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Role of the protonmotive force and of the state of the lipids in the in vivo protein secretion in *Corynebacterium glutamicum*, a Gram-positive bacterium

Nicolas Bayan [a], Silja Schrempp [a], Gwennaël Joliff [b], Gérard Leblon [c] and Emanuel Shechter [a]

[a] *Laboratoire des Biomembranes, CNRS (UA 1116 and GDR 961), Université de Paris-Sud, Orsay (France),* [b] *Orsan Recherche, Les Ulis (France) and* [c] *Laboratoire de Biologie Moléculaire des Corynébactéries, CNRS (UA D1354 and GDR 961), Université de Paris-Sud, Orsay (France)*

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PS1 is a protein translocated across the cytoplasmic membrane of *Corynebacterium glutamicum*, a Gram-positive bacterium. Western blots of whole cell extracts showed the presence of two bands associated with the mature and the precursor forms. Addition of chloramphenicol led to the disappearance of the precursor form while dissipation of the protonmotive force ($\Delta\tilde{\mu}_H$) prior to the addition of chloramphenicol prevented the maturation of the precursor. Dissipation of $\Delta\tilde{\mu}_H$ prior to a pulse chase experiment resulted in a complete block on translocation; regeneration of $\Delta\tilde{\mu}_H$ allowed the translocation of PS1 synthesized in its absence. On the other hand, dissipation of $\Delta\tilde{\mu}_H$ immediately after a pulse period had little effect on PS1 secretion. Lowering the temperature to 10°C at the end of the pulse period completely inhibited secretion. The efficiency of secretion as a function of increasing temperature followed closely the order-to-disorder transition of the membrane lipids as detected by fluorescence anisotropy of diphenylhexatriene. Taken together, the results show that $\Delta\tilde{\mu}_H$ and the state of the lipids affect different steps of PS1 secretion.

Introduction

The molecular mechanism of protein translocation across the cytoplasmic membrane of *Escherichia coli*, a Gram-negative bacterium, is well documented, in vivo and in vitro. The translocated proteins are synthesized as precursors with an N-terminal extension (signal-sequence) [1]. In vivo, the translocation may be either co- or post-translational, depending on the conditions and on the nature of the protein [2]. Various soluble and membrane proteins participate in the translocation. SecB, a soluble chaperonin which retards the structuration of the translocated protein, is usually (although not always) required [3–5]. SecA, a membrane protein loosely bound to the cytoplasmic side of the membrane, possesses an ATPase activity and is absolutely required for translocation [6–8]. Similarly, a set of transmembrane polytopic proteins is also required: SecY [9], secD [10], secE [11] and secF [12], which together may form part of the translocation machinery (possibly the channel) [13]. During translocation, the precursor is cleaved by a signal-peptidase located in the cytoplasmic membrane to give the mature protein [14,15]. In terms of energy, the in vivo translocation is inhibited by the dissipation of the protonmotive force ($\Delta\tilde{\mu}_H$) [16–18] and by an inactivation of SecA by sodium azide (for a review, see Ref. 19). In vitro, ATP is an absolute requirement [20,7], while $\Delta\tilde{\mu}_H$ is generally, although not always, required [21,22].

In Gram-negative bacteria, the translocation of a protein across the cytoplasmic membrane leads to a protein located in the periplasm between the cytoplasmic and outer membranes: complete export requires that the protein crosses both the cytoplasmic and the external membranes. Various special mechanisms for this process have been identified [23–26]. On the other hand, the cell envelope of Gram-positive bacteria contains only the cytoplasmic membrane. Thus, proteins translocated across that membrane are, in principle, directly secreted into the external medium. Gram-posi-

Correspondence to: E. Shechter, Laboratoire des Biomembranes, Université de Paris-Sud, Bâtiment 432, 91405 Orsay, France.

Abbreviations: Cam, chloramphenicol; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DPH, diphenylhexatriene; TPP^+, tetraphenylphosphonium ion.

tive bacteria are therefore of interest as potential candidates for the industrial production of secreted proteins.

Far less is known regarding the mechanism of protein secretion across the cytoplasmic membrane by Gram-positive as compared to Gram negative bacteria. In particular, no in vitro translocation system has yet been developed. In vivo studies have shown that proteins secreted by Gram-positive bacteria are synthesized as precursors with an N-terminal extension (reviewed in Ref. 27). This signal sequence bears some resemblance to those of the precursor proteins translocated across the cytoplasmic membrane of *E. coli*. There has not, as yet, been any genetic or biochemical characterization of the protein components involved in secretion. Recently, genes coding for *Bacillus subtilis* proteins similar to SecA [28] and SecY [29,30] of *E. coli* have been cloned. The *B. subtilis* protein homologous to *sec*Y is able to complement the translocation defect of an *E. coli* SecY mutant [31]. In common with *E. coli*, the in vivo translocation of proteins across the cytoplasmic membrane of Gram-positive bacteria requires the presence of $\Delta\tilde{\mu}_H$ [32–34]. These similarities may suggest similar mechanisms of translocation across the cytoplasmic membrane in Gram-positive and Gram-negative bacteria. This however, remains to be proven.

Corynebacterium glutamicum is widely used for the industrial production of secreted metabolites (amino acids, in particular). Recently we have shown that this bacterium secretes two proteins (designated PS1 and PS2) into the external medium; PS1 is synthesized as a larger precursor and the corresponding gene has been cloned and sequenced [35]. We have studied PS1 to determine some of the characteristics of its translocation across the cytoplasmic membrane in vivo and its subsequent secretion. We report here on the influence of the electrochemical proton gradient and of the temperature on the translocation and secretion of PS1.

Materials and Methods

Growth of bacteria

For all the secretion experiments, *Corynebacterium glutamicum* (strain ATCC 17965) cultures were grown at 34°C and pH 6.9 on a rotary shaker (260 rpm) in the following medium (per liter): 14 g $K_2HPO_4 \cdot 3H_2O$; 5.3 g KH_2PO_4; 2 g $(NH_4)_2SO_4$; 2 mg thiamin; 30 μg biotin; 200 μg deferoxamine; 250 mg $MgSO_4 \cdot 7H_2O$; 8 mg $FeCl_3$; 6 mg $FeCl_2$ and trace amounts of Ca, Mn, Zn, Co, Cu, Mo, Ni, B. Glucose (0.4%) was included as a carbon source.

SDS extraction of PS1 from the cell wall

1 ml of a cell culture ($OD_{650} = 1$) was centrifuged at 12000 × *g* for 3 min. The pellet was resuspended in 30 μl of a Tris-HCl buffer (60 mM, pH 6.8) containing 2% SDS. The suspension was boiled for 5 min and centrifuged as above. The supernatant was collected and contained PS1 and PS2 initially associated with the cell wall. The same results were obtained if the extraction was performed directly in 30 μl of Laemmli buffer, used for SDS-PAGE (60 mM Tris-HCl, 2% SDS, 5% glycerol, 0.001% Bromophenol blue indicator).

Measurement of $\Delta\psi$, ΔpH and ATP content

$\Delta\psi$ was determined from the distribution of tritiated tetraphenylphosphonium bromide ([^{3}H]TPP$^+$) according to Houssin et al. [36]. [^{3}H]TPP$^+$ (10 GBq/mmol, 10 μM final concentration) was added to 1 ml culture ($OD_{650} = 1$, i.e., 0.2 mg cell dry weight per ml). The steady-state level of intracellular [^{3}H]TPP$^+$ accumulation was attained within 10 min. 100 μl of the cell suspension were filtered through Whatman GF/F glass fiber filters. The filters were washed twice with 4 ml of phosphate buffer (pH 6.9) and counted for radioactivity. [^{3}H]TPP$^+$ accumulation was corrected for non-specific binding by subtracting a blank obtained under identical conditions, but using cells pretreated with 20 μM of the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP).

ΔpH was estimated from the distribution of [^{14}C]benzoate according to Houssin et al. [36]. 1 ml of culture ($OD_{650} = 1$) was incubated 5 min with [^{14}C]benzoate (19.7 MBq/mmol, 10 μM final concentration) in the presence of ^3H_2O (0.11 MBq/ml). After centrifugation, the pellet and the supernatant were counted for radioactivity. Intracellular [^{14}C]benzoate accumulation was corrected for non-specific binding by subtracting a blank obtained under identical conditions, but using cells pretreated with 20 μM of the protonophore CCCP.

Internal ATP was measured by bioluminescence using a Biocounter M2010 (Lumac) and a Boehringer kit assay (Mannheim, Germany) [37]. 10 μl of the bacterial suspension were diluted with 90 μl of dimethylsulfoxide, then with 4.9 ml distilled water. 100 μl of the final suspension were assayed for ATP content.

$\Delta\psi$ and ΔpH and ATP concentration were calculated assuming a cell volume of 2.7 μl/mg cell dry weight [38].

Preparation of membrane vesicles

Cells were grown as described under 'Growth of bacteria'. Penicillin G (0.3 unit/ml) was added when OD_{650} reached 0.5. The incubation was then continued for 2 h. The cells were harvested and resuspended in the same growth medium containing 2 mg/ml lysozyme and 10% succinate. The suspension was gently agitated for 20 h at 34°C. At this stage, protoplasts were formed [39]. The protoplasts were passed through a French press at 2000 psi. The resulting suspension was cen-

trifuged at 16000 × *g* to remove unbroken cells and debris. The supernatant was then centrifuged for 20 min at 100000 × *g*. The resulting pellet was resuspended in 100 mM phosphate buffer (pH 7.5).

Fluorescence anisotropy

Membrane vesicles were suspended in 100 mM phosphate buffer (pH 7.5) at a concentration of 100 μg protein/ml. They were labelled with the fluorescent probe (DPH, final concentration: 0.5 μM) by incubation for 1 h at 37°C. The sample was then cooled to 5°C.

Fluorescence polarization was measured using a SLM 4000 apparatus equipped with a cell thermostated compartment and a magnetic stirrer according to Le Grimellec et al. [40]. The temperature was increased stepwise from 5°C to 30°C. Results of the steady-state depolarization experiments are expressed in terms of fluorescence anisotropy, r, where:

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}}$$

The excitation wavelength was 362 nm; the emission wavelength was 430 nm.

SDS-PAGE and Western blot

SDS-PAGE was carried out as described by Laemmli [41] with a 4% stacking gel and a 7.5% separation gel. Samples were denatured in the presence of 2% SDS in 50 mM Tris-HCl (pH 6.8). After electrophoresis, gels were stained with Coomassie blue gel and were dried and autoradiographed.

For Western blot experiments, proteins were blotted onto nitrocellulose sheets at 50 V for 1 h in a Tris (25 mM)-glycine (193 mM) buffer (pH 8.35) containing 20% methanol. Immunodetection was performed as described by Pierce and Capron [42] using goat anti-rabbit IgG conjugated with alkaline phosphatase. Immunoblots were developed with Nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indoxylphosphate *p*-toluidine (BCIP).

Densitometry of the autoradiographs and immunoblots was performed using a Desaga CD60 densitometer.

Results

Distribution of PS1 between the cell wall and the external medium; time course of PS1 secretion

Few proteins are secreted by *Corynebacterium glutamicum*. Fig. 1 (left lane) displays a Coomassie blue-stained SDS gel of the supernatant of a culture during the exponential phase of growth. Mainly two major bands were observed. They corresponded to PS1 and PS2 as evidenced by Western blotting using anti-PS1 and anti-PS2 antibodies [35]. Their apparent molecular weights were 67000 and 63000, respectively.

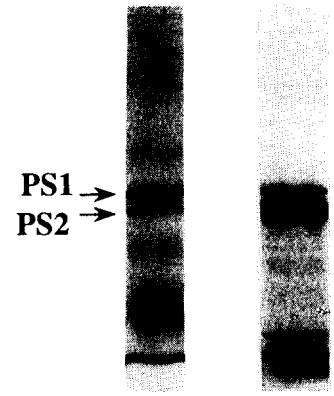

Fig. 1. PS1 distributes between the external medium and the cell wall. Supernatant: Coomassie blue-stained profile of SDS-PAGE of 1 ml equivalents of external culture medium during exponential growth ($OD_{650} = 1$). Cell wall: Coomassie blue-stained profile of SDS-PAGE of the soluble extract of a cell pellet (corresponding to 1 ml culture during exponential growth (OD = 1)) treated with SDS as described in Materials and Methods. The higher and lower bands correspond to PS1 and PS2, respectively.

A sizeable fraction of PS1 and PS2 remained associated with the cell and could be detached by SDS treatment: An exponentially growing culture was centrifuged and the pellet was treated with SDS as described in Materials and Methods (extraction of PS1 from the cell wall). Fig. 1 (right lane) displays an SDS-PAGE of the solubilized extracted material. Mainly the two major bands (PS1 and PS2) were observed. The absence of any significant other band indicated that under the conditions used, the SDS treatment neither lysed the cells, nor solubilized the cytoplasmic membrane. This was confirmed by freeze-fracture electron microscopy: only whole cells were found (data not shown). Therefore, it appears that PS1 and PS2 are parietal proteins which have an affinity for the cell wall and which are in equilibrium between the cell wall and the external medium. Most probably, the forms of PS1 and PS2 associated with the cell wall have been completely translocated across the cytoplasmic membrane. The following will focus only on PS1.

The SDS treatment extracts practically all cellular PS1. First, no further PS1 could be extracted by repeated treatment of the cell pellet with SDS. Second, when a total cell extract obtained from cells which had been pretreated with SDS was analyzed by SDS-PAGE and immunoblot, only a faint band associated with PS1 could be detected. In terms of intensity, it represented at most 10% of the intensity of a similar PS1 band observed on a cell extract obtained from cells which had not been submited to the SDS treatment (data not shown).

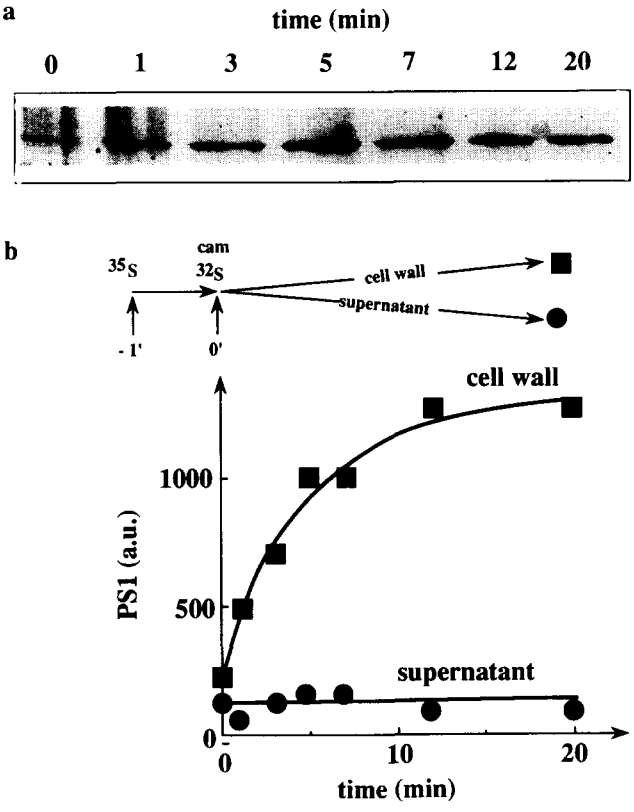

Fig. 2. Time course of the appearance of PS1 in the external medium and in the cell wall. Exponentially growing cells were pulsed with [^{35}S]methionine (37 TBq/mmol, 16 nM final concentration) for 1 min at 34°C, then chased by addition of [^{32}S]methionine (0.5 mM final concentration) in the presence of chloramphenicol (100 μg/ml). The incubation was continued at 34°C. Aliquots (1 ml) were withdrawn at a series of times, rapidly chilled and centrifuged at 0°C. The proteins of the supernatant were precipitated with trichloroacetic acid. The pellets were treated as described in Materials and Methods to extract cell wall PS1. The soluble extracts (cell wall PS1) and the supernatants (supernatant PS1) were submitted to SDS-PAGE and autoradiography. The amount of PS1 was determined by densitometry. (a) Autoradiography of cell wall PS1 as a function of time. (b) Quantification of PS1 (the units are arbitrary).

The data presented in Fig. 2 show that, under the experimental conditions used (1 min pulse, 20 to 30 min chase), the direct release of radioactive PS1 from the cell wall into the external medium is negligible. A growing bacterial culture was pulsed at 34°C for 1 min with [^{35}S]methionine and then chased with an excess of [^{32}S]methionine in the presence of 100 μg/ml chloramphenicol (Cam) in order to prevent any further protein synthesis. Aliquots were withdrawn after a series of times, rapidly frozen at −20°C and subsequently centrifuged at 0°C. The proteins of the supernatant were precipitated with trichloroacetic acid. The pellets were treated with SDS as described in Materials and Methods. Both supernatants and SDS extracts of the pellet were submitted to SDS-PAGE and autoradiography. The autoradiography of the SDS extracts is shown in Fig. 2a. The amount of PS1 directly released in the external medium (supernatant PS1) or released in the external medium following the SDS treatment of the pellet (cell wall PS1) was quantified by densitometry (Fig. 2b). The amount of PS1 directly released in the supernatant during a 20 min chase was negligible compared to the amount of PS1 released following the SDS treatment. Therefore, we analyzed the PS1 fraction released from the cell by SDS. We will refer to this fraction as 'secreted PS1'.

Under growing conditions at 34°C, a steady-state level of PS1 secretion was attained within 15 to 20 min after the beginning of the pulse with a half-time of secretion of approx. 3 min.

Collapse of $\Delta\tilde{\mu}_H$ prior to PS1 synthesis inhibits its secretion

At the growth temperature of 34°C, *Corynebacterium glutamicum* cells develop an electrochemical gradient of protons ($\Delta\tilde{\mu}_H$) across the membrane generated by the respiratory chain. We determined the values of the two components of $\Delta\tilde{\mu}_H$ ($\Delta\psi$ and ΔpH) as described in Materials and Methods. $\Delta\psi$ was 130 mV (negative inside the cell) and ΔpH was 0.5 unit (alkaline inside the cell) giving a total $\Delta\tilde{\mu}_H$ of 160 mV. $\Delta\tilde{\mu}_H$ can be decreased by the addition of the protonophore CCCP; $\Delta\tilde{\mu}_H$ collapses completely at a CCCP concentration of 20 μM.

The effect of the collapse of $\Delta\tilde{\mu}_H$ on PS1 secretion prior to its synthesis was investigated (Fig. 3). 20 μM CCCP were added to an exponentially growing cell suspension ($OD_{650} = 1$) at 34°C in order to collapse $\Delta\tilde{\mu}_H$. 5 min later, the cells were pulsed for 1 min with [^{35}S]methionine and then chased with an excess of [^{32}S]methionine in the presence of Cam (100 μg/ml). The cell suspension was further incubated for 15 min. PS1 secretion was negligible (Fig. 3, line b) as compared to a control which had been incubated in the absence of CCCP (Fig. 3, lane a). The absence of PS1 secretion was not the result of a decrease in the internal ATP concentration secondary to the collapse of $\Delta\tilde{\mu}_H$; it varied only from 3.9 mM to 3 mM upon a 45 min incubation with CCCP. Also, the absence of PS1 secretion was not the result of a large inhibition of protein synthesis during the pulse in the absence of $\Delta\tilde{\mu}_H$. Indeed, [^{35}S]methionine is taken up by the cells during the pulse and we could show that under these conditions the total radioactive proteins synthesized in the absence of $\Delta\tilde{\mu}_H$ amounts to some 65% of radioactive proteins synthesized during an equivalent pulse in the presence of $\Delta\tilde{\mu}_H$ (data not shown). PS1 synthesized during the pulse in the absence of $\Delta\tilde{\mu}_H$ and not secreted during a following chase in the absence of $\Delta\tilde{\mu}_H$ could be secreted post-translationaly upon reformation of $\Delta\tilde{\mu}_H$. This was demonstrated as follows. The cell suspension which had been pulsed in the presence of CCCP and further incubated 15 min in the presence

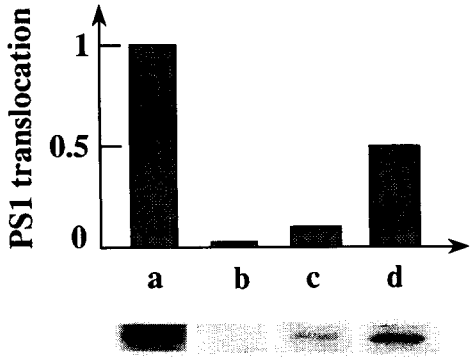

Fig. 3. Post-translational translocation of PS1. 3 ml of exponentially growing culture ($OD_{650} = 1$) at 34°C were treated for 5 min with 20 μM CCCP. The suspension was then pulsed for 1 min with [^{35}S]methionine (37 TBq/mmol, 16 nM final concentration) then chased with [^{32}S]methionine (final concentration: 0.5 mM) in the presence of Cam (100 μg/ml). The incubation was continued for 15 min at 34°C. 1 ml was withdrawn and treated as described in Materials and Methods in order to extract cell wall PS1 (a control was treated in the same way except that CCCP was omitted). The remaining suspension was divided into two parts which were centrifuged. The pellets were resuspended in 1 ml of the growth medium containing [^{32}S]methionine (final concentration: 0.5 mM) and Cam (100 μg/ml) at 34°C in the presence and in the absence of 20 μM CCCP, respectively. The incubation was continued for 45 min and cell wall PS1 was then extracted. The extracted material was submitted to SDS-PAGE and autoradiography. a, Control; b, cells incubated for 15 min with CCCP; c, cells incubated 15 min with CCCP, then centrifuged and incubated 45 min in the presence of CCCP; d, cells incubated 15 min with CCCP, then centrifuged and incubated 45 min in the absence of CCCP. The intensity of the bands were determined by densitometry and are shown as histograms in arbitrary units. Secretion in (d) corresponds to 50% of the control (a). Since total protein synthesis in the absence of $\Delta\tilde{\mu}_H$ represented 65% of that observed in the presence of $\Delta\tilde{\mu}_H$ (see text), the efficiency of secretion in (d) as compared to (a) is 75%.

of CCCP (Fig. 3, lane b) was divided into two aliquots which were centrifuged and resuspended, respectively, in a medium containing 20 μM CCCP and in a medium devoid of CCCP. All solutions contained Cam and an excess [^{32}S]methionine to prevent any synthesis of radiolabelled protein. Resuspension in the medium devoid of CCCP allowed the formation of a sizeable $\Delta\tilde{\mu}_H$ (120 mV). The aliquots were incubated for 45 min at 34°C. The amount of secreted protein was then determined (Fig. 3c and d). Labelled PS1 secretion was observed in the aliquot incubated in the absence of CCCP (lane d). The smaller secretion as compared to the control which has been pulsed and chased from the beginning in the presence of $\Delta\tilde{\mu}_H$ (lane a) could largely been explained by the smaller synthesis of PS1 in the absence of $\Delta\tilde{\mu}_H$ (see above). Little secretion of labelled PS1 was observed in the aliquot incubated in the presence of CCCP (lane c).

Maturation of PS1 is $\Delta\tilde{\mu}_H$ dependent

We have reported previously that PS1 can be expressed in *E. coli* and we have shown that the precursor is matured and translocated across the cytoplasmic membrane into the periplasm [35]. We could further show by immunoblots of cell extracts that the maturation of PS1 is dependent on $\Delta\tilde{\mu}_H$ [35]. We report here similar results for PS1 secretion in *C. glutamicum*.

Exponentially growing culture ($OD_{650} = 1$, 34°C) was cooled at 0°C and sonicated at that temperature (see legend of Fig. 4). It was then centrifuged at 16000 × *g* for 3 min to remove unbroken cells and debris. The supernatant (membrane and soluble components) was analyzed by SDS-PAGE and immunoblotting (Fig. 4, control). The immunoblot shows two bands: an intense band of lower molecular weight; a weaker one of higher molecular weight. A second experiment was performed on exponentially growing culture which had been pretreated with Cam (100 μg/ml) for 5 min before chilling, and sonication (Fig. 4: +Cam): only the lower molecular weight band was observed. In a third experiment, 20 μM CCCP were added 1 min before the addition of Cam (Fig. 4: +CCCP, + Cam): under these conditions the two bands were again observed.

The difference in molecular weight between the high- and low-molecular mass bands is approx. 5000 Da. The putative cleavage site between the signal-sequence and the mature part of the protein lies between residues 43 and 44 of the precursor [35]. Therefore, the two bands probably represent the precursor and mature forms of PS1, respectively. The disappearance of

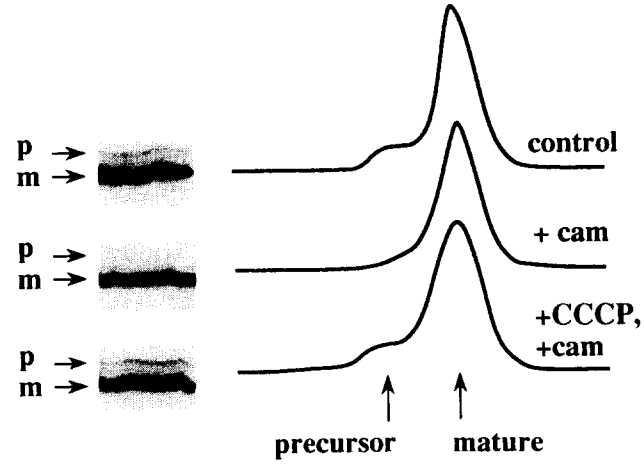

Fig. 4. Immunoblot of a cell extract. 3 ml of exponentially growing culture ($OD_{650} = 1$) were sonicated at 0°C in the presence of 20 μM CCCP. The total sonication time was 5 min. An integrated impulse timer allowed successive sonication and cooling periods of 0.5 s. The sonicated material was centrifuged at 16000 × *g* for 3 min. The supernatant containing the soluble material and the cytoplasmic membrane was submitted to SDS-PAGE and Western blot using anti-PS1 antibodies. a, Control; b, Cam (100 μg/ml) was added to the cell culture 5 min before sonication; c, CCCP (20 μM) was added to the cell culture 1 min before the addition of Cam (100 μg/ml) and the incubation continued for 5 min before sonication. The tracings represent the densitometry plots of the immunoblot. p, precursor form of PS1; m, mature form of PS1.

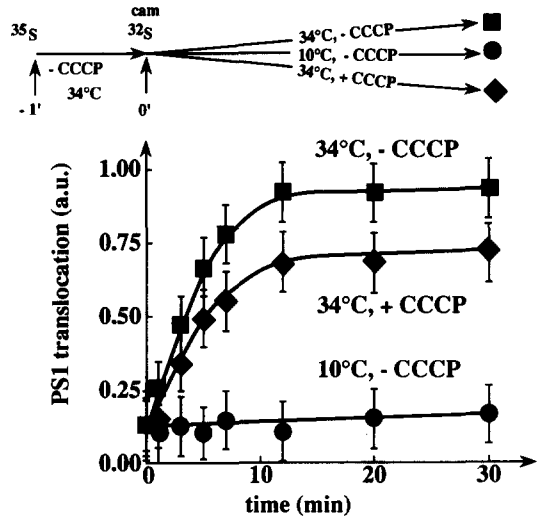

Fig. 5. Effect of $\Delta\tilde{\mu}_H$ and temperature on PS1 secretion. 30 ml of exponentially growing culture ($OD_{650} = 1$) at 34°C were pulsed with [^{35}S]methionine (37 TBq/mmol, 16 nM final concentration) for 1 min. During the pulse the suspension was divided into three samples. At the end of the pulse period [^{32}S]methionine (final concentration: 0.5 mM) and Cam (100 μg/ml) were added to the first sample and the incubation was continued at 34°C (34°C, −CCCP). To the second sample, [^{32}S]methionine (final concentration: 0.5 mM) and Cam (100 μg/ml) were added together with CCCP (20 μM) and the incubation continued at 34°C (34°C, +CCCP). To the third sample, [^{32}S]methionine (final concentration 0.5 mM) and Cam (100 μg/ml) were added while at the same time the suspension was rapidly cooled to 10°C; the incubation was then continued at 10°C (10°C, −CCCP). Aliquots (1 ml) were withdrawn at a series of times and cell wall PS1 was extracted. The extracted material was submitted to SDS-PAGE and autoradiography. The intensities of the bands were determined by densitometry and plotted as a fraction of the most intense band.

the precursor form upon arrest of protein synthesis is not the result of its degradation since it does not occur in the presence of CCCP. Thus, the data indicate that the precursor form can be transformed post-translationally into the mature form when protein synthesis is blocked. In addition, the data indicate that the maturation (i.e., a step involved in translocation) is inhibited when $\Delta\tilde{\mu}_H$ is dissipated by addition of CCCP.

Collapse of $\Delta\tilde{\mu}_H$ after PS1 synthesis has a minor effect on PS1 secretion

Although the collapse of $\Delta\tilde{\mu}_H$ blocked the maturation and the translocation of PS1, the addition of CCCP after a 1 min pulse with [^{35}S]methionine had only a minor effect on PS1 secretion. (Fig. 5). Exponentially growing culture at 34°C was pulsed with [^{35}S]methionine for 1 min followed by a chase with an excess [^{32}S]methionine in the presence of Cam (100 μg/ml) and CCCP (20 μM). Aliquots were then taken at a series of time and the amount of secreted PS1 was determined by SDS-PAGE and autoradiography (Fig. 5: 34°C, +CCCP). PS1 secretion was compared to that observed in a control experiment where the pulse was performed in the absence of CCCP (Fig. 5: 34°C, −CCCP). Each value is the mean of three independent experiments. Clearly, the collapse of CCCP shortly after PS1 synthesis only slightly inhibits the secretion of PS1.

Effect of temperature on PS1 secretion and relationship with the order-to-disorder transition of the lipids

Fig. 5 shows the time course of PS1 secretion at 34°C and also the time course of secretion of PS1 at 10°C. Exponentially growing cells (34°C) were pulse labelled with [^{35}S]methionine for 1 min. Cam (100 μg/ml) and an excess of [^{32}S]methionine were then added (time 0). The temperature of the cell suspension was then rapidly brought to 10°C and aliquots were taken at a series of times and secreted PS1 was detected by SDS-PAGE and autoradiography and quantified by densitometry (Fig. 5: 10°C, −CCCP). Clearly, little secretion took place at 10°C. The absence of secretion at low temperature was not the result of a decrease of $\Delta\tilde{\mu}_H$. Indeed, we have determined that at 10°C $\Delta\tilde{\mu}_H$ was still 140 mV (instead of 160 mV at 34°C) and that decreasing artificially at 34°C $\Delta\tilde{\mu}_H$ from 160 mV to 140 mV had little effect on PS1 secretion (data not shown). These data indicate that there is a temperature-dependent secretion step after the $\Delta\tilde{\mu}_H$-dependent step.

We determined the steady-state level of PS1 secretion as a function of temperature. Exponentially growing cells (34°C) were pulse labelled with [^{35}S]methionine for 1 min. Cam (100 μg/ml) and an excess of [^{32}S]methionine were then added (time 0). The temperature of the cell suspension was then rapidly brought to the desired temperature and the incubation was continued at that temperature for 30 min. PS1 secretion was determined by SDS-PAGE and autoradiography (Fig. 6a) and quantified by densitometry (Fig. 6b). Secretion of PS1 was clearly temperature-dependent: no secretion took place below 10°C; it increased rapidly above that temperature to a maximum at around 30°C.

The lipids of *Corynebacterium glutamicum* display an order-to-disorder transition of their hydrocarbon chains as a function of temperature. This was followed by changes in the fluorescence anisotropy of diphenylhexatriene (DPH). Membrane vesicles prepared from cells grown as described in Materials and Methods were labelled with DPH. The fluorescence anisotropy was determined as a function of temperature. (Fig. 6b). A transition is clearly apparent between 10°C and 25°C. At low temperatures the fluorescence anisotropy is high indicating limited movement of the probe and hence a viscous membrane (ordered lipids); at high temperatures the fluorescence anisotropy is low indicating a rapid movement of the probe, and hence a fluid membrane (disordered lipids).

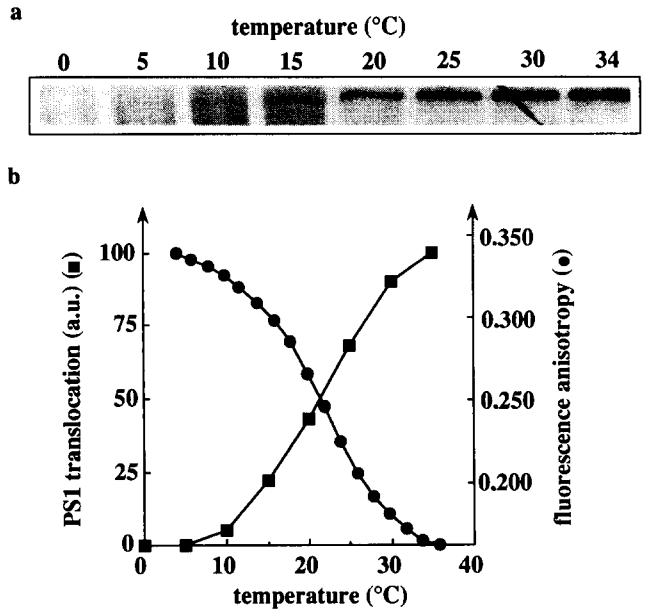

Fig. 6. PS1 secretion and order-to-disorder transition of the membrane lipids. PS1 secretion: 10 ml of exponentially growing culture (OD_{650} = 1) at 34°C were pulsed with [^{35}S]methionine (37 TBq/mmol, 16 nM final concentration) for 1 min. At the end of the pulse period [^{32}S]methionine (final concentration: 0.5 mM) and Cam (100 μg/ml) were added. 1-ml aliquots were withdrawn and rapidly cooled to the indicated temperature. The incubation was continued at that temperature for 30 min and cell wall PS1 was then extracted. The extracted material was submited to SDS-PAGE and autoradiography (a). The intensity of the bands were determined by densitometry (b: lefts axis) and are given in arbitrary units, a value of 100 being assigned at 34°C. Order-to-disorder transition: Membrane vesicles were prepared from whole cells and labelled with DPH as described in Materials and Methods. Fluorescence anisotropy was determined as a function of temperature from 5°C to 30°C (b: right axis).

The increased efficiency of PS1 secretion parallels the decrease in fluorescence anisotropy, and thus the increased fluidity of the membrane lipids.

Discussion

Corynebacterium glutamicum naturally secretes a protein designated PS1 into the external medium. Actually, PS1 is a parietal protein associated with the cell wall from which it can be dissociated by treatment with SDS under conditions which do not solubilize any cytoplasmic membrane protein or lyse the cell.

PS1 is synthesized in a precursor form. The analysis of the amino-acid sequence of the protein deduced from its nucleotide sequence reveals an amino-terminal signal-sequence with characteristics similar to those of Gram-positive bacteria and a putative cleavage site between residues 43 and 44 [35].

The classical way to investigate in vivo translocation across the cytoplasmic membrane of a protein carrying a signal-sequence is to analyze the conversion of the precursor form into the mature form. We have determined in this way the role of $\Delta\tilde{\mu}_H$. At optimal growth temperature (34°C) and at pH 7, the cells develop a $\Delta\tilde{\mu}_H$ of 160 mV across the cytoplasmic membrane composed of a $\Delta\psi$ of 130 mV (negative inside the cell) and a ΔpH of 0.5 (alkaline inside the cell). We could show by immunoblots of cell extracts that the maturation (i.e., a step involving the translocation across the cytoplasmic membrane) of PS1 is $\Delta\tilde{\mu}_H$-dependent (see in Fig. 4 the effect of the addition of CCCP). We could confirm the immunoblot experiments by the pulse chase experiments described in Fig. 3. The collapse of $\Delta\tilde{\mu}_H$ prior to a pulse with [^{35}S]methionine blocks PS1 secretion. Since $\Delta\tilde{\mu}_H$ is across the cytoplasmic membrane, it is reasonable to assume that its collapse prior to the pulse affects a translocation step. In addition, we show that the regeneration of $\Delta\tilde{\mu}_H$ in the absence of de novo synthesis allows the secretion of previously synthesized PS1 indicating that a post-translational translocation is possible. These results are similar to those reported for protein secretion in some other Gram-positive bacteria [32–34] and also to those reported for the in vivo translocation of periplasmic proteins in *E. coli* [16–18].

We were not able to follow the time course of the conversion of the precursor form into the mature form. Indeed, *Corynebacterium glutamicum* has a thick cell wall. As a consequence, lysis needed in order to gain access to the non-translocated precursor form requires harsh treatment (prolonged incubation at high temperature with lysozyme, prolonged sonication) and complete and reproductive lysis is difficult to attain. This precludes a more quantitative and comparative analysis of cytoplasmic and membrane proteins (precursor form of PS1, non-translocated mature form of PS1) present in the cell under various experimental conditions. We were therefore led to follow the translocation by the appearance of PS1 in the cell wall and its extraction by SDS.

Under normal conditions (34°C, presence of $\Delta\tilde{\mu}_H$), the half-time of PS1 secretion is 2–3 min with a steady-state level of PS1 secretion attained within 15 min. These characteristics of the time course of PS1 secretion would reflect those of PS1 translocation across the cytoplasmic membrane if the translocated PS1 is immediately located in a region of the cell wall from which it can be extracted by SDS. While most of PS1 is extracted by the SDS treatment (a second treatment with SDS does not extract any further PS1), it cannot be excluded that PS1 which has just been translocated across the cytoplasmic membrane is located in a region close to the cytoplasmic membrane inaccessible to SDS. Therefore, it might be that the time course of secretion reflects, at least partially, the time course of diffusion of PS1 in the cell wall from a SDS-inaccessible to a SDS-accessible location.

We show that the collapse of $\Delta\tilde{\mu}_H$ immediately

after a 1 min pulse has only a minor effect on the time course of PS1 secretion (see Fig. 5). At the end of a 1 min pulse, PS1 secretion of the control is still negligible (see time point 0 in the control experiment in Fig. 5). Thus, during the pulse period most of the radiolabelled PS1 underwent the $\Delta\tilde{\mu}_H$ dependent step and later steps are largely independent of $\Delta\tilde{\mu}_H$. These later steps may be $\Delta\tilde{\mu}_H$ independent steps involved in the translocation across the membrane, if the translocation and not the diffusion in the cell wall is the rate limiting step in secretion. This result would be at variance with those reported for the in vivo and the in vitro translocation of periplasmic proteins across the cytoplasmic membrane of *E. coli*. There it was shown that the early steps, including maturation of the precursor, are independent on $\Delta\tilde{\mu}_H$ while later steps, possibly the translocation *per se* of the polypeptide chain across the translocator (secY/secE), are dependent on $\Delta\tilde{\mu}_H$ [43–46]. Alternatively, if the translocation is not the rate limiting step in secretion, complete translocation might be practically accomplished at the end of the pulse period and the similarity of the secretion in the presence and absence of $\Delta\tilde{\mu}_H$ during the pulse would reflect the absence of an effect of $\Delta\tilde{\mu}_H$ on the diffusion of PS1 in the cell wall from the SDS inaccessible to the SDS accessible location.

The temperature experiments which we report in Figs. 5 and 6 and which relate the efficiency of PS1 secretion and the order-disorder transition of the cytoplasmic membrane lipids favor the former possibility. In has been clearly demonstrated that the nature of the lipids greatly affects in *E. coli* the translocation of proteins across the cytoplasmic membrane [47], while numerous membrane functions, in particular transport, depend on the 'fluidity' of the hydrocarbon chains of the lipids [48]. We show that the temperature greatly affects the secretion of PS1, no secretion being observed when the temperature is decreased to 10°C at the end of a 1 min pulse period (Fig. 5). There is a strong parallel between the efficiency of secretion and the fluidity of the membrane (ordered or disordered state of the lipids). Therefore, it would appear that the temperature dependent (and thus the $\Delta\tilde{\mu}_H$ independent) late step is associated with the cytoplasmic membrane and thus with the translocation rather than with the diffusion within the cell wall. The effect may be direct if the protein passes the membrane through the lipid bilayer, as it has been proposed for at least one protein in *B. subtilis* [49]; it may be indirect if the protein crosses the membrane via a protein translocator, the structure of the lipids modulating the activity of the translocator.

The results presented here should be substantiated by in vitro translocation studies. However, at this stage no in vitro translocation system has been obtained for Gram-positive bacteria.

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