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Amphipathic helical ordering of the flagellar secretion signal of *Salmonella* flagellin

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ABSTRACT

Export of external flagellar proteins requires a signal located within their N-terminal disordered part, however, these regions do not share any significant sequence similarity suggesting that the secondary/tertiary structure might be important for recognition by the export gate. NMR experiments were performed to reveal the conformational properties of the flagellin signal sequence *in vitro*. It assumed a largely disordered fluctuating structure in aqueous environment, but acquired a folded structure containing an amphipathic helical portion in 50% MeOH or upon addition of SDS micelles which are known to promote hydrophobic interactions. Our observations raise the possibility that the signal sequence may partially undergo amphipathic helical ordering upon interaction with the recognition unit of the flagellar export machinery in a similar way as revealed for protein import into intracellular eukaryotic organelles mediated by targeting signals of high diversity.

1. Introduction

The bacterial flagellum is a protein-based rotary nanomachine for locomotion which contains a membrane embedded molecular motor rotating a long helical filament [1]. The helical filament is connected to the basal structure via a short, highly curved segment called the hook. While the hook is a helical assembly of ~130 copies of FlgE subunits, the filament may comprise tens of thousands of flagellin (FliC) subunits and can grow up to 20 μm. Other components of the filamentous axial portion of the bacterial flagellum are the five rod proteins (FliE, FlgB, FlgC, FlgF, FlgG) forming the axis of the motor, and the hook associated proteins FlgK, FlgL and FliD. FlgK and FlgL are junction proteins that connect the hook to the filament, while FliD forms a capping structure at the distal end of the flagellar filament, which helps the incorporation of flagellin monomers into the filament at the tip.

Flagellar axial proteins forming the structures lying beyond the cytoplasmic membrane are synthesized in the cell and exported by the flagellum-specific protein export apparatus from the cytoplasm to the site of assembly at the distal end of the growing filament [2]. Thousands of subunits must be translocated through the narrow (20–25 Å wide) central channel of the flagellum in a largely unfolded conformation. The flagellar protein export system is located at the cytoplasmic side of the basal part of the flagellum to distinguish flagellar proteins from other cytoplasmic proteins and to facilitate their transportation. The membrane-associated FlhA-FlhB complex of the export apparatus is thought to be responsible for export substrate recognition. The flagellar export machinery belongs to the family of the type III secretion systems, which also include those for secretion of virulence factors by a wide variety of pathogenic bacteria [3–5]. Recent studies have demonstrated that the proton motive force across the cytoplasmic membrane is responsible for driving the export process [6,7].

In spite of intensive efforts, the nature of the signal directing flagellar protein secretion is still mysterious [8–10]. The protein substrates do not possess a classical cleavable signal sequence or do not share any obvious consensus sequence. Secretion was reported to require a disordered N-terminal secretion signal, but an mRNA encoded signal within the 5′-untranslated region may also play a role in facilitating the export process [10]. A growing number of evidence indicates a critical role for the recognition signal located in the N-terminal region of the secreted proteins [11–14]. The

Abbreviations: FliC, flagellin; TS, targeting signal.

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identified signal sequences of different flagellar proteins, however, do not show any significant sequence similarity. It is an open question how such dissimilar signals interact with a single export apparatus.

Terminal disorder is a common structural feature of the axial proteins of the bacterial flagellum [1,15]. In the case of *Salmonella* flagellin the disordered terminal regions span the first 66 and the last 44 amino acid residues [16]. The disordered regions of the axial components are highly conserved and predicted to have a potential to fold into amphipathic α-helical structures. Signal sequences of the axial proteins lie exclusively within their disordered N-terminal region.

In this work, conformational properties of the export signal of *Salmonella* flagellin, comprising residues 26–47, were investigated *in vitro* under various conditions. Although the major portion (residues 32–44) of the signal sequence forms the extended and rather irregular spoke region in the filament core [19], connecting the inner and outer ring structures, our NMR studies demonstrate that it can readily adopt a largely helical conformation in a solvent promoting amphipathic helix formation.

2. Materials and methods

2.1. Materials

Deuterated d_{25}-SDS and CD_3OH were from Cambridge Isotope Laboratories (Andover, MA, USA). Natural abundance amino acid derivatives were purchased from Novobiochem (Laufelfingen Switzerland) or Reanal (Budapest, Hungary). The 4-methylbenzhydrylamine (MBHA) resin was obtained from Novobiochem (Laufelfingen Switzerland). N,N′-diisopropylcarbodiimide (DIC), 1-hidroxybenztriazole (HOBt), 1.8-diazabicyclo[5.4.0]undec-7-ene (DBU), and trifluoroacetic acid (TFA) were products from Fluka (Buchs, Switzerland). Solvents (DMF, DCM, acetonitrile, diethyl-ether) for peptide synthesis and purification were purchased from Reanal (Budapest, Hungary). All other reagents were from Sigma (St. Louis, MO, USA).

2.2. Peptide synthesis and purification

The flagellin signal peptide (GTAIERLSSGLRINSAKDDAAG) was synthesized manually by solid phase peptide synthesis on Rink-amide MBHA resin using standard Fmoc/^{t}Bu chemistry. After assembly, the peptide was cleaved from the resin by 10 mL TFA using 0.75 g phenol, 0.5 mL distilled water, 0.5 mL thioanisole and 0.25 mL ethandithiol as scavengers. Crude products were precipitated by dry diethyl-ether, dissolved in 10% acetic acid and freeze-dried. The freeze-dried crude product was purified on a semi-preparative Phenomenex Jupiter C18 column (250 × 10 mm I.D.) with 10 μm silica (300 Å pore size) (Torrance, CA, USA). Flow rate was 4 mL/min. Linear gradient elution was generated using 0.1% TFA in water as eluent A and 0.1% TFA in acetonitrile-water (80:20, v/v) as eluent B. Peaks were detected at $\lambda = 220$ nm.

Analytical HPLC was performed on a Knauer (Herbert Knauer GmbH, Berlin, Germany) system using a Phenomenex SYNERGI MAX-RP column (Torrance, CA, USA) as a stationary phase. Linear gradient elution, 0 min 0% B; 5 min 0% B; 50 min 90% B, was used. Flow rate of 1 mL/min was applied at ambient temperature. Peaks were detected at $\lambda = 220$ nm. The samples were dissolved in eluent B. The molecular mass of the peptide was measured by ESI-MS. Positive ion electrospray ionization mass spectrometric analysis was performed on a Bruker Esquire 3000 plus (Germany). The sample were dissolved in acetonitrile-water (50:50, v/v), containing 0.1% acetic acid.

2.3. NMR experiments

Aqueous samples with no micelles and samples in methanol were prepared by dissolving ~5 mg of the lyophilized peptide (MW = 2202) in *either* 700 μL buffer containing 20 mM potassium-phosphate, 50 mM potassium-chloride, 0.05% NaN_3 and 10% D_2O at pH = 6.3 *or* in 700 μL CD_3OH/H_2O (1:1, v/v) mixture. Samples containing SDS micelles were prepared by dissolving ~3 mg peptide in 700 μL of 10 mM sodium-phosphate buffer (pH = 5.8), containing 200 mM d_{25}-SDS and 10% D_2O.

One- and two-dimensional solution NMR experiments were carried out on a *Varian NMR SYSTEM*™ (600 MHz for ^{1}H) four-channel spectrometer using a 5-mm indirect detection triple resonance ($^1H^{13}C^{15}N$) z-axis gradient probe. Experiments were performed at either 25 °C (aqueous buffer, 50% methanol) or 40 °C (SDS). The ^{1}H resonances were assigned and analyzed as described in Ref. [20].

3. Results

3.1. Solution NMR spectroscopy

The conformation of the signal peptide was investigated in aqueous buffer, in 50% methanol, and in negatively charged SDS

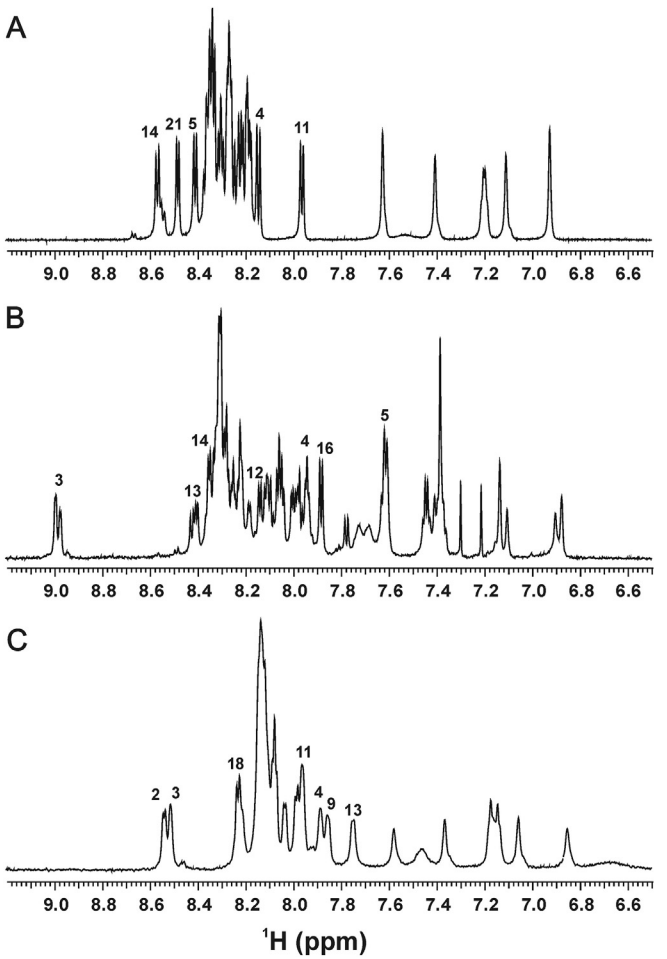

Fig. 1. Amide proton regions of one-dimensional ^{1}H spectra of the export signal in (A) 20 mM potassium phosphate, 50 mM potassium chloride, 0.05% NaN_3 at pH = 6.3 and 25 °C, (B) CD_3OH/H_2O (1:1, v/v) at 25 °C, and (C) 200 mM d_{25}-SDS, 10 mM sodium phosphate at pH = 5.8 and 40 °C. Resonances for which chemical shift changes are most easily seen are labeled with the corresponding residue number.

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