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Formation of MgF_3^--dependent complexes between an AAA^+ ATPase and σ^{54}

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ABSTRACT

The widely distributed bacterial σ^{54}-dependent transcription regulates pathogenicity and numerous adaptive responses in diverse bacteria. Formation of the σ^{54}-dependent open promoter complex is a multi-step process driven by AAA^+ ATPases. Non-hydrolysable 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 σ^{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.

1. Introduction

The high-energy phosphoryl transfer reaction is a principle mechanism exploited by mechanochemical enzymes such as the AAA^+ ATPases (*A*TPases *a*ssociated with various cellular *a*ctivities). 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_x, which occupies the γ-phosphate position within the ATP catalytic site) and are reported to represent different ATP states (AMP–AlF_x and ADP–BeF_3^- for the ATP ground state and ADP–AlF_x for the ATP transition state). Being the most frequently used γ-phosphate analogue, the AlF_x moiety shows complexity in binding to the catalytic site. Schlichting et al. [3] surveyed the majority of the AlF_x-containing crystal structures and demonstrated that the pH of the crystallographic experiment determined whether AlF_3 or AlF_4^- was present in the crystal (thus abbreviated as AlF_x in this paper). The AlF_3 moiety adopts a trigonal bipyramidal arrangement with the axial coordination sites being occupied by oxygens from the β-phosphate and hydrolytic water, which is thought to closely mimic the γ-phosphate at the point of hydrolysis in geometry [4]. The AlF_4^- moiety adopts an octahedral arrangement with a net negative charge, complementary to the transition state γ-phosphate [4]. The fact that both AlF_x 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 *b*acterial *e*nhancer *b*inding *p*rotein (bEBP) called the *Escherichia coli p*hage *s*hock *p*rotein 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_x 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 σ^{54}. We demonstrated that this novel MgF_3^--dependent complex was more homogeneous than the previously described complexes with AlF_x 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

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structural information on co-complexes of some AAA$^+$ ATPases with their remodelling targets.

2. Materials and methods

2.1. Protein expression

E. coli $PspF_{1-275}$ was purified as previously described [9]. *Klebsiella pneumonia* σ^{54} was purified as previously described [10].

2.2. Native gel mobility shift assay

Reactions were performed in 10 μl volumes containing 10 μM $PspF_{1-275}$, 2.35 μM σ^{54}, ±$AlCl_3$, ±$MgCl_2$, ±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 $PspF_{1-275}$ and 4.7 μM σ^{54} were formed after 15 min incubation with reagents at 37 °C and run with gel filtration buffer (20 mM Tri–HCl pH 8.0, 50 mM NaCl, 15 mM $MgCl_2$) in a Superdex 200 Column (10/30, 24 ml, GE Healthcare) at room temperature.

2.4. In vitro RP_O formation assay

The RP_O formation assay was conducted as previously described [9]. Typically in 10 μl volumes, 4 μM $PspF_{1-275}$, 100 nM holoenzyme (1:4 ratio of E: σ^{54}), 4 mM dATP and 20 nM linear *Sinorrhizobium 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-^{32}P GTP] (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 Mg^{2+}-promoted $PspF_{1-275}$–σ^{54} complex requires ADP but not Al^{3+}

In the presence of the ATP transition state analogue ADP–AlF_x, the PspF surface-exposed L1 loops extend to stably engage σ^{54} [11]. The resulting $PspF_{1-275}$•σ^{54}•ADP–AlF_x trapped species represents a sub-complex of one of the intermediate states en route to open complex formation (RP_O) [12] to support transcription initiation by making the start site available [13]. However, heterogeneity is often observed in the population of ADP-AlF_x 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, Mg^{2+} can replace Al^{3+} in transforming the G protein α 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 $PspF_{1-275}$–σ^{54} trapped complexes, possibly with new geometrical and functional features, we performed the trapping experiment by in situ formation of MgF_3^- 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 Mg^{2+}–acetate. We initially assessed whether the intrinsic Mg^{2+} concentration from the reaction buffer was sufficient to support the formation of MgF_3^- moieties. Indeed, without any added Al^{3+}, a more homogeneous population of Mg^{2+}-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 Mg^{2+} ions to the $PspF_{1-275}$–σ^{54} interaction assay did not seem to increase the yield of complexes, even though the concentrations of $PspF_{1-275}$ and σ^{54} were not limiting (Fig. 1 compare lane 8 with lane 6). When the Mg^{2+} ions were removed from the STA buffer, the $PspF_{1-275}$–σ^{54} complex formation was completely abolished (Fig. 1 lane 12) but restored once the Mg^{2+} ions were added back (Fig. 1 lane 10), further confirming the Mg^{2+}-dependent nature of this newly trapped complex. The gel filtration data (Fig. 2) demonstrated that the ADP–MgF_3^--dependent complex eluted as a single homogenous peak (at 10.06 ml) before the doubly peaked ADP–AlF_x-dependent complexes (at 10.16/10.91 ml), suggesting a different intermediate state is likely to be represented by the ADP–MgF_3^--dependent complex.

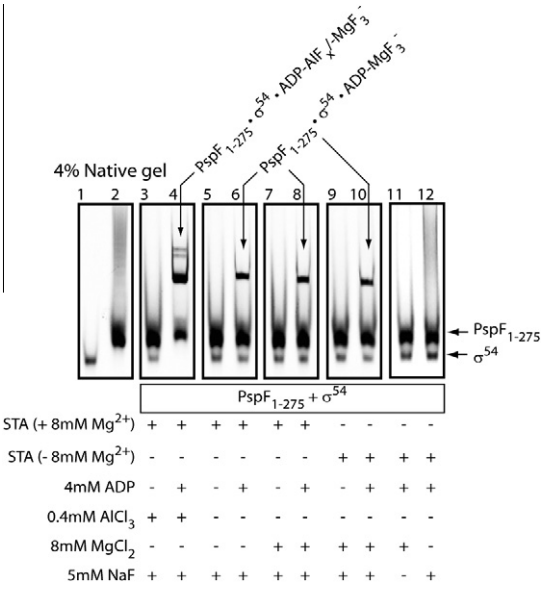

Fig. 1. $PspF_{1-275}$ forms nucleotide- and MgF_3^--dependent trapped complexes with σ^{54}. The STA (+ 8 mM Mg^{2+}) buffer has been routinely used in various native gel shift assays and transcription activation assays and contains 2.5 mM Tris–acetate pH 8.0, 8 mM Mg^{2+}–acetate, 10 mM KCl, 1 mM DTT, and 3.5% PEG 8000. To assess whether the trapped complex formation was dependent on the intrinsic Mg^{2+} ions, the STA (−8 mM Mg^{2+}) buffer was used in which the 8 mM Mg^{2+}–acetate was replaced by 8 mM K^+–acetate.

The above observations suggest that the AlF_x-dependent trapped complexes formed in the presence of Mg^{2+} ions are likely to be a mixture of $PspF_{1-275}$•σ^{54}•ADP–AlF_x/–MgF_3^- with ADP–AlF_x species dominating.

3.2. ADP–AlF_x stabilises the $PspF_{1-275}$–σ^{54} complex more strongly than does ADP–MgF_3^-

Since trapped complexes formed in STA buffer contain a mixture of $PspF_{1-275}$•σ^{54}•ADP–AlF_x/–MgF_3^- due to the presence of both Mg^{2+} and Al^{3+} ions in the reaction, we examined whether or not the AlF_x-dependent complexes could form in the absence of Mg^{2+} ions. As shown in Fig. 3A, adding NaF and Al^{3+} ions to the Mg^{2+}–acetate free STA buffer shifted nearly all the σ^{54} into the trapped complex (Fig. 3A lane 6). The addition of 0.4mM Mg^{2+} ions (same concentration as Al^{3+} ions) or a 20-fold higher concentration of Mg^{2+} ions yielded 16% and 37% ADP–MgF_3^- trapped complexes compared to the Al^{3+}-dependent assays (Fig. 3A compare lanes 4 and 5 with 6). Furthermore, a titration experiment revealed that

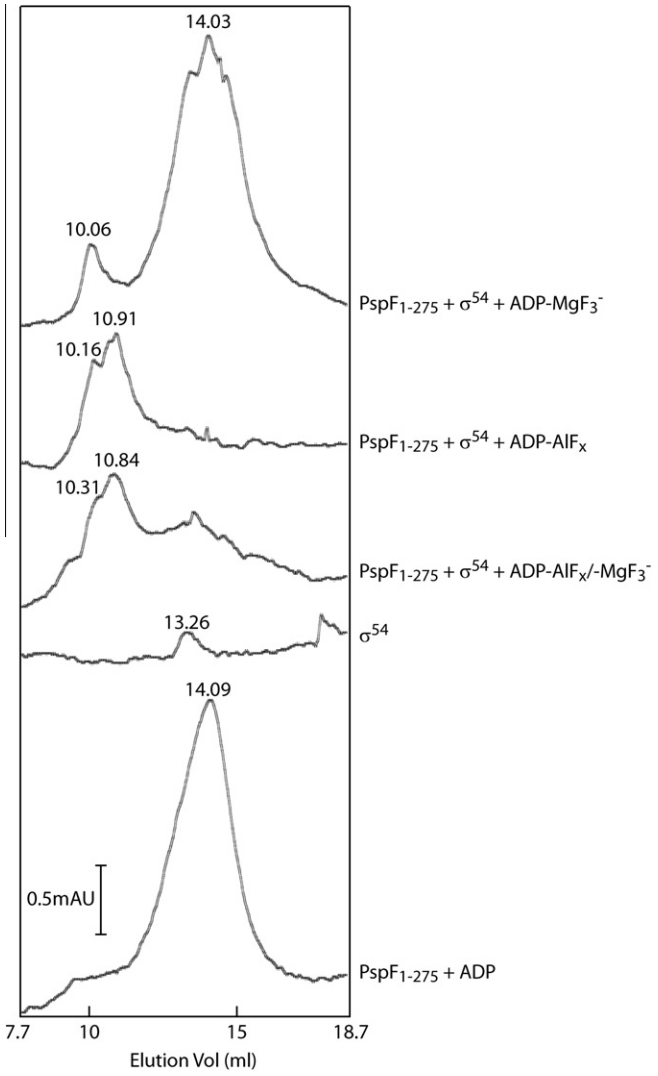

Fig. 2. Gel filtration of the ADP–AlF_x/–MgF_3^- dependent trapped complexes at room temperature.

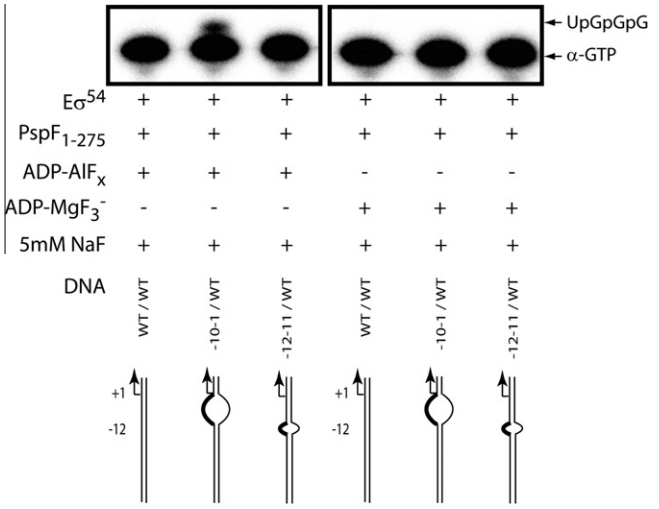

Fig. 4. Comparison of the ADP–AlF_x and ADP–MgF_3^- dependent complexes in the RP_O formation assay. The amount of abortive tetra-nucleotides (UpGpGpG) generated from this assay directly correlates with the amount of open complex formation. Three DNA sequences with mismatches on the non-template strand (thick curves in cartoons) were used: the homoduplex WT/WT, the early-melted −12−11/WT (mimicking the DNA conformation in the closed promoter complex), and the late-melted −10−1/WT (mimicking the DNA conformation in the open promoter complex).

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^- is a functional analogue of ADP–BeF_3^- in RP_O 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 Eσ54 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 −1). However, the ground state ADP–BeF_3^- complex failed to do so [13]. Here we employed the spRNA assay to assess whether the MgF_3^--dependent complexes are transcriptionally active and/or carry any functional

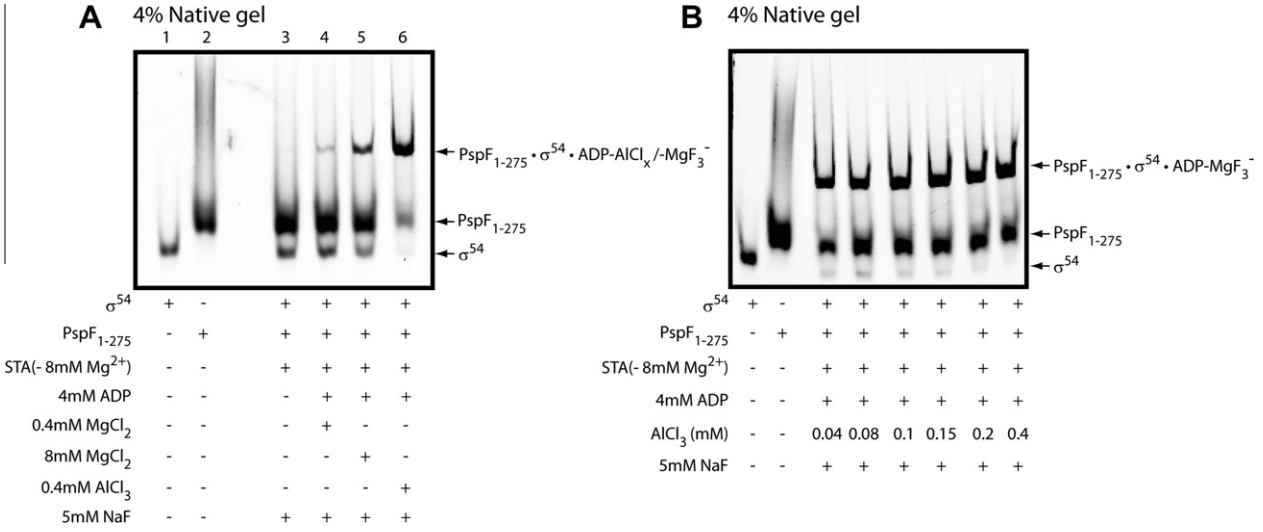

Fig. 3. (A) Al^{3+} ions are more efficient at promoting the trapped complex formation. (B) Titration of the amount of Al^{3+} ions required for trapped complex formation.

similarities to either ADP–AlF_x or ADP–BeF_3^- dependent complexes.

In the presence of a dinucleotide primer UpG and radio-labelled GTP, abortive tetra-nucleotides UpGpGpG are generated on the linear *nifH* promoter by preventing $E\sigma^{54}$ from transcribing beyond the +3 site. The amount of UpGpGpG formed reflects the amount of RP_O-like activity in the presence of ADP-metal fluorides. Consistent with the previous data, the ADP–AlF_x dependent complex was able to generate an RP_O-like activity from the pre-melted DNA probe (Fig. 4). However, the ADP–MgF_3^--dependent complex failed to yield RP_O-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_x.

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 σ^{54}. The MgF_3^--dependent complexes can co-exist in solution with the AlF_x-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_x 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_x may interfere with protein crystallisation. Based on the pH effect [3], we reason that the AlF_x moiety under the trapping conditions in this work (pH 8.0) is more likely to assume a trigonal bipyromidal $AlCl_3$ 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_4^- 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 ATPases.

Vincent et al. suggested that additional mechanistic roles could be assigned to the MgF_3^- moiety. Their observation of MgF_3^--dependent GTPase–GAP complex formation in the absence of GDP challenges the widely held γ-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 σ^{54}, suggesting that MgF_3^- in AAA^+ ATPases is confined to function solely as a γ-phosphate mimick. We reason that in contrast to the relatively 'simple' GTP catalytic site between the GTPase–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 $PspF_{1-275}$–σ^{54} complex formation than is the AlF_x moiety (14% by BeF_3^- and 16% by MgF_3^- in comparison to 100% by AlF_x), and are unable to productively reorganise RP_C to yield an RP_O-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 bipyromidal 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 $PspF_{1-275}$–σ^{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^+ ATPases and their targets in pre-hydrolysis state.

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