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Artificial Binding Proteins (Affitins) as Probes for Conformational Changes in Secretin PulD

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The DNA-binding protein Sac7d was previously modified to bind with high affinity to the N domain of the outer membrane secretin PulD from the bacterium *Klebsiella oxytoca*. Here, we show that binding of the Sac7d derivatives (affitins) to PulD is sensitive to conformational changes caused by denaturant and by the zwitterionic detergent Zwittergent 3–14 routinely used to extract secretins from outer membranes. This sensitivity to the conformational state of PulD allowed us to use the affitins as probes for the native structure of PulD and to devise protocols for examining *in vitro* synthesized protein in nonionic detergent and for the affinity purification of native PulD using affitins as ligands. When fused to periplasmic PhoA, three affitins inhibited PulD multimerization *in vivo* and caused loss of function. In two cases, this was likely to be due to dimerization of the affitin by the bound PhoA, as the effect was absent when the affitins were fused to monomeric MalE. In the third case, the MalE and PhoA moieties probably interfered sterically with PulD protomer interactions and, thereby, inhibited multimerization. None of the affitins tested interacted with PulD at sites of protomer interaction or blocked the secretin channel through which exoproteins cross the outer membrane in the Type II secretion pathway of which PulD is a key component.

Keywords: Sac7d; outer membrane protein; secretin; Type II protein secretion; protein folding

Introduction

Type II protein secretion systems (T2SSs) are widespread in Gram-negative bacteria, where they facilitate the movement of specific proteins from the periplasm to outside of the cell. The T2SS is composed of 12–15 different proteins that are associated with either the inner or outer membrane and that function together as a membrane potential- and ATP-dependent[1,2] protein secretion machinery, the secreton.[3] Plant and animal pathogens such as *Xanthomonas campestris*, *Erwinia* spp., and *Vibrio cholerae* make extensive use of the T2SS to secrete hydrolytic enzymes and virulence factors essential for pathogenesis.[4] Common to all studied T2SSs is a homomultimeric outer membrane protein complex (secretin) that probably represents the channel through which exoproteins are secreted.[5] The *Erwinia chrysanthemi* secretin OutD binds the exoprotein pectate lyase directly,[6] suggesting an additional role in substrate recognition. Secretins also play major roles in the elaboration of other bacterial outer-membrane-spanning structures such as those of the Type III injectisome,[7] Type IV pili,[8,9] and the filamentous bacteriophage secretion apparatus.[10]

The pullulanase (PulA)-specific T2SS of *Klebsiella oxytoca* is one of the most extensively studied secretons. Cryo-electron microscopy (EM) of purified protein solubilized in detergent revealed that the

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Abbreviations used: T2SS, Type II protein secretion system; EM, electron microscopy; NRMSD, normalized root-mean-square deviation; POE, polyoxyethylene; EDTA, ethylenediaminetetraacetic acid.

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secretin (PulD) of this system assembles into a dodecameric structure composed of two rings sandwiching a central disk.[11] Tryptic digestion showed that the PulD polypeptide has two major domains: a trypsin-sensitive N domain and a trypsin-resistant C domain. While the C domain can assemble into membrane-associated dodecamers, the N domain, which forms part of the periplasmic region of the PulD multimer,[11] remains soluble and monomeric when produced separately.[12] The related secretin OutD from *E. chrysanthemi* interacts with the inner-membrane secreton component OutC and contains specificity determinants for some, but not all, secreted proteins,[6,13] but an equivalent interaction of PulD with either the secreted protein PulA or the OutC homologue PulC could not be demonstrated (unpublished data). Despite extensive studies, neither the high-resolution structure nor the specific function of PulD, and specifically its N domain, is known in detail.

To overcome problems inherent in using antibody fragments as inhibitors of cellular processes, as promoters of protein crystallization, and as probes for changes in protein conformation, we recently used a mutagenesis and selection approach to alter the specificity of the small (66 amino acids), highly stable DNA-binding protein (Sac7d) from the hyperthermophilic archaeon *Sulfolobus acidocaldarius*[14] to enable it to bind to the PulD N domain.[15] Sac7d has a well-characterized oligonucleotide/oligosaccharide-binding fold comprising a five-stranded β-barrel capped by an α-helix. Proteins containing this structural motif specifically recognize a wide range of substrates such as oligosaccharides, oligonucleotides, proteins, and metal ions.[16] Specificity is mediated by variations of specific amino acid residues located at conserved positions on the binding face, which result in a potential binding area of around 1200 Å^2. Sac7d variants, hereafter called affitins, which bind to the N domain, were previously selected by ribosome display from a combinatorial library of variants in which 14 residues exposed on the original DNA binding face[17,18] were permutated by gene synthesis. This resulted in a large collection of affitins (Sac7*) that all bound specifically to the PulD N domain with high affinity and with different amino acids at most of these 14 positions. When fused to alkaline phosphatase and exported to the periplasm, these affitins inhibited PulA secretion and PulD multimerization to varying extents.[15] The present study further explores the PulD-N/affitin interaction and demonstrates the use of affitins as valuable tools for probing protein structure *in vivo* and *in vitro*.

Results

In vivo effects of monomeric affitins on PulD multimerization

Coexpression of the genes encoding any of three different PhoA-Sac7* chimeras (Sac7*6, Sac7*33, and Sac7*40) with the *K. oxytoca* T2SS (*pul*) genes in *Escherichia coli* inhibits PulD multimerization and, consequently, PulA secretion, but the chimeras nevertheless bound to full-length PulD dodecamers in Far Western blot experiments.[15] Since PhoA is dimeric, PulD multimerization could also have been affected by simultaneous binding of two PulD monomers, rather than by occlusion of sites required for PulD multimerization. Previously, PulD was only detected in strains producing periplasmic Sac7*6-, Sac7*33-, or Sac7*40-PhoA chimeras in the absence of cell envelope proteases, suggesting that PulD bound by these bifunctional affitins was degraded by periplasmic proteases. In the absence of these proteases, more monomeric PulD was detected than in the wild-type strain, but very little multimer was detected.[15] The form of PulD targeted for degradation was therefore likely to be the affitin-bound monomers, rather than the fully assembled multimer.

To study the *in vivo* binding of affitins to PulD further and to avoid possible complications caused by PhoA protein dimerization, chimeras of monomeric maltose binding protein (MalE) and Sac7*6, Sac7*33, or Sac7*40 were targeted to the periplasm of *E. coli* carrying the *K. oxytoca* T2SS genes on the chromosome (PAP7232). Only the MalE-Sac7*40 chimera inhibited PulD multimerization and PulA secretion. The levels of PulD monomer were higher in strains expressing this construct than in the wild-type strain (or strains producing MalE-Sac7* constructs that did not show decreased levels of multimer). The total levels of PulD, estimated from the amount of monomer after phenol dissociation of the multimers, were comparable in all strains (Fig. 1a). Incubating purified MalE-Sac7*40 with membranes from strain PAP7232 allowed cosedimentation of MalE-Sac7*40 with the membranes, while no such cosedimentation occurred when membranes lacking PulD (from strain PAP7447) were used (Fig. 1b), indicating that at least part of the binding site remains accessible to MalE-Sac7*40 in the PulD multimer.

The Sac7*40 protein was produced as a periplasmic construct carrying only a short eight-amino-acid extension (Strep-tag) at the C-terminus to test the possibility that MalE-Sac7*40 inhibited multimerization by steric hindrance by the bulky MalE protein. This construct did not affect multimerization or secretion in strain PAP7232 (Fig. 1c) but still bound to the membrane-inserted PulD multimer in these cells, as indicated by the specific cosedimentation of Sac7*40-Strep with membranes derived from them (Fig. 1b).

PulD-derived peptides are not recognized by Sac7*40

The PulD-N2 protein used as bait to select affitins recognizing the PulD N domain comprised residues 28–262 (EEFSAS-KQLDRQ) of the PulD preprotein following the hexahistidine tag and thrombin cleavage site of pET15b (MGSSHHHHHHSSGLVPRGSHM). Partial proteolysis of the PulD-N2