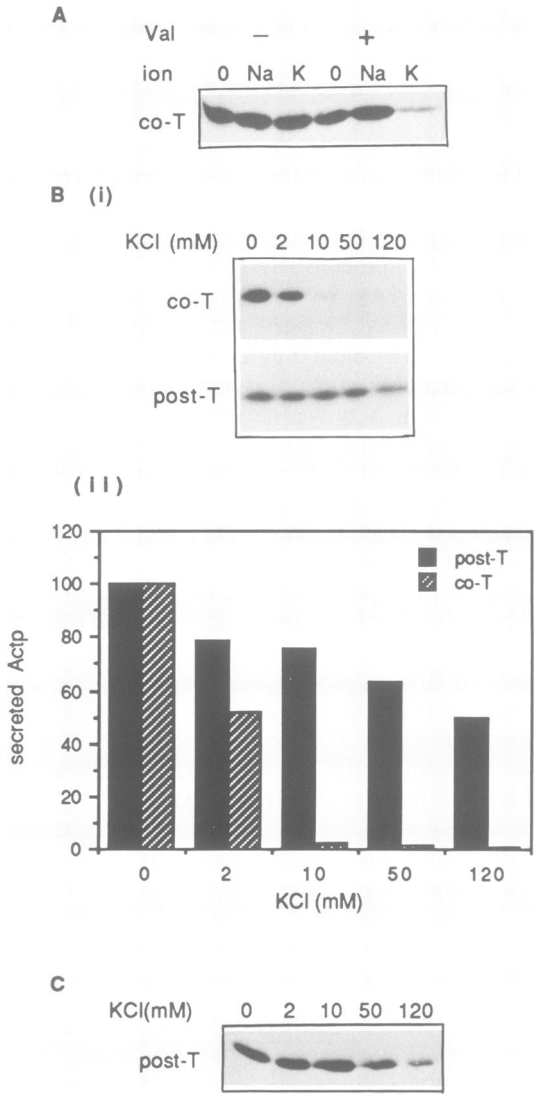

Fig. 5. (**A**) Effect on Actp secretion of co-translational addition of 50 μg/ml valinomycin alone or with NaCl (120 mM) or KCl (10 mM). (**B**) Effect on Actp secretion of valinomycin (70 μg/ml) and increasing amounts of KCl added co-translationally (co-T) or post-translationally (post-T) at external pH 7.2. (i) An example of a single secretion series, and (ii) the means of three independent experiments, expressed as percentages of the Actp secretion at zero KCl. (**C**) Effect on Actp secretion of the combined post-translational addition of valinomycin (70 μg/ml), CCCP (10 μM) and KCl (0–120 mM) to K0.6 medium, pH 7.2.

was seen (even at 120 mM), nevertheless in all subsequent experiments the total concentration of monovalent Na^+ and K^+ ions was held constant at 120 mM, as indicated by Pfanner and Neupert (1985). These initial data indicated that secretion is strongly inhibited by the depolarization of the membrane caused by the combined effect of valinomycin and potassium, i.e. by valinomycin acting specifically as a potassium ionophore.

To investigate this more precisely we chose 70 μg/ml valinomycin, a level equivalent to 6.3 nmol/mg dry weight cells which does not affect Actp translation and lies within previously published concentrations shown to allow the measurement of the effect of ΔP changes without severe ATP depletion (Bakker and Randall, 1984). We assayed the inhibition of Actp secretion in the presence of a range of

KCl concentrations up to 120 mM as previously described for both bacterial and mitochondrial systems (Pfanner and Neupert, 1985; Bakker and Randall, 1984), at the single external pH of 7.2. The effects on Actp secretion observed with co-translational and post-translational addition of valinomycin/KCl (Figure 5B) were analogous to those obtained following CCCP addition. Valinomycin/KCl caused a strong reduction in secretion when present during Actp synthesis, with ~96% inhibition at 10 mM KCl and >99% at 120 mM; in contrast, post-translational addition resulted in only a marginal reduction in Actp secretion to 70% at 10 mM KCl and to 50% at 120 mM. Post-translational addition of increasing amounts of KCl in the presence of both 10 μM CCCP and 70 μg/ml valinomycin resulted in only a marginal reduction in Actp secretion comparable with that achieved by either inhibitor alone (Figure 5C). There was no reduction in secretion up to 10 mM KCl and only 10% and 20% reduction at 50 mM and 120 mM, respectively.

Varying ΔpH indicates ΔP and not Δψ is the requirement at the early stage of Actp secretion

The effects observed following addition of CCCP and valinomycin/KCl indicate that an energized membrane is required for HlyB/HlyD-dependent protein secretion and that this requirement is primarily or totally determined at the early stages and not the later stages of secretion. The results obtained with valinomycin/KCl demonstrate that a loss of membrane potential (Δψ) blocks HlyB-dependent secretion but this could reflect either a specific requirement for membrane potential or an overall requirement for the total proton motive force, which is concomitantly depleted by a loss of membrane potential. To assess which of these possibilities is applicable, we examined the ability of the other component of ΔP, pH gradient ΔpH, to compensate for the loss of membrane potential. The earlier results on pH dependence showed secretion increased in both co- and post-translational assays as an external pH was raised from 6.0 up to 7.5. As the ΔpH is zero at external pH 7.5 and is large at low external pH, this suggests that ΔpH is not a critical factor *per se* in HlyB/HlyD-dependent secretion. This does not, however, exclude the possibility that it might serve experimentally to compensate a loss in Δψ. Varying the ΔpH was performed by changing the external pH; as the external pH is lowered ΔpH is increased.

Figure 6 shows the inhibition of Actp secretion following co- and post-translational addition of 70 μg/ml valinomycin and a range of KCl concentrations which was carried out at six different external pH values, from 7.5 to 6.0 (Figure 6A and B). The post-translational addition of valinomycin/KCl had only a very limited inhibitory effect, even at 120 mM KCl, and this was observed to a similar degree at all six external pH values, with only a marginal indication that secretion at lower pH was less inhibited by the potassium ionophore. When valinomycin/KCl was added co-translationally there was at each external pH a drop in secretion as KCl was increased, but the degree of inhibition appeared to be alleviated as external pH was reduced. Calculations were complicated by the inherent effect of external pH (as shown in Figure 3), but when the amount of Actp secreted at zero KCl at each pH was taken as 100% and plotted against log[KCl] the point at which secretion was inhibited by 50% was seen quite clearly to shift towards higher KCl

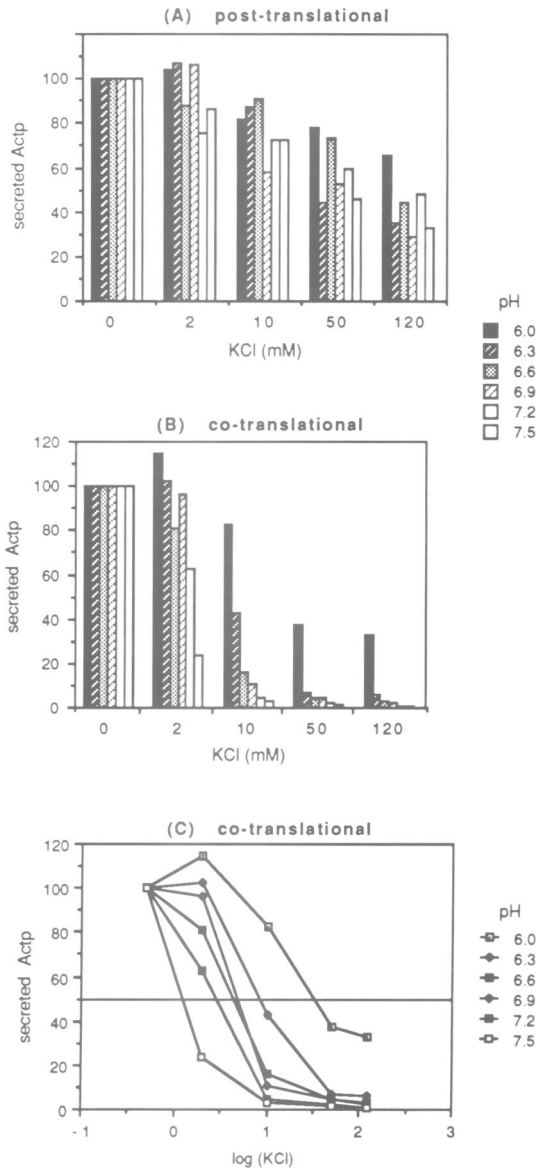

Fig. 6. An increase in ΔpH partly restores secretion of Actp after disruption of membrane potential. Δψ was disrupted by the post-translational (**A**) or co-translational (**B**) addition of valinomycin (70 μg/ml) and various amounts of KCl. The secretion assays were performed at different external pH values. In both cases (co- and post-translational assay) the means of three experiments are shown as percentages of the secretion at zero KCl for each external pH. Panel (**C**) shows that in the co-translational assay in the presence of valinomycin/KCl, an increase in ΔpH causes a shift of the 50% inhibition point towards higher KCl concentrations. The amount of Actp (expressed as percentage of the secretion level at zero KCl at pH 7.5) was plotted against log[KCl]. The horizontal line indicates 50% inhibition of secretion.

concentrations (Figure 6C): at pH 7.5 50% inhibition required 1.5 mM KCl, at pH 7.2 3 mM, at pH 6.9 7 mM, at pH 6.6 6 mM, at pH 6.3 10 mM and at pH 6.0 it was over 20 mM. These data indicate that increasing ΔpH is compensating for diminishing Δψ and suggest indirectly that it is ΔP, and not specifically Δψ, which is needed at the early stages of Actp secretion.

Early stage inhibition of Actp secretion by nigericin-induced depletion of ΔpH

The effect on the secretion process of depleting the pH

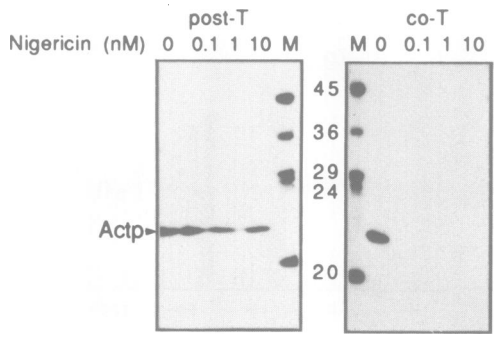

Fig. 7. Effect of nigericin on Actp secretion. The concentrations of nigericin in the post-translational (post-T) and co-translational assays (co-T) are given above the lanes. M, radioactively labelled molecular weight markers (sizes in kDa). Secreted Actp was precipitated from the supernatant by addition of 10% TCA.

gradient was assayed directly by adding nigericin, a neutral K^+/H^+ exchanger which specifically depletes this component. Tris/EDTA-treated cells were suspended in K0.6 medium containing nigericin at concentrations ranging from 0 to 10 nM before and after induction of radiolabelled Actp synthesis. In the co-translational assay secretion was completely inhibited by all concentrations of nigericin used (Figure 7, co-T). However, when nigericin was added after Actp synthesis no inhibition was observed (Figure 7, post-T). The strong inhibitory effect of nigericin in the co-translational assay supports the earlier results which showed that the ΔpH can contribute to the energy requirement for secretion by partially compensating for the loss of membrane potential. The disruption of the proton motive force brought about by the depletion of its ΔpH component again inhibited secretion only at an early, and not at the later stage of the process.

Localization of the ΔP-independent pool of translocating Actp

The above results show that the HlyB/D-dependent secretion process can be divided into at least two sequential steps, the first is early and ΔP-dependent, the second is late and ΔP-independent. Localization of the Actp translocation intermediate in the later ΔP-independent stage was addressed by assessing protease accessibility of the protein. *E.coli* BL21(DE3) (pT7Actp/pEK110) cells were induced, pulse-labelled and harvested directly or following post-translational treatment with CCCP. Aliquots of CCCP-treated and untreated cells were converted into spheroplasts by lysozyme treatment and both whole cells and spheroplasts were then treated in parallel with 1 mg/ml trypsin as outlined in Figure 8A. After neutralizing the trypsin activity with trypsin inhibitor the cells were assayed for secretion of Actp. In the 'trypsin control' experiment, secretion of Actp was allowed to proceed in the presence of a 100-fold lower concentration of trypsin (Figure 8B), which showed that Actp under these assay conditions is inherently protease sensitive. The amount of extracellular Actp was the same whether whole cells were treated with trypsin or not, suggesting that the translocation intermediate was not exposed on the surface of the intact cells. The same results were obtained with and without trypsin treatment of spheroplasts, indicating that the ΔP-independent pool of Actp is not exposed to the periplasmic face of either the inner or outer membrane.

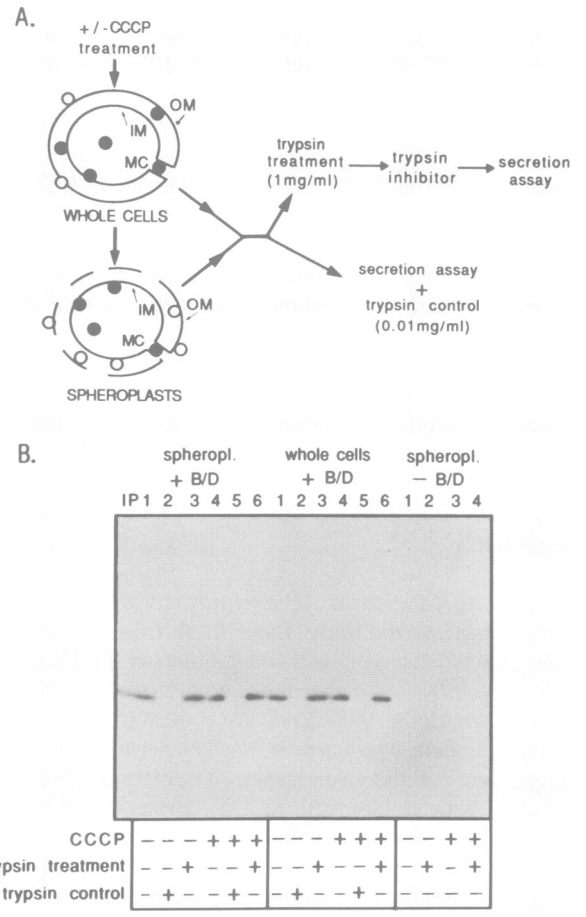

Fig. 8. Accessibility of the ΔP-independent Actp translocation intermediate to trypsin treatment. (**A**) An outline of the experiment including the possible locations of trypsin-accessible (open circles) and trypsin-inaccessible (closed circles) Actp in the cell envelope of whole cells and spheroplasts. IM, inner membrane; OM, outer membrane; MC, putative membrane contact site (HlyB/D 'secretion channel'). (**B**) Trypsin accessibility of Actp in whole cells and spheroplasts. Cells of *E.coli* BL21(DE3) carrying HlyB/D and pT7Actp were induced, pulse-labelled and harvested directly or following post-translational treatment with CCCP (shown as ±CCCP). Aliquots were converted to spheroplasts and both these and the whole cells were incubated with or without 1 mg/ml trypsin (±) as indicated in the cage. Trypsin control: secretion of Actp in the presence of a low concentration of trypsin (10 μg/ml) in the medium to show that Actp is inherently trypsin sensitive. Spheroplasts without HlyB/D were treated in parallel as a control. IP, Actp immunoprecipitated by anti-HlyA antiserum from the extracellular medium of spheroplasts analysed in lane 1.

Spheroplasts of cells lacking HlyB/D were treated in the same way to show that there was no non-specific release of Actp during the manipulations. When spheroplasts were subjected to osmotic lysis and the cellular extract was treated with trypsin (10 μg/ml), all of the Actp was digested (data not shown).

To confirm that Actp was indeed engaged with the membranes during the later ΔP-independent stage of secretion, we analysed the membranes of cells in the post-translational assay, both directly and following treatment with CCCP. After post-translational CCCP treatment of cells, secretion was allowed to proceed for 5 min before the cells were converted into spheroplasts and lysed by freezing in liquid nitrogen and thawing. The total membrane fraction was isolated from a two step sucrose gradient and its content of radiolabelled Actp was analysed directly on a 12%

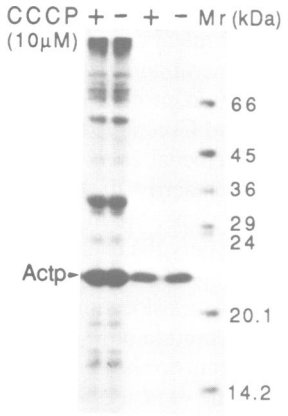

Fig. 9. The ΔP-independent pool of post-translationally secretable Actp is associated with the membrane. Cells were pulse-labelled and post-translationally treated with CCCP, secretion of Actp was allowed for 5 min. The total membrane fraction was isolated from a sucrose step gradient. The two left-hand lanes show total membranes of cells treated or not treated with 10 μM CCCP (shown as ±). The two right-hand lanes show the same samples folowing immunoprecipitation with antiserum raised against HlyA. M_r, molecular weight markers (in kDa).

acrylamide−SDS gel or after immunoprecipitation with antibodies raised against HlyA (Figure 9). The data show that the Actp was associated with the membrane fraction and the same amount of Actp was associated in the presence and absence of CCCP treatment.

Discussion

The HlyB/D-dependent secretion of hemolysin (HlyA) by *E.coli* transfers protein across both cytoplasmic and outer membranes, apparently without a periplasmic intermediate or dependence on either the normal cellular *sec* system or conventional N-terminal processing at the inner membrane. The proteins HlyB and HlyD are assumed to afford secretion specificity in conjunction with the HlyA C-terminal secretion signal and the limited existing data on hemolysin secretion are compatible with the existence of a membrane secretion channel based on the HlyB and HlyD proteins, possibly analogous to the membrane contact sites observed in mitochondria (Schleyer and Neupert, 1985). Another aspect of the hemolysin secretion process which relates specifically to other translocation systems is the close homology shared by HlyB and proteins involved in the transport of various relatively small molecules such as sugars, anti-tumour drugs, ions, peptides and pheromones across membranes of bacteria, yeast, parasites and mammalian cells (Gerlach *et al.*, 1986; Rommens *et al.*, 1989; Monaco *et al.*, 1990; Hyde *et al.*, 1990). These proteins have particularly extensive homology in regions thought, and in some cases shown, to bind NTPs and it is felt that a capacity to hydrolyse ATP or use it for phosphorylation is central to the ability of these proteins to act as membrane pumps, indeed they have been grouped as members of the ATP-Binding Cassette (ABC) family (Hyde *et al.*, 1990). ATP has been shown to be required for *in vitro* bacterial uptake of amino acids and sugars (Dean *et al.*, 1989; Prossnitz *et al.*, 1989) and ATP hydrolysis occurs, both *in vitro* and *in vivo*, during this transport (Mimmack *et al.*, 1989; Bishop *et al.*, 1989; Davidson and Nikaido, 1990). The conventional export of protein from the bacterial cytosol across the cytoplasmic membrane requires energy derived from ATP binding and hydrolysis by the SecA ATPase (Chen and Tai, 1985; Geller *et al.*, 1986; Schiebel *et al.*, 1991), and it seems that this requirement relates to several steps in chaperone function and early translocation (Geller and Green, 1989; Schiebel *et al.*, 1991). Work on the import of eukaryotic pre-pro-alpha factor and the ADP/ATP carrier into, respectively, microsomes and mitochondria (Chirico *et al.*, 1988; Pfanner *et al.*, 1987) also suggests a need for ATP hydrolysis in maintaining translocation competence. The belief that HlyB-directed ATP hydrolysis or phosphorylation is necessary for hemolysin secretion has not yet been supported by direct evidence, although amino acid substitutions in the ATP binding region of HlyB do debilitate secretion (Koronakis *et al.*, 1988).

In addition to the requirement for ATP, a strong dependence on the energy of the cytoplasmic membrane's electrochemical gradient has been demonstrated *in vivo* for several examples of conventional bacterial protein secretion to the periplasm (Date *et al.*, 1980; Wolfe and Wickner, 1984; Enequist *et al.*, 1981; Pages and Lazdunski, 1982; Daniels *et al.*, 1981; Zimmermann and Wickner, 1983; Bakker and Randall, 1984). The requirement for ΔP is distinct from that for ATP and relates to the transloction of subsequent intermediates (Geller, 1990; Schiebel *et al.*, 1991).

In this paper we describe the differentiation of two stages within the HlyB/HlyD-directed process on the basis of the requirement for the membrane electrochemical gradient during secretion of HlyA C-terminal peptide (Actp). We demonstrate the essential role of the proton motive force ΔP by uncoupling it with the protonophore CCCP or depleting individually each of its components, the membrane potential ($\Delta\psi$) and the pH gradient ΔpH, with valinomycin/K^+ or nigericin, respectively. That the energy requirement could be met by either of the ΔP components was also indicated by assay of valinomycin/K^+ inhibition of secretion at different ΔpH values. The dependence on ΔP, not a specific component, equates to the requirement already established for the processing and transfer of β-lactamase across the *E.coli* cytoplasmic membrane. The electrochemical gradient is also required for mitochondrial import of protein to the inner membrane or matrix (Gasser *et al.*, 1982; Schleyer *et al.*, 1982; Pfanner and Neupert, 1985; Hartl and Neupert, 1990) but the need is specifically for the $\Delta\psi$ component (Pfanner and Neupert, 1985).

In our experiments a collapse of ΔP was only inhibitory when carried out early in the Actp secretion process. When ΔP was withdrawn several minutes after the completion of Actp translation, the secretion proceeded at levels substantially unchanged from control. This was observed even at high levels of the inhibitors, alone or in combination, demonstrating that there is no requirement for ΔP in the late stage of Actp secretion, which was observed to be both pH- and temperature-sensitive. The secretion of hemolysin and its derivative Actp are by necessity post-translational as the process is dependent upon a signal located at the extreme C-terminus of the secreted protein. We assumed that in the time immediately following its translation the Actp would associate with the cytoplasmic membrane, and begin insertion and possibly undergo partial translocation before depletion of ΔP was carried out in the post-translational assay. The moderate and HlyA-equivalent translocation rate of Actp measured by pulse−chase suggested that the

secretion process would not be near completion in the post-translational assay and that a translocation intermediate could be identified in the late stage. The ΔP-independent Actp translocation intermediate of the late stage was indeed shown to be located in the membrane fraction, i.e. it had engaged in the inner membrane, and the amount of Actp associated with the membrane was comparable in the presence and absence of CCCP in the post-translational assay, suggesting that at least association with the cytoplasmic membrane has been achieved by the time of CCCP addition. The Actp intermediate in the late stage was not accessible to trypsin in either whole cells or spheroplasts, although it was completely sensitive in preparations of osmotically lysed cells, indicating that the Actp intermediate is not located on the outside of the cell, nor in the periplasm, nor on either periplasmic face. Our conclusion is that it is associated with the inside of the cytoplasmic membrane or an early translocation complex located at the cytoplasmic membrane, e.g. at a HlyB/D-induced inner/outer membrane contact site.

The different effects of collapsing ΔP during and after Actp translation suggest that the early stage of HlyB/D-directed secretion which requires ΔP is binding or some other interaction at the cytoplasmic membrane. The subsequent translocation of the Actp, to the outer membrane and beyond, is ΔP independent. This process might be driven directly by HlyB function, e.g. by ATP binding and possibly hydrolysis or phosphorylation, but it is also possible that it is an energetically favourable transfer, requiring no additional energy. The persistence of Actp secretion in the presence of combined high levels of CCCP and valinomycin K^+ would appear to support the second possibility as, at least in the presence of the higher concentrations of uncoupler, intracellular ATP would be depleted. That HlyB may not be energetically involved in the late stage is also suggested by the function of HlyB analogues in the many eukaryotic examples of translocation across a single membrane, and in addition the analogy with the early functions of the SecA ATPase in conventional secretion. It may be that the various 30−170 kDa proteins of the family secreted by this alternative mechanism will impose individual requirements for folding and unfolding, but these will be additional to the HlyB/D core requirements, as might the influence of post-translational modification of the toxins (Issartel *et al.*, 1991).

Parallels may be drawn between our data and the requirement for ΔP in protein movement across the bacterial cytoplasmic membrane and the requirement for membrane potential at a relatively early stage of mitochondrial protein import. In the latter case once the targeting sequence has traversed the inner membrane the bulk of the protein is translocated independently of $\Delta\psi$ (Schleyer and Neupert, 1985; Pfanner and Neupert, 1987). It may be that the ΔP requirement in the early stage of HlyB/D-dependent secretion applies when the HlyA protein enters a close association with the HlyB/HlyD secretion machinery after release from the initial inner membrane complex, in a way similar to that outlined for passage into the periplasm during conventional bacterial secretion (Geller, 1990). Another alternative to the prospect of HlyA entering a preformed membrane 'pore' (with or without preliminary association with the lipid bilayer) is to postulate an earlier cytosolic association between HlyA and one or both HlyB/D proteins which then insert into the inner membrane. In this case the ΔP dependence could reflect the energy requirement for HlyB insertion into the inner membrane (the specificity of HlyD to the bacterial outer membrane systems suggests HlyB as the common 'recognition protein'). *In vitro* and *in vivo* experiments (Geller and Green, 1989; Thom and Randall, 1988; Geller, 1990; Schiebel *et al.*, 1991) have indicated that protein translocation across the cytoplasmic membrane comprises consecutive steps, the membrane association of the pre-protein needing only the energy of the 'translocase'-bound SecA ATPase, while ΔP is a requirement for a subsequent step once the translocation intermediate has been released from SecA. Secretion of M13 procoat, which does not utilize the SecA/Y translocase, requires ΔP for insertion into the membrane (Date *et al.*, 1980). It seems likely that one or more of these requirements for ΔP is shared by the early stage of hemolysin export but release of protein into the periplasm is replaced in this case by entry into some form of energetically primed membrane fusion channel or complex, independent of a periplasmic intermediate. In this way the outer membrane barrier is not confronted directly, in contrast to the movement of periplasmic aerolysin across this membrane, which is suggested to need ΔP (Wong and Buckley, 1989).

A subdivision of the secretion process and identification of a secretion intermediate allows new experimental approaches in the study of HlyB/HlyD-dependent secretion. The late stage of translocation may be studied *in vitro*, following association of hemolysin with the inner membrane, as 'initiated' ΔP-independent secretion into HlyB/HlyD-containing vesicles. In parallel the early stage in the process can be studied as ΔP-dependent insertion into the cytoplasmic membrane. Protein fusions of Actp to dihydrofolate reductase, the globular structure of which can be stabilized by covalently bound ligand (Eilers and Schatz, 1986), will provide specific non-translocatable intermediates at the cytoplasmic membrane and this could be extended to the isolation of membrane-spanning intermediates. An advantage of the Hly system is that secretion genes can, in contrast to conventional bacterial and mitochondrial translocation (Wickner and Lodish, 1985; Baker *et al.*, 1990), be deleted without pleiotropic effects on the cell. It can therefore be exploited to differentiate *in vivo* between binding to the lipid bilayer and the specific secretion machinery (possibly a membrane contact site) formed by HlyB and HlyD. Such analyses could also have relevance to understanding the recognition function of prokaryotic and eukaryotic ABC transporters.

Materials and methods

Inducible synthesis and secretion of the HlyA C-terminal peptide (Actp)

The HlyA C-terminal 194 amino acid sequence (Figure 10A) was generated as a 205 residue fusion peptide Actp from the plasmid pT7Actp, a derivative of pAR3039 (Studier and Moffatt, 1986) in which the *hlyA* sequence of pEK50 (Koronakis *et al.*, 1989) was fused to the T7 phage promoter, ribosome binding site (RBS) and the in-frame 11 N-terminal residues of the T7 gene 10 product. Specific high level expression from the T7 promoter followed induction of *lac* UV5-directed transcription of the chromosomal T7 RNA polymerase gene1 of lysogen *E.coli* BL21(DE3) cultures with 0.5 mM isopropyl-β-D-thiogalactoside (IPTG). The full size HlyA protein was generated in the same way from pT7HlyA, a derivative of the vector pAR2529, except that the native *hlyA* RBS was used and thus HlyA was not part of a protein fusion. The details of the recombinant DNA manipulations are shown in Figure 10B. The secretion functions were supplied constitutively by the compatible pACYC184 plasmid pEK110, which

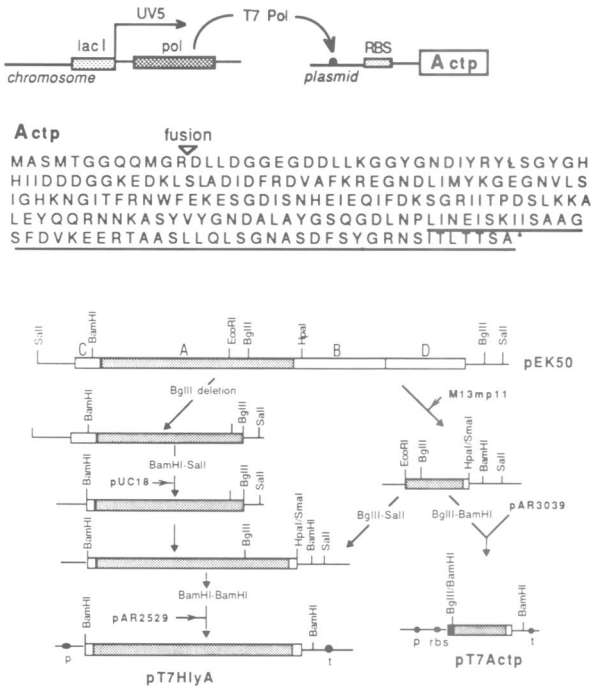

Fig. 10. (**A**) Expression of the HlyA C-terminal fusion peptide (Actp) sequence by the T7 expression plasmid pT7Actp. Actp comprises the 194 amino acid C-terminal sequence of the 1024 residue HlyA, fused in-frame to the 11 N-terminal residues of the T7 gene 10 product. The previously proposed uncleaved C-terminal secretion targeting signal of HlyA is underlined. (**B**) Construction of recombinant plasmids pT7HlyA and pT7Actp encoding, respectively, the proteins HlyA and Actp. The T7 expression components (promoter, terminator and ribosome binding site) are indicated by black circles. The four hemolysin genes are shown as C, A, B and D.

expresses the *E.coli* secretion genes *hlyB* and *hlyD*. *E.coli* BL21 derivatives were grown at 37°C in M9 minimal medium containing 0.2% glucose, 50 μg/ml ampicillin and 20 μg/ml chloramphenicol where appropriate.

HlyB/HlyD-dependent secretion of radiolabelled Actp

Cultures of *E.coli* BL21(DE3) strains were concentrated 8-fold and synthesis of Actp was induced either before or after the imposition of varied external pH or the addition of CCCP, valinomycin/K^+ or nigericin. Changes were thus imposed either during or after synthesis of Actp and accordingly, we refer throughout to co-translational and post-translational treatment and assay of Actp secretion.

Post-translational treatment with valinomycin/KCl. The experimental procedures were based closely on those previously established for the analysis of β-lactamase secretion by Bakker and Randall (1984). Bacterial cultures were grown to A_{600} 0.5 (±0.05) and were concentrated 8-fold before synthesis of Actp was induced for 20 min with 0.5 mM IPTG prior to radioactive labelling with L-[^{35}S]methionine (1000 Ci/mmol, 2–3 μCi added per ml original culture). Labelling proceeded for 4 min with agitation in a 37°C water bath after which excess unlabelled methionine was added to 10 mM followed by spectinomycin to 1 mg/l. All solutions used from this point onwards contained 1 mg/ml spectinomycin to ensure protein synthesis remained inhibited. Cells were then centrifuged for 2 min at 15 000 *g* and resuspended in 120 mM Tris, pH 8.0 to a concentration of 10 mg dry weight of cells per ml. After incubation for 2 min at 37°C EDTA, pH 7.5 was added to 1 mM and the cells incubated for a further 2 min to render the cytoplasmic membrane accessible to the ionophore (Bakker and Randall, 1984). At this stage samples of the Tris/EDTA-treated cells were taken as controls, while to the remaining suspension valinomycin was added to 70 μg/ml (stock 10 mg/ml in ethanol) prior to a further incubation at 37°C for 10 min. This represents 6.3 nmol valinomycin/mg dry weight of cells and is therefore within concentrations previously used in studies of cytoplasmic membrane protein secretion (Bakker and Randall, 1984). The controls and the Tris/EDTA/valinomycin-treated cells were then split again in corex tubes, washed and diluted 4-fold in the supplemented minimal growth medium K0.6 described by Bakker and Randall (1984) set at pH values ranging from pH 6.0 to 7.5. Cells were pelleted by centrifugation and resupsended in fresh medium of the same pH, each further aliquoted in six portions to prepipetted samples of KCl at concentrations ranging up to 120 mM. NaCl was present in all cases such that the monovalent cation concentration was constant at 120 mM, as previously established (Pfanner and Neupert, 1985). The valinomycin/KCl-treated cell suspensions were then incubated at 37°C for a maximum of 25 min to allow secretion before separating the cells from the medium by centrifugation at 15 000 *g*. To avoid breakdown of the ionophore by the cells, this length of time was never exceeded (Bakker and Randall, 1984).

Co-translational treatment with valinomycin/KCl. This was entirely analogous to the post-translational disruption of membrane potential except that the Tris/EDTA treatment, valinomycin treatment, washing and addition of KCl to the concentrated cells preceded the induction of radiolabelled Actp synthesis.

Other treatments. The procedures for co-translational and post-translational treatment of cells with carbonylcyanide-*m*-chlorophenyl hydrazone (CCCP) and nigericin were the same as those described above except that following Tris/EDTA treatment, cells were washed and resuspended in fresh K0.6 medium, pH 7.2 containing various concentrations of the protonophore CCCP or nigericin, respectively. Identical procedures were adopted for the imposition of different external pH values. In all comparative inhibition experiments equal amounts of cells were taken from the same batch of bacterial growth culture.

Quantification of Actp secretion

After both co- and post-translational treatments extensive centrifugation was used to remove cells and the proteins present in the supernatants were precipitated by TCA addition on ice (10% final volume). The TCA-precipitated proteins were dissolved in Tris-buffered SDS loading buffer, heated for 2 min at 90°C and separated on a 15% polyacrylamide–SDS gel with a 5% stacking gel. Gels were stained with Coomassie blue R250, impregnated for 20 min in 0.5 M sodium salicylate, dried and exposed to RX Fuji X-ray film. The autoradiograms were scanned using a Transidyne 2955 densitometer. Every set of experiments was repeated three times.

Protease accessibility of translocating Actp

Trypsin accessibility of the innately trypsin-sensitive Actp was assessed in whole cells and in spheroplasts, again at bacterial cell concentrations of 10 mg/ml dry weight. Cell cultures were induced in the presence of radiolabelled methionine, before chasing and addition of spectinomycin as described above. Where CCCP was added it was to a final concentration of 10 μM. All the subsequent buffers and solutions, with or without CCCP, contained spectinomycin. The cells were washed twice with cold medium pH 7.2 and converted to spheroplasts by resuspending 10 mg dry weight cells in 0.25 ml 0.1 M Tris acetate, pH 7.5, containing 0.5 M sucrose and 5 mM EDTA. Lysozyme was added (20 μl of 2 mg/ml) followed by addition of 0.25 ml ice cold water. After incubation for 5 min on ice, trypsin was added to 1 mg/ml final concentration before continuing incubation for a further 60 min on ice. At the end of the incubation trypsin inhibitor was added to 2 mg/ml to halt proteolysis, $MgCl_2$ was added to stabilize the spheroplasts at a final concentration of 18 mM. Cells were centrifuged for 40 s at 15 000 *g* and resuspended in fresh K0.6 medium, pH 7.2 containing spectinomycin and 12% sucrose (plus CCCP and 200 μg/ml trypsin inhibitor where appropriate). The cells were further incubated at 37°C for 30 min, centrifuged twice for 10 min at 13 000 *g* and the supernatant was precipitated by TCA (final concentration 10%). Secreted Actp was assayed on a 12% acrylamide–SDS gel followed by autoradiography.

Preparation of membranes

Cells were post-translationally treated with CCCP and secretion of Actp was allowed for 5 min before the cells were converted into spheroplasts as described. Spheroplasts were suspended in 50 mM Tris–Cl, pH 7.5 containing 2.5 mM EDTA and lysed by freezing in liquid nitrogen and thawing on ice. The viscosity of the samples was reduced by sonication and whole cells were removed by 10 min centrifugation at 15 000 *g*. The supernatant was loaded on a two-step sucrose cushion (2 ml 2 M sucrose, 2 ml 0.6 M sucrose, in 2.5 mM EDTA) and spun for 3 h at 190 000 *g*. The membrane fraction was isolated at the interface of the two sucrose steps, precipitated with 10% TCA and analysed on a 12% acrylamide–SDS gel followed by autoradiography. Membrane-associated Actp was immuno-precipitated with antibodies raised against HlyA as described (Randall and Hardy, 1986; Koronakis *et al.*, 1989).

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