Membrane potential ($\Delta\psi$) depolarizing agents inhibit maturation

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Precursor forms of exported proteins were first accumulated in the envelope of phenethyl alcohol (PEA)-treated cells. After removal of PEA, a complete processing could be obtained in a few minutes. In this work, we demonstrate that colicins A and E_1, that act on the electrical gradient in the cytoplasmic membrane, prevent the processing of precursor forms previously accumulated. Concentrations of colicins accounting for ~1 killing unit (50–3000 molecules/cell) were found to be sufficient for inhibition of processing. Therefore our results strongly suggest that in intact cells the electrical gradient across the cytoplasmic membrane is required for maturation of exported proteins.

Precursor form *Signal peptide* *Colicin* *Local anesthetic*

1. INTRODUCTION

Periplasmic and outer membrane proteins of *Escherichia coli* are produced in membrane-bound polysomes [1–3] in precursor form containing short-lived signal sequences. These signal peptides, of characteristic structure [4], have to be located at or near the N-terminus for efficient export [5,6] but their processing is not strictly required for the transfer step across the cytoplasmic membrane [5,7]. Furthermore, this processing can occur either during or shortly after synthesis of nascent proteins [8]. The electrochemical gradient of protons in the cytoplasmic membrane is required for maturation of exported proteins [9–12]. This protonmotive force (Δp) being composed of a concentration gradient (ΔpH) and an electrical gradient ($\Delta\psi$) we ask here which of these two components of the protonmotive force is required for the processing of precursor forms.

2. MATERIALS AND METHODS

[^{35}S]Methionine (1200 Ci/mmol) was purchased from Amersham. Phenethyl alcohol (PEA) and carbonyl cyanide *m*-cholorophenyl hydrazone (CCCP) were obtained from Fluka AG and Calbiochem, respectively.

2.1. *Bacterial strains and growth conditions*

The strain *E. coli* K12 CW3747 Met, B_1, PhoR, that constitutively produces alkaline phosphatase was used throughout this study. Cells were grown at 37°C in a Tris-based minimal medium as in [13], supplemented with 16 mM maltose.

2.2. *Conditions for radiolabelling of proteins, immunoprecipitation and gel electrophoresis*

Cells were grown to 2×10^8 cells/ml, centrifuged and resuspended in 1/20th of the original volume in fresh medium. After 15 min at 37°C, the culture was divided, a small aliquot was kept without addition as a control, in the other fraction 0.5% (40 mM) phenethyl alcohol was added. After 3 min [^{35}S]Methionine (60 μCi/ml, 60 mmol) was added in each fraction and incubation done for 15 min at 37°C. Control cells were then centrifuged and the pellet frozen. Phenethyl alcohol-treated cells were divided into several fractions and centrifuged. Each fraction was resuspended in fresh medium to the original volume. A fraction was kept without addition as a control during the chase. In other fractions, colicin A or colicins E_1, E_2, E_3 were added, as indicated in figure legends. Unlabelled methionine (70 mM) was added and samples were removed at various times during the chase and rapidly centrifuged. Cell pellets were solubilized

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and immunoprecipitations with the desired antisera were done as in [14]. Immunoprecipitated proteins were analyzed by sodium dodecyl sulfate gel electrophoresis in 11% polyacrylamide gels (SDS–PAGE) according to [15]. The gels were impregnated and fluorographed as in [14]. Densitometer scannings of fluorographs were done with a Vernon densitometer. The extent of lysis during pulse and chase experiments was evaluated by assaying β-galactosidase (induced by 1 mM isopropyl β-D-thiogalactoside). The lysis was never $> 5 - 7\%$.

2.3. *Colicins purifications and assays*

Colicins were purified and assayed as in [16,17].

3. RESULTS

3.1. *Specific effect of colicins A and E_1 on maturation of exported proteins*

A functional class of colicins sometimes referred to as the E_1 type, as opposed to E_2 and E_3 types, includes colicins E_1, K, I_a, I_b, A and L. All members of the E_1 class have been shown to depolarize the cytoplasmic membrane [18–20] very likely by forming ion-permeable channels in this membrane [21,22].

We have therefore used colicins A and E_1 as tools for depolarization of the cytoplasmic membrane instead of CCCP which is supposed to act on the total Δp [23]. Precursor forms of exported proteins were first accumulated in the envelope of PEA-treated cells as in [9,10]. After removal of PEA, a complete processing can be obtained after a few minutes [10]. A time of chase after PEA removal was chosen such that about half of the pre-LamB was matured (42% mature form) so that both mature and precursor forms could be seen (fig. 1). When colicins A or E_1 were present during the chase no maturation at all was observed. In contrast, colicins E_2 and E_3 that have DNase [24] and RNase [25] activities, respectively, did not prevent processing to occur. With these colicins 35–36% maturation was observed close to the 42% found in the absence of colicins.

3.2. *Colicin A dose-dependence of pre-LamB and pre-PhoA maturation*

The dependence of pre-LamB maturation upon colicin A concentration is shown in fig. 2. Colicin A at 5 or 0.5 μg/ml prevented efficiently the maturation which increased to the control level (no colicin A added) at 0.05 μg/ml. The same result was observed for a periplasmic protein, the alkaline phosphatase (fig. 2B).

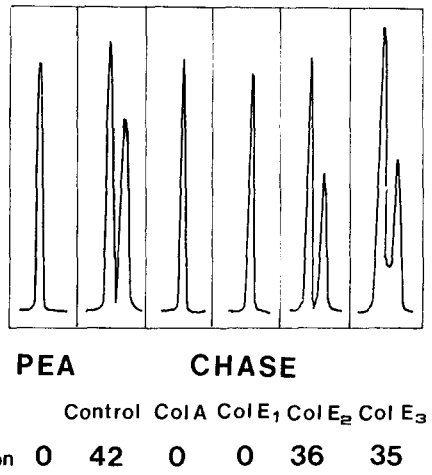

Fig. 1. Specific effect of E_1-type colicins on maturation of pre-LamB protein. Cells were labelled with [^{35}S]-methionine in the presence of PEA and they were chased for 5 min in absence or in presence of various colicins (1 μg/ml). Immunoprecipitates were analyzed by SDS–PAGE. Densitometer scannings of fluorographs are shown.

3.3. *Kinetics of establishment of processing inhibition by colicin A*

The maturation of the precursor form of OmpA, another major outer membrane protein, was also inhibited (table 1). However, the establishment of this inhibition was not instantaneous in this case

Table 1

Kinetics of establishment of pro-OmpA processing inhibition by colicin A (0.5 μg/ml)

Time of chase (min)	Percentage of maturation[a]	
	− col A	+ col A
1	40	40
3	60	41
60	100	41

[a]Percentages of pre-OmpA maturation were determined from fluorograph densitometer scannings

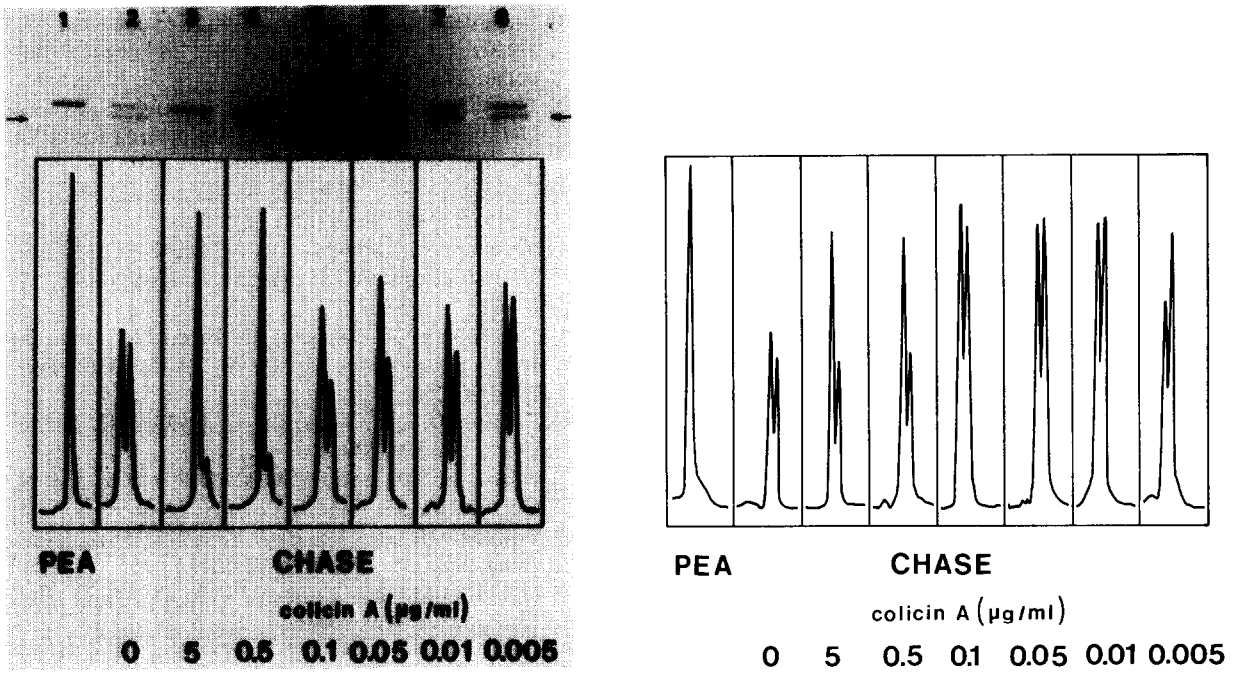

Fig. 2. Colicin A dose-dependence of pre-LamB and pre-PhoA maturation. Cells were labelled with [^{35}S]methionine in the presence of PEA and they were chased for 5 min in the absence or in the presence of various amounts of colicin A: (A) immunoprecipitates obtained with an antiserum directed against LamB were analyzed by SDS–PAGE (top) and fluorographs were scanned with a densitometer (bottom); (B) immunoprecipitates obtained with an antiserum directed against alkaline phosphatase were analyzed.

since after 1 min in the presence or absence of colicin A, 40% of pre-OmpA was already matured. Similarly, in the presence of CCCP we could not totally prevent the processing [10]. Both of these observations might be related to the fast processing of OmpA [26].

4. DISCUSSION

Our results clearly demonstrate that colicins E_1 and A can prevent the in vivo processing of both precursor forms of periplasmic and outer membrane proteins. A similar result was obtained by using 30 μM CCCP [9–12] a condition that collapses both the electrical gradient ($\Delta\psi$) and the concentration gradient (ΔpH), the two components of the proton electrochemical gradient across the cell membrane:

$$\Delta p = \Delta\psi - \frac{2.3\ RT}{F}\ \Delta\mathrm{pH} \qquad [23]$$

Colicin A (0.5 μg/ml) corresponding to an average of 4800 molecules/cell is sufficient to completely prevent maturation. This amount is compatible with reports of colicin preparations in which a killing unit consists of 50–3000 colicin molecules [27]. This poor efficiency might be due either to inactive colicin molecules or to receptors inactivated by the PEA-treatment. In any event, colicin A and E_1 only collapse the electrical gradient ($\Delta\psi$) without affecting the concentration gradient (ΔpH) [18–22]. Therefore our results clearly demonstrate that in intact cells the electrical gradient across the cytoplasmic membrane is required for maturation of exported proteins. It is likely that the correct orientation of the dipole constituted by the signal peptide in the electrical gradient is needed for the signal peptidase to reach the strategic bond to be cleaved.

A similar requirement of electrical gradient across the inner mitochondrial membrane has been evidenced for post-translational transfer of pro-

teins into mitochondria [28,29]. Further investigations will indicate if there is any similarity with the co-translational protein export in prokaryotes in terms of membrane potential-dependent orientation of specific polypeptide fragments.

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REFERENCES

[1] Randall, L.L. and Hardy, S.J. (1977) Eur. J. Biochem. 75, 43–53.
[2] Varenne, S., Piovant, M., Pagès, J.M. and Lazdunski, C. (1978) Eur. J. Biochem. 86, 603–606.
[3] Smith, W.P., Phonj, C., Thompson, R.C. and Davis, B. (1977) Proc. Natl. Acad. Sci. USA 74, 2830–2834.
[4] Inouye, M. and Halegoua, S. (1980) Crit. Rev. Biochem. 10, 339–371.
[5] Baty, D., Mercereau-Puijalon, O., Perrin, D., Kourilsky, P. and Lazdunski, C. (1981) Gene 16, 79–87.
[6] Talmadge, K., Brosuis, J., Gilbert, W. (1981) Nature 294, 176–178.
[7] Lin, J.J.C., Kamazawa, A., Ozols, J. and Wu, A.C. (1978) Proc. Natl. Acad. Sci. USA 75, 4891–4895.
[8] Josefsson, L.G. and Randall, L.L. (1981) Cell 25, 151–157.
[9] Pagès, J.M. and Lazdunski, C. (1981) CR Acad. Sci. Paris 293, 27–30.
[10] Pagès, J.M. and Lazdunski, C. (1982) Eur. J. Biochem. 124, 561–566.
[11] Enequist, H.G., Hirst, T.R., Harayama, S., Hardy, S.J.S. and Randall, L.L. (1981) Eur. J. Biochem. 116, 227–233.
[12] Daniels, C.J., Bole, D.G., Quay, S.C. and Owender, D.L. (1981) Proc. Natl. Acad. Sci. USA 78, 5386–5400.
[13] Lazdunski, A., Murgier, M. and Lazdunski, C. (1979) J. Mol. Biol. 128, 127–141.
[14] Varenne, S., Knibiehler, M., Cavard, D., Morlon, J. and Lazdunski, C. (1982) J. Mol. Biol. 159, 57–70.
[15] Lugtenberg, B., Meijers, J., Peters, R., Van der Hoek, P. and Van Alphen, L. (1975) FEBS Lett. 58, 254–258.
[16] Cavard, D. and Lazdunski, C. (1979) Eur. J. Biochem. 96, 519–524.
[17] Schwartz, S.A. and Helsinski, D.R. (1971) J. Biol. Chem. 246, 6318–6327.
[18] Tokuda, H. and Konisky, J. (1978) Proc. Natl. Acad. Sci. USA 75, 2579–2583.
[19] Gould, J.M. and Cramer, W.A. (1977) J. Biol. Chem. 252, 5491–5497.
[20] Weiss, M. and Luria, S.E. (1978) Proc. Natl. Acad. Sci. USA 75, 2483–2487.
[21] Schein, S.J. Kagan, B.L. and Finkelstein, A. (1978) Nature 276, 159–163.
[22] Pattus, F., Cavard, D. and Lazdunski, C. (1982) Toxicon 20, 263.
[23] Mitchell, P. (1966) Biol. Rev. 41, 445–502.
[24] Schaller, K. and Nomura, M. (1976) Proc. Natl. Acad. Sci. USA 73, 3989–3993.
[25] Boon, T. (1971) Proc. Natl. Acad. Sci. USA 69, 549–552.
[26] Crowlesmith, I., Gamon, K. and Henning, U. (1981) Eur. J. Biochem. 113, 375–380.
[27] Holland, I.B. (1975) Adv. Microb. Physiol. 12, 55–139.
[28] Schleyer, M., Schmidt, B. and Neupert, W. (1982) Eur. J. Biochem. 125, 109–116.
[29] Morita, T., Miura, S., Mori, M. and Tatibana, M. (1982) Eur. J. Biochem. 122, 501–509.