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Effect of divalent ions on the minimal relaxase domain of MobA

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ABSTRACT

The MobA protein encoded by plasmid R1162 plays an important role in conjugative mobilization between bacterial cells. It has two functional domains, the N-terminal relaxase domain and C-terminal primase domain. The N-terminal 186 residues (minMobA) is the minimal domain required for relaxase activity. We investigated the effects of different divalent metallic cations on minMobA activity measuring DNA binding, DNA nicking, and protein denaturation experiments. The results show that divalent cations are not required for DNA binding but are required for DNA nicking. The range of metals that function in minMobA suggests the cation role is largely structural. The most tightly binding cation is Mn^{2+}, but the expressed protein shows roughly equal amounts of Mg^{2+} and Ca^{2+}, both of which facilitate substrate binding and catalysis. Surprisingly, Zn^{2+} does not facilitate DNA binding nor allow nicking activity.

Introduction

Bacterial conjugation is the direct transfer of genetic material between bacterial cells through temporary cell-to-cell contact. It is not only an important mechanism in the evolution of bacteria but is also one of major causes of antibiotics resistance. Bacteria acquire resistance to antibiotics by the conjugative transfer of plasmids or transposable elements.

There are two classes of plasmids involved in conjugative transfer. One is called the self-transmissible element; the other is the mobilizable plasmid [1–3]. The self-transmissible element encodes a self-transmissible conjugative transfer system, while the mobilizable plasmid is transmissible only in the presence of a type IV secretion system (T4SS), which can be encoded by a self-transmissible plasmid. Interestingly, the mobilization plasmids are exceptionally promiscuous, in that they can be transferred into a very diverse range of organisms including yeast, plant, and animal cells [4–6]. This promiscuity of mobilizable plasmids can constitute a great danger to human health, by spreading antibiotic resistance among potential pathogens [7,8].

Among the most studied mobilizable plasmids is R1162, which is isolated from *Pseudomonas aeruginosa* [9]. The R1162 plasmid is the archetype for the so-called incompatibility group Q (IncQ), whose members are characterized by their broad-host-range and relatively small size [2]. The plasmid encodes resistance to the antibiotics streptomycin (*strA* and *strB*) and sulfonamide (*sulII*). It also contains an origin of transfer (*oriT*), and encodes three proteins: MobA, B and C, which are all required for plasmid mobilization.

The *oriT* DNA sequence of R1162 is a 35mer oligonucleotide, which was mapped by Becker and Meyer (see Fig. 1) [10,11]. Sequence alignments of *oriT* with other IncQ family members reveals that a 12-base core region is highly conserved. The 23-base oligonucleotide 5′ to the core region is an imperfectly inverted repeat, which is commonly found in this family of plasmids. The Mob proteins assemble at *oriT* to form a complex called the relaxosome [9].

The most important protein of the relaxosome is the 78 kDa (709 residues) MobA protein, which has two functional domains. The C-terminal domain encodes a 43 kDa protein, called primase, which can also be expressed separately. Both forms of primase have been found in host cells. The function of the primase is to lay down primers within the origin of replication, *oriV*, which can be further extended by host cell DNA polymerases. The N-terminal domain of MobA is a dsDNA-nicking enzyme, called a nickase or relaxase. This 250 residue enzyme cleaves one of the DNA strands within *oriT* and forms a covalent adduct with the 5′ end. Tyr25 of the N-terminal domain has been identified as the critical residue [10]; it carries out a nucleophilic attack on the target phosphodiester bond at the nick site and forms a covalent phosphodiester bond with the 5′ end of the nicked product. Once the covalent adduct is formed, the DNA–protein complex is recognized by a T4SS system. The cleaved strand is unwound from its complement strand and then transferred in the 5′–3′ direction to the host cell. When the transfer is completed, the MobA protein carries out a second transesterification reaction that rejoins the two DNA ends and releases the protein. Once the circular plasmid DNA is released in the host cell, a complementary strand is synthesized by the host polymerase [10].

The nickase enzymes are part of the HUH superfamily, in which the amino acid motif His-hydrophobe-His is conserved and partic-

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Fig. 1. OriT sequence of R1162. The nicking site is marked by a black arrow.

ipates in cation binding [12,13]. Nickases may also exhibit a second amino acid motif near their N termini, called a YYXXXYY sequence. The first Tyr is the nucleophile in the nicking reaction, and the first Y in the second cluster may function in the release and re-circularization of the nicked DNA [14]. MobA contains the HUH motif, but lacks a second Tyr and is the only member of this subgroup for which an X-ray structure currently exists.

Like other HUH proteins, MobA is known to have a requirement for divalent cations, initially thought to be Mg^{2+}, although Mn^{2+}, Ca^{2+}, or Ba^{2+} could substitute at lesser efficiencies [15]. The related relaxase TraI is also said to have Mg^{2+} in the active site of its crystal structure [16]. This contrasts with the situation reported for the relaxase TrwC. An X-ray structure and binding data suggested that the most likely active site ion was Zn^{2+}, although Ni^{2+} and Cu^{2+} could also sustain nickase activity. These authors saw no evidence that Mg^{2+} or Mn^{2+} could bind [14].

It has been shown that the N-terminal 186 residues of R1162 MobA, referred to as minMobA, is competent to cleave DNA [10]. The X-ray structure of minMobA has been solved [17]. It consists of five antiparallel β-strands connected by four helices lying on both sides of the sheet. The critical active site residue, Tyr25, lies on the first helix on the front side of the molecule, close to an electron-dense metal ion. This metal ion is bound on the front side of the beta sheet, chelated by His120 and His122 (the HUH motif), His112, and a water molecule in a pseudo-tetrahedral geometry. The His cluster of residues is conserved in other relaxase structures [14,18]. Since the minMobA crystallization condition included 10 mM $MnCl_2$, the metal ion in the crystal structure was thought to be Mn^{2+}. Anomalous X-ray scattering confirmed that this was the case. However, the physiologically relevant metal for relaxase is still unknown. It is also uncertain if the metal is structural or catalytic in nature. To further explore the role of a metal ion in mobA's cleavage reaction, we have assessed the ability of minMobA to cleave single-strand oligo-DNA substrate and to bind ssDNA. We have also examined its circular dichroism spectrum in the presence of Ni^{2+}, Mg^{2+}, Zn^{2+}, Mn^{2+} and Ca^{2+}. The results suggest that the protein flexibility and the conformational change of active site residues produced by divalent ion binding are essential for the catalytic cycle to occur.

Materials and methods

Protein purification

Wild-type and mutant minMobA were produced and purified as described [17].

Construction of minMobA variants

Site directed mutations were introduced according to the Stratagene protocol (Stratagene, La Jolla, CA). Around 50 ng of plasmid and 150 ng of each primer were combined with reaction buffer (20 mM Tris–HCl (pH 7.5), 8 mM MgCl2, 7.5 mM DTT, 50 μg/ml of bovine serum albumin (BSA)[1]), 150 μM dNTP mix, 1 U of KOD Hot Start DNA Polymerase (Novagen), and deionized water to final volume of 50 μl. Reactions were cycled with a denaturing temperature of 95 °C for 50 s, a re-annealing temperature of 65 °C for 50 s, and an extension temperature of 72 °C for 9 min for 18 cycles on a GeneAmp 2400 thermocycler (Perkin-Elmer, Norwalk, CT). The reaction mixture was further treated with 10 U of DpnI (New England Biolabs, Ipswich, MA) at 37 °C for 2 h. Then 1 μl of treated reaction mixture was transformed into *Escherichia coli* DH5α competent cells. The presence of the expected minMobA mutations was confirmed by DNA sequencing.

Fluorescence polarization assay

All fluorescence measurements were made using an Envision spectrofluorometer (Perkin-Elmer). A 35mer oligonucleotide (Integrated DNA Technologies) with sequence CCAGTTTCTGAAGA GAAACCGGTAAATGCGCCCT was labeled at the 3′ end with fluorescein. Samples were excited at 490 nm, and emitted light was collected through orange glass filters (OG 515, Schott). The binding affinities for the minMobA (Y25F) and oligo-DNA complexes were determined in the presence and absence of 1 mM metallic cations by measurement of the steady-state anisotropy of fluorescence as a function of added protein. The binding buffer contained 10 mM HEPES (pH 7.5), 20 mM KCl. The concentration of protein was plotted against anisotropy of fluorescence and fit to a hyperbolic equation in order to compute the dissociation constants. The nonlinear regression analysis was performed with the program GraFit 5.0 (Erithacus Software).

DNA cleavage assay

DNA cleavage assays were performed as described [17]. Briefly, a 35mer oligonucleotide (Integrated DNA Technologies) with sequence CCAGTTTCTGAAGAGAAACCGGTAAATGCGCCCT was (3′-^{33}P)-labeled with terminal transferase, according to the manufacturer's instructions (New England Biolabs). The assay reaction mixtures contained 0.03 μM labeled 35mer oligonucleotide, 0.1 μM minMobA, 40 mM Tris–HCl (pH 8.0), 20 mM NaCl, and in the presence and absence of 1 mM metallic cations. For minMobA mutants, the reaction mixtures also contained 50 mM $MgCl_2$. The reactions were terminated by addition of EDTA to 40 mM after 0, 1, 2, 4, 6, 9, 11, 15, 20, 25, 28, 36, 45, 55, and 60 min. Reaction products were separated by 12% SDS–PAGE and imaged on a Molecular Imager FX system (BioRad Laboratories, Inc., Hercules, CA) to quantify the bound radiolabeled adduct. The concentration of protein–DNA covalent adduct was plotted against time and fit to a single exponential equation: [protein–DNA] = A∗(1-exp(−kobs∗t)) + C. The nonlinear regression analysis was performed with the program GraFit 5.0 (Erithacus Software).

To explore the nicking activity over a range of metallic cations concentrations, DNA cleavage assay was also carried out by using a 49mer oligonucleotide (Integrated DNA Technologies) with sequence CCAGTTTCTCGAAGAGAAACCGGTAAGTGCGCCCTCCCCTTCAAAGTAG. Cleavage experiments were performed by incubation of 5 μM 49mer oligonucleotide with 10 μM minMobA at 37 °C in 10 mM HEPES (pH 7.5), 20 mM KCl plus a one of the divalent metal salts ($CaCl_2$, $MgCl_2$, $MnCl_2$, $NiCl_2$ and $ZnCl_2$). Metal ion concentrations ranged from 1 μM to 1 mM (1 μM, 10 μM, 1000 μM and 1000 μM). The reactions were terminated by addition of EDTA to 40 mM after 60 min. Reaction products were separated on 12% SDS–PAGE, followed by silver staining with SilverXpress staining kit (Invitrogen), and imaged on a Molecular Imager FX system (BioRad Laboratories, Inc., Hercules, CA) to quantify the protein and protein-18mer oligonucleotide adduct.

Isothermal titration calorimetry

A MicroCal VP–ITC calorimeter (MicroCal, Northampton, MA) was used to measure the binding affinity of minMobA to metallic

[1] *Abbreviation used:* BSA, bovine serum albumin; ICP-MS, inductively coupled plasma mass spectrometry.