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Protein Expression and Purification 40 (2005) 336–339
Protein Expression & Purification
www.elsevier.com/locate/yprep

Purification of a functional mature region from a SecA-dependent preprotein

Catherine Baud[a,1], Efrosyni Papanikou[a,1], Spyridoula Karamanou[a], Giorgos Sianidis[a], Andreas Kuhn[b], Anastassios Economou[a,*]

[a] *Institute of Molecular Biology and Biotechnology, FORTH and Department of Biology, University of Crete, P.O. Box 1527, GR-711 10 Iraklio, Crete, Greece*

[b] *Institute of Microbiology and Molecular Biology, University of Hohenheim, D-70593 Stuttgart, Germany*

Received 30 September 2004, and in revised form 8 December 2004
Available online 5 January 2005

Abstract

Most of the bacterial proteins that are active in extracytoplasmic locations are translocated through the inner membrane by the Sec translocase. Translocase comprises a membrane "pore" and the peripheral ATPase SecA. Where preproteins bind to SecA and how they activate translocation ATPase remains elusive. To address this central question we have purified to homogeneity the mature and preprotein parts of an exported protein (pCH5EE). pCH5EE satisfies a minimal size required for protein translocation and its membrane insertion is SecA-dependent. Purified pCH5EE and CH5EE can form physical complexes with SecA and can functionally suppress the elevated ATPase of a constitutively activated mutant. These properties render pCH5EE and CH5EE unique tools for the biochemical mapping of the preprotein binding site on SecA.

Keywords: Signal peptide; Mature preprotein; M13 procoat; SecA; ATPase; Translocase

Most exported proteins are transported from the cytosol across the inner membrane in bacteria by the Sec translocase [1]. Translocase core comprises a membrane-embedded channel-like structure formed by the SecY/SecE/SecG trimer [2] and the peripheral ATPase SecA [1]. Secretory preproteins are tagged by N-terminal signal peptides, targeted to the membrane by chaperone/pilot-like factors such as the signal recognition particle [3] and SecB [4], bind to membrane-bound SecA [5], trigger multiple rounds of ATP hydrolysis [6,7], and lead to processive preprotein translocation [1].

Mapping of preprotein binding surfaces on SecA has not been possible because traditional model preproteins like proOmpA display low affinity for soluble SecA [6,8,9]. Synthetic signal peptides were shown to bind tightly to SecA [10] and mutations that prevent this binding have been characterized [11]. In contrast, binding of mature domains has remained elusive. To address this, we resorted to using the model preprotein pCH5EE, a mutant derivative of the major M13 coat protein (hereafter proM13coat), a model substrate with a minimal size required for secretion. Wild type M13 procoat inserts directly into the lipid bilayer by the YidC pathway [12]. Strikingly, introduction of two glutamyl residues after position +2 in the mature region (Fig. 1) renders pCH5EE SecA-dependent for membrane insertion [13].

We now report stable expression of the mature region peptide CH5EE and its purification to homogeneity (>99%). Like pCH5EE, CH5EE interacts physically and functionally with SecA. This peptide can now be used for mapping the mature domain binding site on SecA.

* Corresponding author. Fax: +30 2810 391166.
E-mail address: aeconomo@imbb.forth.gr (A. Economou).
[1] These authors contributed equally.

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doi:10.1016/j.pep.2004.12.009

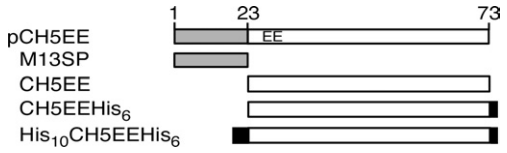

Fig. 1. pCH5EE and derivative constructs.

Materials and methods

Cloning and expression of CH5EE

CH5EE was constructed in two steps. PCR was performed using plasmid pJQ8 (pCH5EE) [13] as a template using the oligo 5′CATATGCAAGCCGGC GAGGAAGAAGGTGACGATCCCGC and reverse primer 5′CGCGAATTCGAGCTCGGTACCCCC3′. The *Nde*I/*Bam*HI restriction fragment was cloned into the expression vector pT7-7. A 0.2 kb *Nde*I/*Bam*HI restriction fragment was subcloned into the pET22b (Invitrogen) giving rise to pIMBB285 that expresses CH5EE-His_6 (pIMBB278). The same product was also subcloned to pET16b where 10 additional histidines were added to the N-terminus giving rise to His_{10}-CH5EE-His_6 (pIMBB285). The resulting constructs were transformed in three different *Escherichia coli* Table 1 strains for expression: BL21.19(DE3) [14], BL21/plysS (Invitrogen), and JM109 (Promega).

Purification of CH5EE using Ni^{2+}–NTA

Escherichia coli strain JM109/pIMBB278 (His-CH5EE) was grown in LB medium up to an optical density of $A_{550} = 0.6$. HisCH5EE expression was induced with IPTG (0.5 mM) for 3 h. Cells were harvested and dissolved in resuspension buffer (RB; 50 mM Tris–Cl, pH 7.6, 10% sucrose, 1 mM PMSF, and 3 ml buffer/g cell pellet) and were lysed by lysozyme (0.4 mg/ml; 1 h; 4 °C) and then treated with DNAse (10 μg/ml; 10 min; 20 °C) after addition of $MgCl_2$ (5 mM). One volume of extraction buffer (ExB; 50 mM Tris–Cl, pH 7.9, 0.5 M NaCl, and 8 M Urea) was added to the resuspended material (final concentration: 25 mM Tris–Cl; 0.25 M NaCl; and 4 M Urea), followed by 15 min incubation (4 °C). Suspensions were treated with a Dounce homogenizer. After centrifugation (4 °C/13,500 rpm, Heraeus rotor 3335), pellets were resuspended in 100 ml of 20 mM Tris–Cl, pH 7.9, 10% sucrose, and 1 mM PMSF and sonicated while the supernatant (Fig 2, lane 3; S1) was kept. After centrifugation (4 °C/13,500 rpm Heraeus rotor 3335), pellets were solubilized in 50 ml of 10 mM triethanolamine, 10% glycerol and the supernatant (lane 4; S2) was kept. After centrifugation (4 °C/13,500 rpm Heraeus rotor 3335), pellets were solubilized in 50 ml 20 mM Tris–Cl, pH 7.9, 10% glycerol, 0.5 M NaCl, 6 M urea, and 1% w/v sodium *N*-lauroyl sarcosine and incubated (15 min; 4 °C), and the supernatant (lane 5; S3) was kept. A final centrifugation (4 °C/13,500 rpm Heraeus rotor 3335) was carried out and the supernatant (lane 6; S4) was incubated (2 h; 4 °C) with nickel–nitrilotriacetic acid-agarose (Ni^{2+}–NTA; ratio: 0.25 ml bed volume/liter of culture) treated with equilibration buffer (EB; 20 mM Tris–Cl, pH 7.9, 10% glycerol, 0.5 M NaCl, 6 M urea, 1% w/v sodium *N*-lauroyl sarcosine, and 5 mM imidazol). The resin was separated from unbound flow through material (lane 7; FT) and washed consecutively with WB1 (20 mM Tris–Cl, pH 7.9, 6 M urea, 0.5 M NaCl, 10% glycerol, 0.5% w/v sodium *N*-lauroyl sarcosine, 5 mM imidazol; 5 volumes), WB2 (20 mM Tris–Cl, pH 7.9, 0.5 M NaCl, 10% glycerol, 0.5% w/v sodium *N*-lauroyl sarcosine, and 5 mM imidazol; 5 volumes), and WB3 (20 mM Tris–Cl, pH 7.9, 10% glycerol, 0.5% sodium *N*-lauroyl sarcosine, and 15 mM imidazol; 10 volumes) (lanes 8–10; W1–W3). Proteins were eluted with two bed volumes of elution buffer (EluB; 20 mM Tris–Cl, pH 7.9, 10% glycerol, 0.5% w/v sodium *N*-lauroyl sarcosine, and 300 mM imidazol) and were collected in half column volume fractions (peak fraction shown in lane 11; CH5EE indicated with a filled arrow). Proteins were dialysed against 20 mM Tris–Cl pH 7.9, 6 M urea (12 h; 20 °C), concentrated by ultrafiltration (Ultrafree-15; Millipore) and stored at −80 °C. Protein concentration was estimated by the DC Protein Assay (Bio-Rad), using bovine serum albumin as a standard. Samples were analysed by

Table 1
Purification of CH5EE from *E. coli* strain JM109 cells

E. coli strain	Culture volume (L)	Wet weight (g)	Total CH5EE in starting material (mg)	Total purified CH5EE (mg)	Purity (%)
JM109	8	8	10.4	1	>99

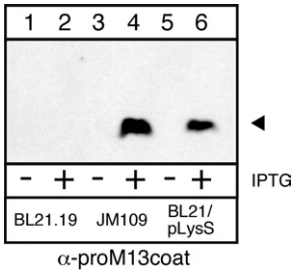

Fig. 2. CH5EE expression. CH5EE protein expression in three different *E. coli* strains (BL21.19, JM109, and BL21/plysS). Protein expression was induced by addition of 0.5 mM IPTG at 37 °C. Samples were collected before and after 3 h of induction. Protein samples have been resolved by SDS–PAGE on 10% High-Tris gel [11] and immunostained with α-proM13coat antibody [13]. Lane 1, BL21.19 uninduced whole cell sample; lane 2, BL21.19 after 3 h induction; lane 3, JM109 uninduced whole cell sample; lane 4, JM109 after 3 h induction; lane 5, BL21/plysS uninduced whole cell sample; and lane 6: BL21/plysS after 3 h induction.