Formation of MgF$_3^-$-dependent complexes between an AAA$^+$ ATPase and $\sigma^{54}$

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The widely distributed bacterial $\sigma^{54}$-dependent transcription regulates pathogenicity and numerous adaptive responses in diverse bacteria. Formation of the $\sigma^{54}$-dependent open promoter complex is a multi-step process driven by AAA$^+$ ATPases. Non-hydrolyzable nucleotide analogues are particularly suitable for studying such complexity by capturing various intermediate states along the energy-coupling pathway. Here we report a novel ATP analogue, ADP–MgF$_3^-$, which traps an AAA$^+$ ATPase with its target $\sigma^{54}$. The MgF$_3^-$-dependent complex is highly homogeneous and functional assays suggest it may represent an early transcription intermediate state valuable for structural studies.

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1. Introduction

The high-energy phosphoryl transfer reaction is a principle mechanism exploited by mechano-chemical enzymes such as the AAA$^+$ ATPases (ATPases associated with various cellular activities). The AAA$^+$ ATPases convert the chemical energy released from ATP hydrolysis to the remodelling of a diverse array of their substrates, to achieve for example protein unfolding, membrane fusion, DNA repair and transcription activation [1,2]. However, this energy transfer process is transient. In order to capture the AAA$^+$ ATPases and their substrates for kinetic and structural studies, nucleotide analogues are widely used. These analogues, in many cases, consist of an ADP and a metallo-halide (e.g., AlF$_3$, which occupies the $\gamma$-phosphate position within the ATP catalytic site) and are reported to represent different ATP states (AMP–AlF$_3$ and ADP–BeF$_3$ for the ATP ground state and ADP–AlF$_3$ for the ATP transition state). Being the most frequently used $\gamma$-phosphate analogue, the AlF$_3$ moiety shows complexity in binding to the catalytic site. Schlichting et al. [3] surveyed the majority of the AlF$_3$-containing crystal structures and demonstrated that the pH of the crystallographic experiment determined whether AlF$_3$ or AlF$_5^-$ was present in the crystal (thus abbreviated as AlF$_3$ in this paper). The AlF$_3$ moiety adopts a trigonal bipyramidal arrangement with the axial coordination sites being occupied by oxygens from the $\beta$-phosphate and hydrolytic water, which is thought to closely mimic the $\gamma$-phosphate at the point of hydrolysis in geometry [4]. The AlF$_3$ moiety adopts an octahedral arrangement with a net negative charge, complementary to the transition state $\gamma$-phosphate [4]. The fact that both AlF$_3$ species are found in the crystal structures suggests the catalytic site has enough flexibility to accommodate either without much reconfiguration and energy loss [3].

Vincent et al. reported a high-affinity dimeric complex formation between the RhoA GTPase and the p190 RhoGAP (Rho GTPase-activating protein) in a fluoride- and magnesium-dependent manner but an aluminium- and GDP-independent manner [5]. To investigate whether the same effect can be observed on AAA$^+$ ATPases and their substrates, we employed a bacterial enhancer binding protein (bEBP) called the Escherichia coli phage shock protein F (PspF) – a Clade 6 hexameric AAA$^+$ ATPase for this study [6]. PspF or its AAA$^+$ domain alone (residues 1–275, PspF$_{1-275}$) can activate the psp operon (pspABCDE and pspG) [7] by reorganising the E$\sigma^{54}$-DNA complex through PspF surface-exposed loops in a nucleotide-dependent manner. Recently, the Cryo-EM contour structure of a PspF$_{1-275}$-E$\sigma^{54}$-ADP–AlF$_3$ complex has been resolved [8]. However, the high-resolution hexameric crystal structure of PspF is yet to be obtained, partially due to the interference from precipitation arising from high concentrations of AlCl$_3$ used.

Here, we report an MgF$_3^-$-dependent complex formation between the ADP-bound PspF$_{1-275}$ and $\sigma^{54}$. We demonstrated that this novel MgF$_3^-$-dependent complex was more homogeneous than the previously described complexes with AlF$_3$ and may represent an intermediate state early along the activation pathway. We propose that MgF$_3^-$ will serve as a new reagent to obtain high-resolution
structural information on co-complexes of some AAA+ ATPases with their remodelling targets.

2. Materials and methods

2.1. Protein expression

*E. coli* PspF1–275 was purified as previously described [9]. *Klebsiella pneumonia* σ54 was purified as previously described [10].

2.2. Native gel mobility shift assay

Reactions were performed in 10 μl volumes containing 10 μM PspF1–275, 2.35 μM σ54, ±AlCl3, ±MgCl2, ±ADP and ±NaF in STA buffer (2.5 mM Tris–acetate pH 8.0, ±8 mM Mg–acetate, ±8 mM K–acetate, 10 mM KCl, 1 mM DTT, 3.5% (w/v) PEG 8000) at 37 °C for 15 min. Complexes were analysed on 4% native gels.

2.3. Gel filtration

The trapped complexes with 20 μM PspF1–275 and 4.7 μM σ54 were formed after 15 min incubation with reagents at 37 °C and run with gel filtration buffer (20 mM Tris–HCl pH 8.0, 50 mM NaCl, 15 mM MgCl2) in a Superdex 200 Column (10/30, 24 ml, GE Healthcare) at room temperature.

2.4. In vitro RPO formation assay

The RPO formation assay was conducted as previously described [9]. Typically in 10 μl volumes, 4 μM PspF1–275, 100 mM holoenzyme (1:4 ratio of E: σ54), 4 mM dATP and 20 mM linear Sinorhizobium meliloti nifH promoter probes (Sigma–Aldrich) were incubated at 37 °C for 15 min before the elongation mixture (0.5 mM dinucleotide primer UpG, 0.2 μCi/μl [α-32P CTP] (3000 Ci/mmol) and 0.2 mg/ml heparin) was added for another 10 min incubation. Reactions were quenched by addition of 4 μl formamide stop dye and run on a sequencing gel.

3. Results

3.1. The Mg2+-promoted PspF1–275–σ54 complex requires ADP but not AlF3

In the presence of the ATP transition state analogue ADP–AlF3, the PspF surface-exposed L1 loops extend to stably engage σ54 [11]. The resulting PspF1–275–σ54–ADP–AlF3 trapped species represents a sub-complex of one of the intermediate states en route to open complex formation (RPO) [12] to support transcription initiation by making the start site available [13]. However, heterogeneity is often observed in the population of ADP–AlF3 trapped complexes (Fig. 1 lane 4), which can lead to potential complications in mass spectroscopic analyses and crystallography. Higashijima et al. have shown that at high F– concentrations, Mg2+ can replace Al3+ in transforming the G protein ζ subunit into a more active state, possibly by associating with three F– ions to mimic the γ-phosphate of GTP [14]. In an attempt to obtain a more homogeneous population of PspF1–275–σ54–trapped complexes, possibly with new geometrical and functional features, we performed the trapping experiment by in situ formation of MgF3– in the absence and presence of nucleotides.

The trapping reaction buffer (STA buffer), which has routinely been used in various binding and transcription activation assays [9], contains 8 mM Mg2+-acetate. We initially assessed whether the intrinsic Mg2+ concentration from the reaction buffer was sufficient to support the formation of MgF3– moieties. Indeed, without any added Al3+, a more homogeneous population of Mg2+-promoted complexes was observed, whose formation was absolutely dependent on the presence of ADP (Fig. 1 lanes 5 and 6) and NaF (Fig. 1 lane 11). Addition of further Mg2+ ions to the PspF1–275–σ54 interaction assay did not seem to increase the yield of complexes, even though the concentrations of PspF1–275 and σ54 were not limiting (Fig. 1 compare lane 8 with lane 6). When the Mg2+ ions were removed from the STA buffer, the PspF1–275–σ54 complex formation was completely abolished (Fig. 1 lane 12) but restored once the Mg2+ ions were added back (Fig. 1 lane 10), further confirming the Mg2+-dependent nature of this newly trapped complex. The gel filtration data (Fig. 2) demonstrated that the ADP–MgF3––dependent complex eluted as a single homogenous peak (at 10.06 ml) before the doubly peaked ADP–AlF3–dependent complexes (at 10.16/10.91 ml), suggesting a different intermediate state is likely to be represented by the ADP–MgF3––dependent complex.

The above observations suggest that the AlF3–dependent trapped complexes formed in the presence of Mg2+ ions are likely to be a mixture of PspF1–275–σ54–ADP–AlF3–/–MgF3– with ADP–AlF3– species dominating.

3.2. ADP–AlF3 stabilises the PspF1–275–σ54 complex more strongly than does ADP–MgF3–

Since trapped complexes formed in STA buffer contain a mixture of PspF1–275–σ54–ADP–AlF3–/–MgF3– due to the presence of both Mg2+ and Al3+ ions in the reaction, we examined whether or not the AlF3–dependent complexes could form in the absence of Mg2+ ions. As shown in Fig. 3A, adding NaF and Al3+ ions to the Mg2+-acetate free STA buffer shifted nearly all the σ54 into the trapped complex (Fig. 3A lane 6). The addition of 0.4 mM Mg2+ ions (same concentration as Al3+ ions) or a 20-fold higher concentration of Mg2+ ions yielded 16% and 37% ADP–MgF3– trapped complexes compared to the AlF3–dependent assays (Fig. 3A compare lanes 4 and 5 with 6). Furthermore, a titration experiment revealed that
a relatively low concentration of the Al\(^{3+}\) ions (0.04 mM) was required to form the AlF\(_x\)-dependent complexes, much lower than the 0.4 mM routinely used (Fig. 3B). The above observations suggest that although both Al\(^{3+}\) and Mg\(^{2+}\) ions can form the ADP-dependent trapped complexes independently of one another’s presence, the Al\(^{3+}\) ions are far more efficient at promoting the complex formation.

3.3. ADP–MgF\(_3\)/C\(_0\) is a functional analogue of ADP–BeF\(_3\) in RPO formation

Burrows et al. [13] devised a short primed RNA (spRNA) synthesis assay and demonstrated that the putative transition state ADP–AlF\(_x\) complex could reorganise E\(_{54}\) to a near open complex state on a pre-opened linear DNA probe (the S. meliloti nifH promoter with the non-template ‘melted’ from \(-10\) to \(-11\))/C\(_0\) (mimicking the DNA conformation in the closed promoter complex), and the late-melted \(-10\)–\(-1\))/C\(_0\) (mimicking the DNA conformation in the open promoter complex).

Fig. 2. Gel filtration of the ADP–AlF\(_x\)/–MgF\(_3\)– dependent trapped complexes at room temperature.

Fig. 3. (A) Al\(^{3+}\) ions are more efficient at promoting the trapped complex formation. (B) Titration of the amount of Al\(^{3+}\) ions required for trapped complex formation.
similarities to either ADP–AlF\(_3\) or ADP–BeF\(_3\) dependent complexes.

In the presence of a dinucleotide primer UpG and radio-labelled GTP, abortive tetra-nucleotides UpGpGpGpG are generated on the linear \(\text{nif}H\) promoter by preventing E\(\text{f}^{\text{54}}\) from transcribing beyond the +3 site. The amount of UpGpGpGpG formed reflects the amount of RPs\(_3\)-like activity in the presence of ADP-metal fluorides. Consistent with the previous data, the ADP–AlF\(_3\) dependent complex was able to generate an RPs\(_3\)-like activity from the pre-melted DNA probe (Fig. 4). However, the ADP–MgF\(_3\)– dependent complex failed to yield RPs\(_3\)-like activity from any linear DNA probes used in this experiment (Fig. 4) – a similar functional phenotype was exhibited by the ADP–BeF\(_3\)– dependent complexes [13]. We propose that MgF\(_3\)– and BeF\(_3\)– may (i) similarly change the organisation of the ATPase catalytic site and (ii) represent functional intermediate states of the AAA\(^+\) domain in ATP hydrolysis which form prior to the state created by the ADP–AlF\(_3\).

4. Discussion

We have identified a new method dependent on the formation of MgF\(_3\)– moieties, to stably trap an AAA\(^+\) ATPase PsPF\(_{1–275}\) with its target substrate \(\sigma^{54}\). The MgF\(_3\)– dependent complexes can co-exist in solution with the AlF\(_3\)– dependent complexes when both metal ions are present – a condition under which most of the previous biochemical, mass spectroscopic and crystallographic experiments were performed. This potential heterogeneity of complex formation with AlCl\(_3\) and NaF in the presence of Mg\(^{2+}\) is not readily detected given the AlF\(_3\) functions more efficiently in trapping conditions, and so could have been easily overlooked. As a potential source of heterogeneity in protein conformation, the presence of MgF\(_3\)– and AlF\(_3\) may interfere with protein crystallisation. Based on the pH effect [3], we reason that the AlF\(_3\) moiety under the trapping conditions in this work (pH 8.0) is more likely to assume a trigonal-bipyramidal AlCl\(_3\) configuration than an octahedral AlF\(_4\) configuration. However, Xiaoxia et al. [15] argue that there is a dominant role of charge in selection of the best bound ATP analogues and thus the AlF\(_3\) moiety might be considered the better binding candidate species compared to AlCl\(_3\), as has been observed in other classes of ATP hydrolysing enzymes. Clearly, further detailed analyses and high resolution structural information are required to determine the precise ATP analogue species bound and roles of charge/geometry relationships in their binding to the bEBP class of ATPasers.

Vincent et al. suggested that additional mechanistic roles could be assigned to the MgF\(_3\)– moiety. Their observation of MgF\(_3\)– dependent GTase–GAP complex formation in the absence of ATP challenges the widely held \(\gamma\)-phosphate mimicking role for MgF\(_3\)– [5]. Our MgF\(_3\)– dependent trapping data revealed an absolute requirement for ADP for PsPF\(_{1–275}\) to interact with \(\sigma^{54}\), suggesting that MgF\(_3\)– in AAA\(^+\) ATPas is confined to function solely as a \(\gamma\)-phosphate mimic. We reason that in contrast to the relatively ‘simple’ GTP catalytic site between the GTase–GAP heterodimer, the ATP catalytic sites at the hexameric interfaces of an AAA\(^+\) ATPase need to be precisely organised and selective for nucleotide analogues in order to productively coordinate the energy relay across subunits [16].

The MgF\(_3\)– and BeF\(_3\)– moieties as trapping reagents displayed similar phenotypic traits at the level of the PsPF\(_{1–275}\) engaging its target. Both moieties are less efficient at promoting the PsPF\(_{1–275}\)–\(\sigma^{54}\) complex formation than is the AlF\(_3\) moiety (14% by BeF\(_3\)– and 16% by MgF\(_3\)– in comparison to 100% by AlF\(_3\)) and are unable to productively reorganise RP\(_c\) to yield an RP\(_3\)-like complex on a pre-opened DNA probe [13] and this work. Graham et al. suggest that the geometry of these two moieties is different at the catalytic site, as BeF\(_3\)– adopts a tetrahedral arrangement and MgF\(_3\)– adopts a trigonal bipyramidal arrangement [4]. Thus, MgF\(_3\)– and BeF\(_3\)– in combination with ADP may represent slightly different intermediate early states of bound ATP prior to ATP hydrolysis. Clearly the MgF\(_3\)– and ADP-dependent PsPF\(_{1–275}\)–\(\sigma^{54}\) complex has novelty and is the first such complex reported for an AAA\(^+\) ATPase, with the potential to advance high-resolution structural studies between nucleotide-bound AAA\(^+\) ATPas and their targets in pre-hydrolysis state.

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