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Autodisplay of the protease inhibitor aprotinin in *Escherichia coli*

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Abstract

The Kunitz type protease inhibitor aprotinin, containing three intramolecular disulfide bonds, was expressed on the surface of *Escherichia coli* by Autodisplay. For this purpose, the aprotinin gene was fused in-frame to the transporter domain encoding DNA region of the AIDA-I autotransporter protein. Culture of cells supplied with the artificial gene at reducing conditions resulted in the translocation of aprotinin to the cell surface. Correct folding of aprotinin was shown by high affinity to its target enzyme HLE. No surface translocation was detectable under non-reducing conditions, indicating the degradation of aprotinin in the periplasm. By the use of periplasmic-protease defective *E. coli* strains PW147, PW151, and PW152, under non-reducing conditions, significant amounts of aprotinin appeared in the periplasm but not at the surface. Our results indicate that aprotinin molecules, reaching stable conformation before transport across the outer membrane, are degraded in the periplasm due to proteolysis. In case folding can be prevented, i.e., by blocking disulfide bond formation in the periplasm, aprotinin is translocated and can adopt its active conformation at the cell surface.

Keywords: Autodisplay; Autotransporter; Aprotinin; Disulfide bond; Periplasm; Protease inhibitor; Flow cytometry; DegP; DegQ; Protein transport

During recent years, bacterial surface display of heterologous proteins has become a versatile biotechnological tool, which can be used to gain basic insights in molecular biology issues as well [1]. Among other systems used, the autotransporter pathway [2] is a very elegant way to translocate a recombinant protein of choice to the cell surface of a Gram-negative bacterium. The name "Autodisplay" was initially introduced for the use of the autotransporter domain of the *Escherichia coli* adhesin involved in diffuse adherence (AIDA-I) [3] for outer membrane translocation of the recombinant protein in combination with the signal peptide of the cholera toxin β-subunit (CTB) and an artificial promoter [4]. Some features of the "Autodisplay"-system are unique among other cellular surface display systems used so far, e.g., more than 10^5 recombinant molecules have been reported per single cell of *E. coli* [5] or stable multimeric proteins were detectable even in case subunits were expressed from monomeric genes [6,7]. In addition, it is an easy to handle system. The recombinant passenger protein is transported by simply introducing its coding sequence in-frame between the signal peptide and the translocator domain (Fig. 1), while the native passenger (the actual adhesin) is detached [8]. Introduction of a multiple cloning site instead of the native passenger allows the in-frame insertion of various passenger domains. This "Autodisplay" was successfully used for the functional surface expression of a wide variety of recombinant proteins [4,5,7,9–11]. In addition to "Autodisplay" other surface display tools have been reported more recently based on various natural autotransporter proteins different from AIDA-I [12–14].

The first characterized autotransporter protein was IgA1 protease from *Neisseria gonorrhoeae* [15]. A concept for its secretion mechanism was proposed

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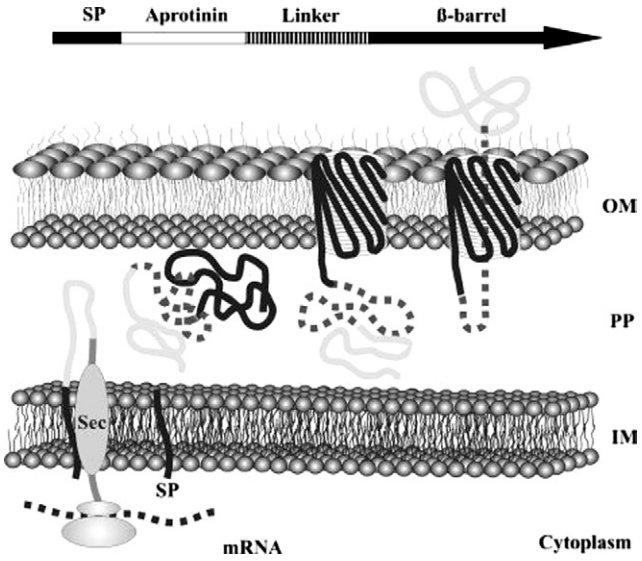

Fig. 1. Autodisplay of recombinant proteins in *E. coli*. Proteins to be transported by the autotransporter pathway to the cells' surface are synthesized as a single polyprotein precursor containing structural requirements sufficient for surface translocation. SP, signal peptide; IM, inner membrane; PP, periplasm; and OM, outer membrane.

concurrently with its discovery. The mechanism included the passengers translocation through an outer membrane β-barrel, quite similar to the well-known porins. In contrast to the porins, the β-barrel is formed by the autotransporter itself, namely by its C-terminus [16]. It was quickly concluded that some surface proteins from other Gram-negative bacteria were obviously transported by similar means [17] and finally these surface proteins and/or secreted proteins were comprised to a new protein family called "autotransporter" [2]. However, the last step in transport, in detail the outer membrane translocation, remained subject to controversial discussions. Whereas the initial concept by Meyer and co-workers restricted the passage through the β-barrel only to proteins in an unfolded stages [18–20], other studies reported the translocation of proteins in a folded confirmation [21,22], which appears to be incompatible with translocation across the pore formed by the β-barrel. This resulted in an alternative model of transport involving a kind of a higher-level pore, build up of oligomers of 9–11 single β-barrels [22]. Most recently the first crystal structure of an autotransporter protein, NalP from *N. gonorrhoeae*, became available [23]. The data from this study were rather consistent with the hypothesis that the passenger-domain is transported across the hydrophilic channel formed by the β-barrel.

In the present study, we used aprotinin, a rapidly folding, disulfide bond stabilized serine protease inhibitor [24,25], as a passenger in Autodisplay. The aim was to learn more about the fate of the recombinant passenger protein during transport. Aprotinin, a serine protease inhibitor, was selected as a passenger, since it should exhibit resistance to degradation in the periplasm caused by several well-known serine proteases, e.g., DegP, DegS or DegQ. This study shows that folded aprotinin was not translocated to the surface but remained in the periplasm. The most probable reason is its three-dimensional structure exceeding the size of the β-barrel lumen. In case that folding of aprotinin during transport was prevented by blocking of disulfide bond formation, it was successfully translocated to the cell surface. Finally it adopted active conformation a posteriori, as indicated by high affinity to one of its known target proteins, human leucocyte elastase.

Materials and methods

Bacterial strains, plasmids, and culture conditions. Escherichia coli strains JK321 [20] (Δ*omp*T *pro*C *leu*6 *trp*E38 *ent*A zih12::Tn10 *dsb*A::*kan)* and UT5600 (F$^-$ *ara*14 *leu*B6 *azi*-6 *lac*Y1 *pro*C14 *tsx*-67 *ent*A403 *trp*E38 *rfb*D1 *rps*L109 *xyl*-5 *mtl*-1 *thi*1 Δ*omp*T-fepC266) [4] have been used in earlier studies on the autotransporter-mediated surface display of recombinant proteins. Periplasmatic-protease negative strains for the expression of autoransporter fusion proteins used in this study, PW147 (HsdR2, *ara*D139, *gal*E15, *gal*K16, *rps*L, *deg*Q1, (Δ*Eco*RV)), PW151 (HsdR2 *ara*D139 *gal*E15 *gal*K16 *rps*L *deg*P41), and PW152 (HsdR2 *ara*D139 *gal*E15 *gal*K16 *rps*L Δ*deg*Q2::*kan* Δ*Eco*RV::*kan*) were kindly provided by H. Kolmar and P. Sauer [26]. *E. coli* TOP10 (F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G) and the vector pCR2.1-TOPO, which were used for subcloning of PCR products, were obtained from Invitrogen (Groningen, the Netherlands). Plasmid pJM1013 is a derivative of pJM7, which encodes the AIDA-I autotransporter domains and both have been described earlier [8,27]. Plasmid pIU27.1.O encoding aprotinin was kindly provided by H. Apeler, A.G. Bayer (Wupperthal, Germany). Bacteria were routinely grown at 37 °C in Luria–Bertani (LB) broth containing 100 mg ampicillin per litre.

Recombinant DNA techniques. For the construction of an aprotinin–autotransporter fusion protein, the aprotinin gene was amplified by PCR from plasmid pIU27.1.O with oligonucleotide primers DZ001 (5′-gcg tcg acc gtc ctg act tct gcc tcg agc cg-3′) and DZ002 (5′-ggg gta cca gca cca ccg caa gta cg-3′). The PCR product was inserted into vector pCR2.1-TOPO and recleaved with *Sal*I and *Kpn*I. The restriction fragment was ligated into pJM7 resulting in plasmid pAT-AP02-7 (Fig. 2). Plasmid pAT-AP02-7 was used as a template for PCR to construct plasmid pAT-AP02-13 with oligonucleotide primers JJ009 (5′-gct cta gac gtc ctg act tct gcc tcg agc cg-3′) and JJ010 (5′-gaa gat cta gca cca ccg caa gta cgc-3′). The PCR product was inserted into vector pCR2.1-TOPO and recleaved with *Xba*I/*Bgl*II. The restriction fragment was ligated into pJM1013, restricted with the same enzymes, resulting in plasmid pAT-AP02-13. Both ligations yielded an in-frame fusion of aprotinin with the AIDA-I autotransporter unit under the control of a constitutive promoter (P_{TK}) [4]. They differed in the length of the linker region, that is necessary between the recombinant passenger protein and the β-barrel to obtain full-surface exposure of the passenger (Fig. 2).

Outer-membrane preparation. E. coli cells were grown overnight and 1 mL culture was used to inoculate fresh LB medium (20 mL). Cells were cultured at 37 °C with shaking (200 rpm) for about 5 h until OD_{578} of 0.7 was reached. Cells were harvested and outer membranes were prepared according to a modification of the rapid isolation method of Hantke [28], as described earlier.