A cell-free protein translocation system prepared entirely from a Gram-positive organism

Karl-Ludwig Schimz[a,*], Gaby Decker[a], Elke Frings[a], Jochen Meens[a], Michael Klein[a], Matthias Müller[b]

[a]*Institut für Biotechnologie, Forschungszentrum Jülich GmbH, D-52425 Jülich, Germany*
[b]*Institut für Physikalische Biochemie, Ludwig-Maximilians-Universität München, Schillerstr. 44, D-80336 München, Germany*

Received 22 December 1994; revised version 15 February 1995

Abstract A cell-free protein translocation system derived exclusively from a Gram-positive bacterium is described here for the first time. Highly efficient in vitro synthesis of plasmid encoded preprolipase of *Staphylococcus hyicus* is accomplished by coupled transcription/translation using either a cytosolic extract of *S. carnosus* alone or in combination with T7-RNA-polymerase. Addition of inside-out cytoplasmic membrane vesicles of *S. carnosus* leads to the partial conversion (processing) of preprolipase to prolipase. In addition, as shown in a protease protection assay, a significant part of preprolipase plus prolipase is translocated in vitro into the lumen of the vesicles. Translocation of preprolipase into the membrane vesicles requires the proton-motive force and the *S. carnosus* SecA protein.

Key words: Protein secretion; Gram-positive bacteria; Cell-free translocation system; SecA; Lipase; *Staphylococcus carnosus*

1. Introduction

By use of genetic as well as biochemical methods, protein translocation across the cytoplasmic membrane of the Gram-negative bacterium *Escherichia coli* has been investigated intensively [1–4]. A number of *sec* genes and their products (SecA, B, D, E, F, G, Y), which are involved in protein translocation, have thus been identified and characterized. The introduction of in vitro translocation systems prepared either from crude subcellular fractions [5,6] or reconstituted from purified Sec proteins [7,8] have rendered possible detailed investigations on the molecular mechanisms of protein translocation in this organism (most recent examples are: [9–11]). In addition to the Sec proteins, the existence of an SRP (signal recognition particle)-dependent pathway of protein export in *E. coli* has recently been suggested (reviewed in [12]).

Despite their importance in industrial enzyme production [13], investigations on protein translocation in Gram-positive bacteria have only recently led to the discovery of Sec and SRP homologues [14–22] of which SecA [23] and SecY [24] were shown to be essential for growth and protein translocation in *B. subtilis*. These findings suggest that only minor variations exist between the molecular mechanisms of protein translocation across the cytoplasmic membranes of Gram-negative and Gram-positive organisms.

However, as pointed out by Simonen and Palva [25] "the fact that no in vitro translocation assay is available for *Bacillus* species has hampered the characterization of its secretion components". In this report, we describe the first in vitro protein translocation system of a Gram-positive bacterium. In vitro synthesis [26,27] and signal sequence cleavage [28,29] by membrane-free extracts of Gram-positive bacteria have been reported previously.

2. Materials and methods

2.1. Plasmids

Plasmid pLipPS1 contains the preprolipase gene of *S. hyicus* on a *Pst*I fragment [30]. Plasmid pCULP was constructed (B. Freundlieb, unpublished) by inserting this fragment into pCU1 [31]. A promoter-free *lip* gene was isolated from pEFO [32] by cleavage with *Pst*I and ligated into pGEM3z (Promega, Madison, WI) so that its expression became dependent on the T7 RNA polymerase promoter (plasmid pCS1).

To obtain plasmid pCS2 encoding an uncleavable ppL a *Bam*HI–*Sal*I fragment of pJM1 [32] corresponding to the 222 NH_2-terminal amino acids of ppL was ligated into double stranded DNA of phage M13mp18. The uracil-containing single stranded template DNA of this phage was used in a mutagenesis reaction with the mismatch primer K1 (5′-TTGTGTTGTCGAATCGTTAACCTCTGCCACGCCCC-3′). DNA from the resulting phage clones was screened for the presence of the desired mutation by sequencing [33]. The *Bam*HI–*Sal*I fragment isolted from double stranded phage DNA was ligated into pJM1 from which the wild-type fragment had been removed. From the resulting plasmid pJM1.1 the *lip* allele (encoding a modified signal sequence cleavage site, −1 Ala to −1 Val) was isolated on a *Bam*HI–*Hin*dIII fragment which was finally ligated into pGEM3z to give plasmid pCS2. Construction of pJM10 encoding *lip* under the *lac* promoter has been described elsewhere [32]. Plasmid DNA was isolated according to published procedures from *E. coli* and *B. subtilis* [34] and *S. carnosus* [35].

2.2. Preparation of an extract from S. carnosus for cell-free transcription/translation

A protocol previously described for *E. coli* [5] was modified as follows. *S. carnosus* strain TM300 [36] grown overnight in Brain-Heart-Infusion medium (Difco) at 30°C was used to inoculate 1 l batches (15 ml/l) which were grown up to an A_{600} of about 1. Cells were rapidly cooled by pumping the suspension through a copper coil immersed in an ice-water bath and then harvested for 15 min at $4{,}400 \times g_{max}$ and 5°C. The cell pellet was resupended in 10 mM TeaOAc pH 8.0, 14 mM $Mg(OAc)_2$, 60 mM KCl, 1 mM DTT (1 g wet weight plus 1.5–2 ml) and cells were broken by 5 passages at 8,000 psi followed by 5 at 16,000 psi through a French Pressure Cell (SLM-Aminco, Urbana/IL) which was kept at 4°C by intermediate coolings. An S-30 was prepared by centrif-

*Corresponding author. Fax: (49) (2461) 612 710.

Abbreviations: lip, *S. hyicus* lipase encoding gene; ppL, preprolipase; pL, prolipase; mL, mature lipase; INV, inside-out cytoplasmic membrane vesicles; DCCD, dicyclohexyl carbodiimide; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenyl-hydrazone; IPTG, isopropyl-1-thio-β-D-galactoside; TeaOAc, triethanolamine acetate; PVDF, polyvinylidene difluoride; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate.

Results of this work were presented in part at the Fall Meeting of the Gesellschaft für Biologische Chemie, Würzburg/Germany, September 19–21, 1994 (Biol. Chem. Hoppe-Seyler, 375 (1994) Supplement 1, p. 88).

SSDI 0014-5793(95)00180-8

ugation for 30 min at 30,000 × g_{max}, 5°C, and subjected to a read-out of the endogenous mRNAs as described [37] followed by dialysis against 1 l batches of S-30 buffer in which KCl had been replaced by KOAc (three times for 1 h each at 5°C). An S-200 was prepared by loading the tubes of rotor A110 of the Beckman Airfuge with 175 μl each, spinning for 5 min at 30 psi (199,000 × g_{max}), and carefully removing 130 μl supernatant from the top. Aliquots of S-30 and S-200 were frozen in liquid nitrogen and stored at −70°C. The S-200 was further freed of small molecules by repeated concentrations and dilutions in dialysis buffer using Centricon 10 microconcentrators (Amicon).

2.3. Inside-out cytoplasmic membrane vesicles from S. carnosus

Gradient-purified inner membrane vesicles were prepared as described [5] with the following modifications. *S. carnosus* strain TM300 was grown to an A_{600} of about 3.5 as detailed above (equally active INV, however, were obtained when cells were harvested at A_{600} ranging from 0.9 to 4.5). Phenylmethylsulfonyl fluoride did not proof to be necessary and was therefore omitted. Breakage was performed at 4°C by 4 passages through the French Press at 8,000 psi followed by 3 at 16,000 psi. INV were finally resuspended to a concentration of 40 A_{280}/ml. Repeated freezing and thawing on ice did not affect the translocation activity of the INV.

2.4. In vitro synthesis

Reactions were performed in 25 μl aliquots containing (in the order of addition): 3.2% (w/v) polyethylene glycol 6000–8000, 40 μM each of 19 amino acids without methionine, 2.5 mM ATP, 0.5 mM each of GTP, CTP, UTP, 16 mM KOH, 2 mM DTT, 5 mM phosphoenol pyruvate, 40 mM TeaOAc, pH 7.5, 200 mM KOAc, pH 7.5, 8.5 mM $Mg(OAc)_2$, pH 7.5, 20 mM NH_4OAc, pH 7.5, 0.8 mM spermidine, 8 mM creatine phosphate, 1 μg creatine phosphokinase, 8 mM putrescine, 20 units placental RNAse inhibitor, 0.15 μg plasmid (pCS1 or pCS2) or 2 μg plasmid (pCULP) DNA, 10 μCi [^{35}S]methionine (1,000 Ci/mMol). Reactions were started by adding 3 μl S-200 and (when pCS1 or pCS2 was used) 20 units T7 RNA polymerase and incubated at 30°C for 120 min. They were stopped with 5% (w/v) TCA. To obtain maximal translocation of ppL, INV were added after 60 min (cotranslationally). In posttranslational assays, INV were added following termination of synthesis after 120 min incubation with 185 μg/ml chloramphenicol (or 185 μM of neutralized puromycin). In these cases, ATP; creatine phosphate, creatine phosphokinase, and DTT were added to the original concentrations in order to compensate for potential consumptions during the previous incubation.

2.5. Partial purification of SecA from S. carnosus

SecA from *S. carnosus* was overexpressed in *E. coli* strain JM109 containing plasmid pMA16 (Klein, M., unpublished results) after induction with 2 mM IPTG for 4 h. After cell disruption in the French Press, SecA-containing inclusion bodies were solubilized with 6 M urea dissolved in buffer A [5] and fractionated by size exclusion chromatography at 15°C using a Superdex 26/60 column (Pharmacia). Fractions enriched in SecA, as detected by Western blot analysis, were pooled and precipitated with $(NH_4)_2SO_4$ (80% saturation according to Green and Hughes [38]) to remove the urea. The precipitate was resuspended in buffer A saturated to 80% with $(NH_4)_2SO_4$ and then successively dialyzed against buffer A saturated with 80%, 40%, 20% $(NH_4)_2SO_4$ for 5 h each. In the final step $(NH_4)_2SO_4$ was omitted. SecA thus renatured in the absence of urea was separated from aggregated material by centrifugation.

2.6. Purification of preprolipase and prolipase

S. hyicus preprolipase accumulates in cells of *B. subtilis* strain DB104 when expression from plasmid pJM10 [32] is induced with IPTG (E. Frings and R. Freudl, unpublished). Cells resuspended in SDS-PAGE loading buffer containing 6 M urea were broken by sonication and the cell extract was subjected to preparative SDS-PAGE. Preprolipase was identified by an activity overlay assay [39] and either electroeluted or extracted by homogenization of the gel matrix. *S. hyicus* prolipase which is secreted from strains of *S. carnosus* without further processing [40] was collected from 8 l of culture filtrate of *S. carnosus* carrying pLipPS1 by adsorption to 200 ml of Fractogel TSK-butyl-650(M) (Merck AG, Darmstadt). After washing with 5 l of 50 mM Tris-HCl, pH 8, bound prolipase was eluted with 200 ml 2% (w/v) SDS in 50 mM Tris-HCl, pH 8, and precipitated with 80% $(NH_4)_2SO_4$. The precipitate was collected by filtration, dissolved in the Tris-glycine buffer used for SDS-PAGE, and further purified by preparative SDS-PAGE.

2.7. Miscellaneous

Published procedures were used with minor modifications for SDS-PAGE [41], fluorography [42], and quantitation of the radioactivity present in individual bands of SDS gels [43].

3. Results and discussion

In cultures of *S. hyicus*, a prolipase (pL) is secreted into the medium followed by conversion to the mature lipase (mL) by removal of the propeptide [44,45]. Prolipase is formed from an intracellular precursor, preprolipase (ppL), as predicted by DNA sequence analysis of the lipase gene [30].

3.1. In vitro synthesis of preprolipase

Following a previously described protocol of a cell-free transcription/translation system from *E. coli* [5] a membrane-free, high speed supernatant (S-200) was prepared from *S. carnosus* and used as cytosolic extract for the in vitro synthesis of ppL. In order to achieve highly efficient incorporation of [^{35}S]methionine into newly synthesized proteins, the S-200 was first completely freed of its endogenous amino acids (as verified by HPLC analysis) by several cycles of dilution and ultrafiltration. When the resulting S-200 of *S. carnosus* was programmed with plasmid pCS1, containing the *S. hyicus* lipase gene under the control of the phage T7 promoter, the time-dependent appearance of a 91 kDa-protein was observed (Fig. 1; note that the X-ray film was exposed for 1.5 h only).

The 91 kDa-polypeptide was also obtained when the lipase gene was expressed from its homologous promoter (plasmid pCULP) using the endogenous RNA polymerase of the *S. carnosus* S-200, but was absent from reactions which had been directed by the vector plasmid alone (not shown). It was identified as ppL by immunoprecipitation with antibodies raised against pL of *S. hyicus* (Fig. 2C, lane 18). Furthermore, the in vitro synthesized 91-kDa protein co-migrated on gels of different acrylamide concentrations with ppL purified from an overexpressing recombinant strain of *B. subtilis* (not shown).

3.2. In vitro translocation of preprolipase into membrane vesicles of S. carnosus

Inside-out plasma membrane vesicles (INV) were prepared from *S. carnosus* by repeated passages of a cell suspension through a French pressure cell followed by sucrose density centrifugation. In order to visualize translocation of in vitro

Table 1
Effect on the translocation efficiency of SecA depletion from the S-200 by immunoaffinity chromatography and its readdition

	% Translocation efficiency	
	Without addition of 6 μg SecA/assay	With
S-200 (control)	3.2	6.7
S-200 depleted	2.1	9.5

Radioactivity of the excised bands (ppL and pL) was determined by liquid scintillation counting. The dpm values were corrected with respect to the different amounts of [^{35}S]methionine being incorporated into ppL (n = 11) and pL (n = 9). Translocation efficiency (%) = $[(ppL + pL)_{\text{after proteinase K}} / (ppL + pL)_{\text{before proteinase K}}] \times 100$.

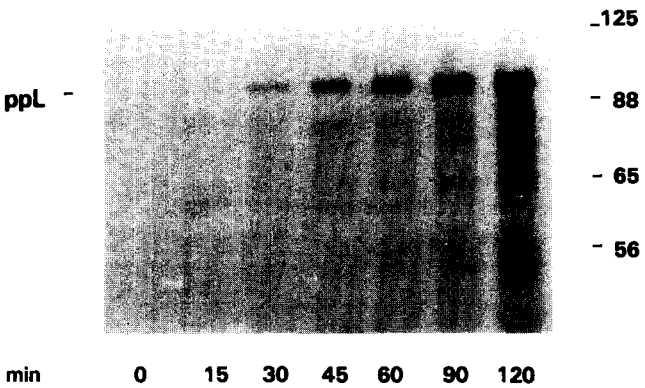

Fig. 1. Time-dependent cell-free synthesis of [^{35}S]methionine-labeled preprolipase (ppL) by an extract (S-200) from *S. carnosus*. Translation products were precipitated with TCA (final concentration 5% w/v), separated by SDS-PAGE, and visualized by fluorography. Masses of prestained standards (SDS7B, Sigma) are given in kDa on the right.

synthesized ppL into INV protease protection experiments were performed (Fig. 2A). In the absence of INV the majority of the translation products were digested by proteinase K leaving a single resistant species of 46 kDa (lane 2), whereas this material was not detected in the absence of proteinase K. Since mL has the same electrophoretic mobility as the 46-kDa species, and is an intrinsically protease-resistant protein [40,46], the 46-kDa protein obtained by digestion with proteinase K (as well as by trypsin and pronase E, not shown), most probably is the result of a cleavage of ppL around the propeptide cleavage site as described also by others [40].

When INV were present during synthesis of ppL two additional proteinase K-resistant peptides appeared in proportion to the amount of INV added (Fig. 2A, lanes 3–8). These peptides co-migrate with ppL (91 kDa) and a protein of 86 kDa. The latter was not observed when a mutated form of ppL (Fig. 2B) was synthesized in which the signal sequence cleavage site was rendered non-functional by changing Ala at the −1 position to Val. The 86-kDa proteinase K-resistant protein therefore must be prolipase generated by the signal peptidase of the INV. This is further demonstrated by its co-migration on SDS-PAGE with prolipase purified from a culture filtrate of *S. carnosus* (Fig. 2C, lanes 18 and 19). Since ppL and pL became accessible to proteolytic digestion by disruption of the membrane vesicles with Triton X-100 (Fig. 2A, lane 11), both species must represent material translocated into the lumen of the vesicles.

In samples not treated with proteinase K (such as on lanes 3,5,7 of Fig. 2A), the formation of pL from ppL by INV could also be seen provided that the X-ray film was exposed for a much shorter time (2.5 h instead of 25 h as was done in Fig. 2A,B,D). Quantification of the extent of pL formed in these cases revealed a processing rate of about 20%. In contrast, the average efficiency of translocation, i.e. the relative amount of protease-resistant ppL and pL, turned out to be 5–7%. It remains to be analyzed why only a fraction of the prolipase that underwent proteolytic processing by the INV-borne signal peptidase became fully translocated, i.e. protease-resistant. Similar situations have been described for in vitro systems of *E. coli* under limitation of ATP, $\Delta\mu_{H^+}$, and SecA [47,48]. It is possible that one of these translocation parameters is not optimally balanced in the cell-free translocation system described here (see below). Alternatively, we cannot rule out the possibility that a portion of the vesicles used have a right side-out orientation allowing for a proteolytic processing of ppL without concomitant translocation.

Translocation of ppL and pL into the lumen of the INV of *S. carnosus* was dependent on the energization of the vesicles. Translocation decreased considerably when 1 mM DCCD was included in the cell-free reaction to inhibit the H^+-translocating ATPase of the INV (Fig. 2D, lane 23,24), while addition of the protonophore FCCP completely blocked translocation at 0.1 mM (Fig. 2D, lane 25,26). We conclude that in vitro translocation of ppL across the plasma membrane of *S. carnosus* requires the H^+-motive force as shown in vivo by others for α-amylase secretion of *B. amyloliquefaciens* [49].

Processing of ppL to pL – but not its translocation – was also achieved by *S. carnosus* membranes disrupted with 1% (v/v) Triton X-100 suggesting that the *S. carnosus* signal peptidase was solubilized under these conditions (not shown).

3.3. *SecA-dependent in vitro translocation of ppL into INV of* S. carnosus

Polyclonal antibodies raised against the *B. subtilis* SecA strongly cross-react with SecA from *S. carnosus* (Klein, M., unpublished results). A fraction of immunoglobulins obtained by chromatography on DEAE-cellulose was covalently coupled to Protein A-Sepharose as described [9,50] and used to deprive the *S. carnosus* S-200 of its endogenous SecA. Depletion was almost complete when examined by immunoblotting (not shown). To restore the SecA content of the depleted S-200, SecA of *S. carnosus* was partially purified. The result of one typical experiment is depicted in Table 1. The efficiency of translocation of ppL into INV decreased by about one third after removal of the soluble SecA from the S-200. The residual translocation activity is most likely due to the INV-bound SecA (verified by immunoblotting) which in *E. coli* amounts to about one half of the total cellular SecA [51]. In comparable experiments performed with an in vitro system of *E. coli* [48], the mere removal of soluble SecA also did not greatly impair translocation of particular precursor proteins as long as the membrane-bound fraction was not reduced. Importantly, as shown in Table 1, SecA purified from *S. carnosus* stimulated translocation of ppL into INV both in conditions where soluble SecA was present (control) as well as after its removal. On the one hand, this is an indication for the limitation of SecA in our in vitro system explaining in part the rather low efficiency of ppL translocation (see above). On the other hand, the results clearly demonstrate that translocation in this novel cell-free system of a Gram-positive organism is a SecA-dependent process.

We believe that in view of the rather low translocation ability of the INV prepared from *S. carnosus* a major clue towards establishing a cell-free synthesis/translocation system was the optimization of the in vitro synthesis. This was achieved by the careful deprivation of the S-200 of its endogenous methionine (see above) and/or of unknown factors of masses smaller than 10 kDa that might be inhibitory to the cell-free synthesis reaction. Crucial was further the finding that a reaction temperature of 30°C was by far superior to 37°C (and 25°C), despite the fact that the organism grows considerably faster at 37°C. Furthermore, using ppL as precursor turned out to be of advantage since an equally efficient translocation into INV added

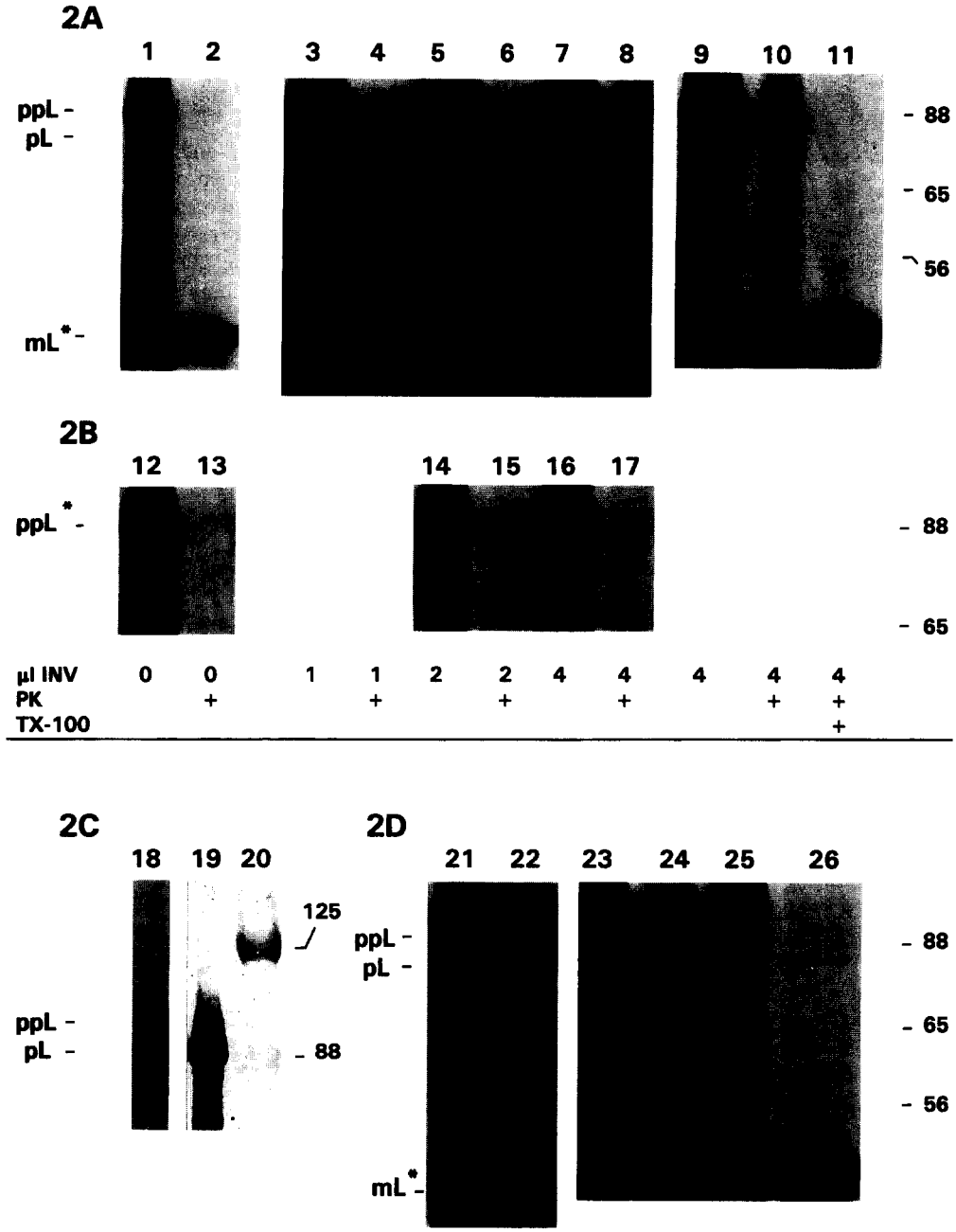

Fig. 2. Translocation of in vitro synthesized preprolipase (ppL) into inside-out plasma membrane vesicles (INV) from *S. carnosus*. Panel A: translocation and processing of ppL to prolipase (pL) is dependent on the concentration of INV and the intactness of the INV. ppL was synthesized as described in the previous figure in the absence or presence of the indicated amounts of INV. Reactions were subdivided into halves and either treated immediately with TCA or incubated at 25°C for 30 min with 250 µg/ml proteinase K (PK) added from a 1 mg/ml stock solution prepared in 5 mM $CaCl_2$. Lane 11, treatment with PK was performed in the presence of 1% (v/v) Triton X-100 (TX-100). mL*, is a non-translocated, protease-resistant breakdown product of ppL and pL of similar size as mature lipase. Panel B: processing of ppL to pL by INV does not occur with a mutant form of ppL (ppL*) replacing Ala at position −1 by Val. Panel C: cross-reactivity of in vitro synthesized ppL and pL with antibodies raised against purified pL. ppL and pL were obtained by in vitro synthesis in the presence of INV. Translation products were immunoprecipitated [43] with anti-pL-antibodies, the cross-reactive proteins were mixed with unlabeled, purified pL, separated by SDS-PAGE and blotted onto a PVDF membrane (according to Applied Biosystems, User Bulletin 36). The membrane was developed with anti-pL-antibodies and alkaline phosphatase-conjugated second antibodies to visualize unlabeled pL (lane 19; lane 20 contains prestained marker proteins). The immunoblot was subsequently autoradiographed to detect the labeled species of lipase (lane 18). Panel D: translocation of ppL is dependent on energized membrane vesicles. Inhibitors dissolved in DMSO were present during in vitro synthesis at final concentrations of 1 mM (DCCD; lanes 23, 24) and 0.1 mM (FCCP; lanes 25, 26). The control samples (lanes 21 and 22) contained an equivalent amount (0.5 µl) of DMSO. Proteinase K treatment was performed on samples of lanes 22, 24, and 26. Quantification of the mL*-band of lanes 22, 24, and 26 revealed that the same amount of material had been loaded onto each lane indicating that the decrease of protected ppL and pL species was in fact the result of inhibited translocation.

co- or posttranslationally suggests an inherently high translocation competence of this protein. This might be due to the presence of the propeptide which in the case of subtilisin has been found to function as an intramolecular chaperone [52,53].

Acknowledgements: We thank Prof. H. Sahm for his interest, continuous support, and for critical discussions. We are grateful to Prof. F. Götz, B. Freundlieb, S. Tschauder, M. Herbort, M. Klose, and R. Freudl for helpful support. Competent technical assistance of K. Stollberg during part of this work is gratefully acknowledged. Thanks are due to L. Eggeling for his help with HPLC analysis of amino acids and J. MacFarlane for a critical reading. This work was supported in part by a grant from the Bundesministerium für Forschung und Technologie, the Sonderforschungsbereich 184, and the Fonds der Chemischen Industrie.

References

[1] Schatz, P.J. and Beckwith, J. (1990) Annu. Rev. Genet. 24, 215–248.
[2] Wickner, W.T., Driessen, A.J.M. and Hartl, F.U. (1991) Annu. Rev. Biochem. 60, 101–124.
[3] Pugsley, A.P. (1993) Microbiol. Rev. 57, 50–108.
[4] Müller, M., and MacFarlane, J. (1994) in: Subcellular Biochemistry, Vol. 22, Membrane Biogenesis (Maddy, A.H. and Harris, J.R. Eds.) pp. 327–359, Plenum Press, New York.
[5] Müller, M. and Blobel, G. (1984) Proc. Natl. Acad. Sci. USA 81, 7421–7425.
[6] Rhoads, D.B., Tai, P.C. and Davis, B.D. (1984) J. Bacteriol. 159, 63–70.
[7] Brundage, L., Hendrick, J.P., Schiebel, E., Driessen, A.J.M. and Wickner, W. (1990) Cell 62, 649–657.
[8] Akimaru, J., Matsuyama, S.J., Tokuda, H. and Mizushima, S. (1991) Proc. Natl. Acad. Sci. USA 88, 6545–6549.
[9] Hoffschulte, H.K., Drees, B. and Müller, M. (1994) J. Biol. Chem. 269, 12833–12839.
[10] Arkowitz, R.A. and Wickner, W. (1994) EMBO J. 13, 954–963.
[11] Hanada, M., Nishiyama, K.-I., Mizushima, S. and Tokuda, H. (1994) J. Biol. Chem. 269, 23625–23631.
[12] Luirink, J. and Dobberstein, B. (1994) Mol. Microbiol. 11, 9–13.
[13] Priest, F.G. (1977) Bacteriol. Rev. 41, 711–753.
[14] Suh, J.W., Boylan, S.A., Thomas, S.M., Dolan, K.M. Oliver, D.B. and Price, C.W. (1990) Mol. Microbiol. 4, 305–314.
[15] Nakamura, K., Nakamura, A., Takamatsu, H., Yoshikawa, H. and Yamane, K. (1990) J. Biochem. 107, 603–607.
[16] Tschauder, S., Driessen, A.J.M. and Freudl, R. (1992) Mol. Gen. Genet. 135, 147–152.
[17] Sadaie, Y., Takamatsu, H., Nakamura, K. and Yamane, K. (1991) Gene 98, 101–105.
[18] Overhoff, B., Klein, M., Spies, M. and Freudl, R. (1991) Mol. Gen. Genet. 228, 417–423.
[19] Jeong, S.M., Yoshikawa, H. and Takahashi, H. (1993) Mol. Microbiol. 10, 133–142.
[20] Meens, J., Klose, M. and Freudl, R. (1994) FEMS Microbiol. Lett. 117, 113–120.
[21] van Dijl, J.M., de Jong, A., Vehmaanperä, J., Venema, G. and Bron, S. (1992) EMBO J. 11, 2819–2828.
[22] Honda, K., Nakamura, K., Nishiguchi, M. and Yamane, K. (1993) J. Bacteriol. 175, 4885–4895.
[23] Takamatsu, H., Fuma, S.-J., Nakamura, K., Sadaie, Y., Matsuyama, S.-J., Mizushima, S., and Yamane, K. (1992) J. Bacteriol. 174, 4308–4316.
[24] Breitling, R., Schlott, B. and Behnke, D. (1994) J. Basic Microbiol. 34, 145–155.
[25] Simonen, M. and Palva, I. (1993) Microbiol. Rev. 57, 109–137.
[26] Roberts, M.W. and Rabinowitz, J.C. (1989) J. Biol. Chem. 264, 2228–2235.
[27] Mahmood, R., Compagnone-Post, P. and Khan, S.A. (1991) Gene 106, 29–34.
[28] Wong, S.-L. and Doi, R.H. (1986) J. Biol. Chem. 261, 10167–10181.
[29] Vehmaanperä, J., Görner, A., Venema, G., Bron, S. and van Dijl, J.M. (1993) FEMS Microbiol. Lett. 114, 207–214.
[30] Götz, F., Popp, F., Korn, E. and Schleifer, K.H. (1985) Nucl. Acids Res. 13, 5895–5906.
[31] Augustin, J., Rosenstein, R., Wieland, B., Schneider, U., Schnell, N., Engelke, G., Entian, K.D. and Götz, F. (1992) Eur. J. Biochem. 204, 1149–1154.
[32] Meens, J., Frings, E., Klose, M. and Freudl, R. (1993) Mol. Microbiol. 9, 847–855.
[33] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[34] Birnboim, H.C. and Doly, J. (1979) Nucl. Acids Res. 7, 1513–1523.
[35] Götz, F., Zabielski, J., Philipson, L. and Lindberg, M. (1983) Plasmid 9, 126–137.
[36] Schleifer, K.H. and Fischer, U. (1982) Int. J. System. Bacteriol. 32, 153–156.
[37] Zubay, G. (1973) Annu. Rev. Genet. 7, 267–287.
[38] Green, A.A. and Hughes, W.L. (1955) in: Methods in Enzymology (Colowick, S.P. and Kaplan, N.O. Eds.) Vol. I, pp. 67–90, Academic Press, New York, London.
[39] Lechner, M., Märkl, H. and Götz, F. (1988) Appl. Microbiol. Biotechnol. 28, 345–349.
[40] van Oort, M.G., Deveer, A.M.T.J., Dijkman, R., Tjeenk, M.L., Verheij, H.M., de Haas, G.H., Wenzig, E. and Götz, F. (1989) Biochemistry 28, 9278–9285.
[41] Lämmli, U.K. (1970) Nature 227, 680–685.
[42] Bonner, W.M. and Laskey, R.A. (1974) Eur. J. Biochem. 46, 83–88.
[43] Ahrem, B., Hoffschulte, H.K. and Müller, M. (1989) J. Cell Biol. 108, 1637–1646.
[44] Ayora, S. and Götz, F. (1994) Mol. Gen. Genet. 242, 421–430.
[45] Ayora, S., Lindgren, P.E. and Götz, F. (1994) J. Bacteriol. 176, 3218–3223.
[46] Wenzig, E., Lottspeich, F., Verheij, B., de Haas, G.H. and Götz, F. (1990) Biochem. (Life Sci. Adv.) 9, 47–56.
[47] Schiebel, E., Driessen, A.J.M., Hartl, F.U. and Wickner, W. (1991) Cell 64, 927–939.
[48] Ernst, F., Hoffschulte, H.K., Thome-Kromer, B., Swidersky, U.E., Werner, P.K. and Müller, M. (1994) J. Biol. Chem. 269, 12840–12845.
[49] Murén, E.M. and Randall, L.L. (1985) J. Bacteriol. 164, 712–716.
[50] Cabelli, R.J., Chen, L., Tai, P.C. and Oliver, D.B. (1988) Cell 55, 683–692.
[51] Cabelli, R.J., Dolan, K.M., Quian, L. and Oliver, D.B. (1991) J. Biol. Chem. 266, 24420–24427.
[52] Ohta, Y., Hojo, H., Aimoto, S., Kobayashi, T., Zhu, X., Jordan, F. and Inoue, M. (1991) Mol. Microbiol. 5, 1507–1510 (and Erratum p. 3090).
[53] Demleitner, G. and Götz, F. (1994) FEMS Microbiol. Lett. 121, 189–198.