Heterogeneous Nucleotide Occupancy Stimulates Functionality of Phage Shock Protein F, an AAA+ Transcriptional Activator*

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The catalytic AAA+ domain (PspF1–275) of an enhancer-binding protein is necessary and sufficient to contact ω54-RNA polymerase holoenzyme (E054), remodel it, and in so doing catalyze open promoter complex formation. Whether ATP binding and hydrolysis is coordinated between subunits of PspF and the precise nature of the nucleotide(s) bound to the oligomeric forms responsible for substrate remodeling are unknown. We demonstrate that ADP stimulates the intrinsic ATPase activity of PspF1–275 and propose that this heterogeneous nucleotide occupancy in a PspF1–275 hexamer is functionally important for specific activity. Binding of ADP and ATP triggers the formation of functional PspF1–275 hexamers as shown by a gain of specific activity. Furthermore, ATP concentrations congruent with stoichiometric ATP binding to PspF1–275 inhibit ATP hydrolysis and E054-promoter open complex formation. Demonstration of a heterogeneous nucleotide-bound state of a functional PspF1–275:E054 complex provides clear biochemical evidence for heterogeneous nucleotide occupancy in this AAA+ protein. Based on our data, we propose a stochastic nucleotide binding and a coordinated hydrolysis mechanism in PspF1–275 hexamers.

The members of the functionally versatile AAA+ (ATPases associated with various cellular activities)3 protein family are found in all kingdoms of life. Activities include cell division, cell differentiation, and transcription activation (1–4). AAA+ proteins form hexameric rings in their active conformation, often assembled from inactive dimers (7–9). The nucleotide binding sites of AAA+ proteins lie at the interface between subunits permitting determinants from adjacent subunits to contribute to nucleotide hydrolysis. The energy derived from NTP hydrolysis is usually coupled to substrate remodeling and functional output (6).

In AAA+ proteins the more general unresolved question is if and how nucleotide binding and hydrolysis are coordinated within hexameric AAA+ ring structures for their biological output. Founded on studies of the nucleotide occupancy, two basic models may serve to distinguish how AAA+ molecular machines function (10). The homogeneous nucleotide occupancy observed in a large number of AAA+ crystal structures (11–13) supports a concerted ATP hydrolysis cycle, where all subunits hydrolyze ATP simultaneously. Other structures showed sub-stoichiometric and mixed nucleotide occupancy within the hexameric ring (14, 15), which supports either a sequential or a rotational hydrolysis mechanism where heterogeneous nucleotide occupancy is coordinated between subunits.

One subfamily of AAA+ proteins, the enhancer-binding proteins (EBPs), also termed ω54 activators, activate bacterial genes transcribed from ω54-dependent promoters (for review, see Refs. 16 and 17)). ATP hydrolysis by the AAA+ domain of EBPs is used to isomerize the E054-closed promoter complex to a transcriptional competent open complex (17, 18).

One well studied example of EBPs, PspF (phage shock protein F), from Escherichia coli comprises (i) a catalytic AAA+ domain (PspF1–275), which is (as for a number of EBPs) sufficient to activate transcription of ω54-dependent promoters in vivo and in vitro (19–22), and (ii) a C-terminal helix-turn-helix domain, which binds upstream activator sequences (20, 23–25). For EBPs XylR (26) and NtrC (27), the helix-turn-helix domain when bound to their respective upstream activator sequences aid high order oligomer formation in combination with nucleotides. The E054 transcriptional activator PspF activates transcription of Psp regulon genes from pspA–E and pspG promoters and is negatively regulated by PspA (28, 29) (for review, see Ref. 30).

Chaney et al. (31) showed that PspF1–275 with ATP hydrolysis transition state analogue ADP-AlF4– (non-hydrolyzable) forms stable hexamers that efficiently engage with ω54 or E054. Bordes et al. (32) provided evidence that the consensus GAFTGΔ loop motif in PspF1–275 was responsible for this binding interaction with ω54. The ADP-AlF4–:PspF1–275:E054 trap complex appears to be an intermediate en route for open complex formation. However, the ADP-AlF4–:PspF1–275:E054 pro-

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3 The abbreviations used are: AAA+ proteins, ATPases associated with various cellular activities; EBP, enhancer-binding protein; E054, ω54-RNA polymerase holoenzyme; ITC, isothermal titration calorimetry; PspF, phage shock protein F; ATPyS, adenosine 5′-O-(thiotriphosphate); AMPPNP, adenosine 5′-i(β,γ-imido)triphosphate.
Heterogeneous Nucleotide Occupancy in a AAA+ Activator

motor complex cannot isomerize to an open complex (4, 31, 33–35). Recent structural studies of a ADP-AlF₃-PspF₁₋₂₇₅-Δ₁⁻⁴ complex revealed that the connecting densities between a hexamer PspF₁₋₂₇₅, and Δ₁⁻⁴ critically involve some but not all of the GAFTGA loops from some but not all AAA+ domains, suggesting an asymmetrical structure (36). A model based on the crystal structures of PspF₁₋₂₇₅ soaked with different nucleotides suggested a molecular mechanism within a PspF subunit by which ATP binding and hydrolysis could coordinate movements of the GAFTGA loop (37). How and if movements between PspF₁₋₂₇₅ subunits are coordinated throughout nucleotide binding and hydrolysis to orchestrate a sequential multiphase structural remodeling of Δ₁⁻⁴-closed promoter complex is unclear.

We now report biochemical data to address relationships between nucleotide occupancy and functionality of PspF₁₋₂₇₅. We determined the relative affinity for different nucleotides and observed only modest cooperative binding of ATP and ADP to PspF₁₋₂₇₅. We show by gel filtration that PspF₁₋₂₇₅ is in equilibrium between different oligomeric states and that either ATP or ADP binding shifts this equilibrium toward higher order oligomers, most probably hexamers. Strikingly, we found that physiologically ADP concentrations stimulate the intrinsic ATPase rates of oligomeric PspF₁₋₂₇₅, suggesting that heterogeneous nucleotide occupancy could play a functional role in the catalytic function of this AAA+ protein. Further support for mixed nucleotide binding comes from our finding that ATP at concentrations above those found in E. coli and where ATP possibly occupies all the binding sites in the PspF₁₋₂₇₅ oligomer inhibit ATP hydrolysis and transcriptional activation. Heterogeneous nucleotide occupancy is also evident in the functionally significant ADP-AlF₃-PspF₁₋₂₇₅-EΔ₁⁻⁴ complex. Simultaneous binding of ADP and ATP within a PspF₁₋₂₇₅ hexamer clearly increases functionality at physiological nucleotide concentrations. Our data support probabilistic nucleotide binding in PspF₁₋₂₇₅ hexamers with a coordinated nucleotide hydrolysis mechanism. We propose that the mechanical actions used for making open promoter complexes arise from asymmetric forms of PspF₁₋₂₇₅ created by differential nucleotide bound states of protomers within a hexamer.

**EXPERIMENTAL PROCEDURES**

Nucleotides—ATP, ADP, AMPPNP, and ATPγS were from Sigma and are at the highest purity level available. Radiolabeled nucleotides were from Amersham Biosciences. Fluorescent DNA probes and oligonucleotides were from Sigma Genosys: WVC7, gaaagaaagccgagatgtttatttttcagacgcgcagctttttgactcgcactagaaggggccgcatcttggtcctgcatctgccattggc; WVC3 HEX, catagtcgcagcagcaggctgtctgtcagcgacagccgctgctagaataaactcgtctttctttc, labeled at 5′.

PspF₁₋₂₇₅-wild type, -K42A, -D107A, and -R168A Plasmids—Plasmid pPB1 encodes E. coli PspF₁₋₂₇₅ with an N-terminal His₉ tag in pET28b (32). Variants of PspF₁₋₂₇₅ were generated from plasmid pPB1 mutagenized to yield pPB1-K42A, pPB1-D107A, and pPB1-R168A (38).

Protein Purification—PspF₁₋₂₇₅-wild type, -K42A, -D107A, and -R168A were purified as described in Bordes et al. (32) from, respectively, pPB1, pPB1-K42A, pPB1-D107A, and pPB1-R168A, respectively. Briefly, 1 liter of LB media was inoculated with an overnight culture (2% v/v) and grown at 37 °C until an A₆₀₀nm of 0.4–0.6. After down-shift of temperature to 25 °C, the protein production was induced with 1 mM final concentration of isopropyl thiogalactoside for 3 h. After centrifugation, cells were resuspended in buffer A (25 mM sodium phosphate buffer, pH 7.0, 50 mM NaCl, and 5% glycerol) and broken by sonication. The supernatant was loaded onto a 5-ml HiTrap™ chelating high performance column (Amersham Biosciences) precharged with nickel and purified as described in Bordes et al. (32). The His tag was removed by thrombin cleavage for 3 h at 23 °C. Finally the protein was dialyzed overnight at 4 °C against final storage buffer (20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM dithiothreitol, 0.1 mM EDTA, and 5% glycerol) and frozen at −80 °C. Δ₁⁻⁴ was purified as described (18). RNA polymerase core enzyme from E. coli was purchased from Epicenter.

**ATPase Activity**—ATPase activity assays were performed in a 10-μl final volume in buffer containing final concentrations of 35 mM Tris acetate, pH 8.0, 70 mM potassium acetate, 15 mM magnesium acetate, 19 mM ammonium acetate, 0.7 mM dithiothreitol, and different concentrations of PspF₁₋₂₇₅. The mix was preincubated at 23 °C for 10 min, and the reaction was started by adding 3 μl of an ATP solution containing 0.6 μCi/μl of [α-³²P]-ATP (3000 Ci/mmol) plus different concentrations of ATP, ADP, AMPPNP, or AMP and incubated for different times at 23 °C. In the case of ADP, AMPPNP, or AMP competition, a fixed concentration of substrate ATP (1 μM) was chosen to minimize the contribution of ATP from [α-³²P]-ATP. Reactions were stopped by adding 5 volumes of 2 M formic acid. [α-³²P]-ADP was separated from ATP by thin-layer chromatography, and radiolabeled ADP and ATP were measured by phosphorimaging (Fuji Bas-1500) and analyzed using the Aida software. Activity is expressed in turnover per minute. Reactions were stopped when around 20% of total ATP was hydrolyzed to keep the same proportion of ADP present in all reactions. The fitting curves were obtained by using the automatic sigmoidal fitting equation on Origin 7.0 software (OriginLab Corp.). All experiments were done at least in triplicate independently and gave the same results. In addition we established (data not shown) that the rate of ATP hydrolysis was linear under assay conditions.

Isothermal Titration Calorimetry (ITC)—ITC experiments were conducted using a MicroCal VP isothermal titration calorimeter. PspF₁₋₂₇₅ was dialyzed overnight at 4 °C immediately before use in 20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 10 mM MgCl₂. After degassing each sample under vacuum, each nucleotide solution (in dialysis buffer) containing ATPγS, ADP, or AMPPNP (8 mM) was titrated into the protein solution (99 μM) in 70 injections of 4 μl (300 s). Raw data for 70 injections at 37 °C were obtained by using the MicroCal VP-VIEWER software. Control titrations of nucleotide into dialysis buffer demonstrated that there was no significant heat of dilution or injection for any of the tested nucleotides (data not shown). ITC data were corrected for heats of injection of nucleotide solution. Binding stoichiometry, enthalpy, entropy and binding constants were determined by fitting the corrected data to a one site binding model. The ITC data were fitted using Origin 7.0
(OriginLab Corp.). All titration were performed at least twice independently. The resultant fitting value was exactly the same.

**ADP-AlF Trapping**—ADP-AlF₆ trapping experiments were performed in a 10–/H9262 l volume with final concentrations of 10 mM Tris acetate, pH 8.0, 50 mM potassium acetate, 8 mM magnesium acetate, 0.1 mM dithiothreitol, 5 mM NaF, and different concentrations of PspF₁–275/H₁₁₀₀₁/H₁₁₀₀₂/H₉₂₆₈₅₄/H₁₁₀₀₁/H₁₁₀₀₂ RNA polymerase core enzyme. The mix was then preincubated at 23 °C for 10 min, and the reaction was started by the addition of 1 μl of mix nucleotide containing 4 mM concentrations of either ADP or ATP or AMPPNP. For radiolabeled trapping experiments, this mix contained 4 mM ATP with either 20 Ci of [γ⁻³²P]ATP (3000 Ci/mmol) or 20 Ci of [α⁻³²P]ATP (3000 Ci/mmol). AlCl₃ (0.4 mM, final concentration) was then added, and the reaction was incubated for 5 min at 23 °C. After adding of 2 μl of loading buffer (50% glycerol, bromphenol blue), all of the sample was loaded onto native 4.5% polyacrylamide gel (acrylamide/bisacrylamide, 37.5:1) and run in 25 mM Tris, pH 8.3, 192 mM glycine (TG buffer). Proteins were detected by Coomassie Blue staining, and radioactivity was measured by phosphorimaging (Fuji Bas-1500) and analyzed using the Aida software.

**Gel Filtration through Superdex 200**—PspF₁–275 wild type or R168A (at different concentrations) were incubated for 3 min at 4 °C in buffer containing 20 mM Tris-HCl, pH 8.0, 50 mM NaCl, and 15 mM MgCl₂. +/− 1 mM ATP or ADP where indicated. Samples (50 μl) were then injected onto a Superdex 200 column (10 × 300 mm, 24 ml) (Amersham Biosciences) installed on an AKTA system (Amersham Biosciences) and equilibrated with the sample buffer. Chromatography was performed at 4 °C at a flow rate of 0.5 ml/min⁻¹, and columns were calibrated with globular proteins: apoferritin (443 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa).

**Native Gel Complex Formation Assays**—Heparin challenge experiments were performed in a 10–/H9262 l reaction volume with final concentrations of 10 mM Tris acetate, pH 8.0, 50 mM potassium acetate, 8 mM magnesium acetate, 0.1 mM dithiothreitol, 0.3 μM RNA polymerase, 0.3 μM PspF₁–275, 0.2 μM fluorescent-labeled DNA heteroduplex (HEX-labeled) assembled from purified oligonucleotides WVC7/WVC3HEX, 2 μM PspF₁–275, and varying concentrations of ATP. The mix was preincubated at room temperature for 10 min, and reactions were then started by the addition of 1 μl of ATP solution at different concentrations and incubated for 1 min at 23 °C; 2 μl of a stop mix containing heparin (1 mg/ml) in loading buffer were then added to the reaction mixture. The competitor challenging reaction was performed for 2 min at 23 °C, and all samples were loaded onto a running (to further stop the reaction) native 4.5% polyacrylamide gel (acrylamide/bisacrylamide 37.5/1) and run in 1× TG buffer. Fluorescence was directly scanned by fluoroimaging (Fuji Bas-1500) and analyzed using the Aida software.

**FIGURE 1.** *ITC. A*, ITC curve after titration of 99 μM PspF₁–275 with ATPγS (8 mM) in 70 injections of 4 μl (300 s) at 37 °C. *B*, ITC curve obtained after titration of 99 μM PspF₁–275 with ADP (8 mM) in 70 injections of 4 μl (300 s) at 37 °C. ITC data were corrected for heats of injection of nucleotide solution (up to 5% of the signal).
RESULTS

PspF1–275 Has Low Binding Affinity for Nucleotides—Nucleoside triphosphate binding has been shown to promote oligomer assembly and/or substrate binding in a number of AAA+ proteins (39–41). For some AAA+ proteins, using ITC, ADP and ATPγS dissociation constants (K_d) have been reported in the low μM range (see Ref. 8 for p97 and Ref. 42 for replication factor C). A much higher K_d (90 μM) for ATPγS was reported for the EBP NtrC (43). A high K_d for EBP would agree with the proposed high nucleotide off-rate for ATP (41, 43). We have used ITC to determine the affinity constant of different nucleotides for PspF1–275 in the presence of magnesium at 37 °C (Fig. 1). We have determined a K_d of 34 μM for ATPγS with a Hill constant of 1.33 (Fig. 1A). The K_d for ADP was 118 μM with a Hill constant of 1.33 (Fig. 1B), indicating that nucleotide binding sites of PspF1–275 within the oligomer have a very low cooperativity for binding ATPγS or ADP. Schumacher et al. (38) showed a 50% loss of PspF1–275 ATPase activity in the presence of a 3 ADP to 1 ATP ratio, in agreement with 3-fold higher affinity for ATP compared with ADP. The 3-fold higher K_d for ADP compared with ATPγS suggests that affinities for ATPγS and ATP are similar. Taken together, these results show that the affinity of EBPs for ADP and ATP are significantly lower than those reported for several other AAA+ proteins. This would explain why we could not obtained faithful heat readings in ITC experiments at low nucleotide concentrations (molar ratio <1). Therefore, we did not determine stoichiometry constants for these nucleotides. We tested AMP binding to PspF1–275 using ITC. No change in energy could be detected, suggesting that PspF1–275 has a very low affinity for AMP. This is consistent with results from AMP competition experiments in ATPase assays with PspF1–275 where a 100,000-fold excess of AMP over ATP was required to partly inhibit ATP hydrolysis (data not shown).

The PspF1–275 Catalytic AAA+ Domain Forms an Apo-hexamer—AAA+ proteins usually function as hexameric ring assemblies (for review, see Ref. 4). ATP binding is thought to promote hexamer assembly from lower oligomers (39), but hexamer formation using a physiologically relevant ATP concentration has not been demonstrated for any EBP, probably due to the high off-rate for ATP (see above) and/or turnover (turnover around 23 min⁻¹; see below). High order oligomer formation from PspF1–275 lower-order oligomers was suggested by the strong concentration-dependent ATPase activity of PspF1–275. To date the only nucleotide reported to lead to stable PspF1–275 hexamer formation is ADP-AlF₆⁻, a non-hydrolyzable ATP hydrolysis transition state mimic used in nucleotide saturating conditions (31, 37).

We performed gel filtration experiments to detect different discrete oligomeric states of PspF1–275 (Fig. 2). At low concentrations of PspF1–275 (9 μM) the major form of PspF1–275 elutes at 13.76 ml, which corresponds, based on reference elution volumes obtain for different protein standards (Fig. 2A), to an elution volume expected for an apparent dimer of PspF1–275 (66 kDa). At increasing concentrations of PspF1–275, the apparent number of PspF1–275 monomers (33 kDa) present in the major complex increases from 2 (66 kDa) to a maximum of 6 (198 kDa) (Fig. 2B).

ATP or ADP Favors Hexamer Formation—To examine the effect of physiologically occurring ATP concentrations on oligomer formation, we performed gel filtration experiments using a column pre-equilibrated with 1 mM ATP at 4 °C. In the presence of ATP, PspF1–275 elutes as an apparent octamer (264 kDa) independently of its concentration (Fig. 2C), demonstrating that ATP promotes and stabilizes a limited high-order oligomer form of PspF1–275. PspF1–275-R168A, a protein that forms constitutive hexameric rings in the absence of nucleotide (deduced from electron microscopy, negative staining) and whose crystal structure is almost indistinguishable from the reported hexameric structure of PspF1–275 (36, 38, 41), also elutes as an apparent octamer (264 kDa) in gel filtration (data not shown). Furthermore, Rappas et al. (36) have established by nanoelectrospray mass spectroscopy that there are six PspF1–275 monomers and one σ₅₄ in the ATP-AlF₆⁻·PspF1–275σ₅₄ complex. This 252-kDa complex elutes with an apparent size of 351 kDa (data not shown). Nucleotide-dependent hexamer assembly has also been shown for the EBP NtrC (44). Therefore, we conclude that ATP-PspF1–275 is hexameric. The differences in retention between high concentrations of apo-PspF1–275 and ATP-PspF1–275 could be due to structural changes induced by nucleotide bound to the oligomer and/or changes in the stability of oligomers formed. Although anticipated, ATP-dependent hexamer formation was not previously shown for an EBP.

As a control experiment we also performed gel filtration of PspF1–275 using a 1 mM ATP pre-equilibrated column. Surprisingly, ADP also promoted high order oligomer formation (Fig. 2D). The elution volumes of PspF1–275 in the presence of either ATP or ADP are equal, clearly demonstrating that ADP triggers hexamer formation of PspF1–275. ADP-dependent higher order oligomer formation has not been reported for any σ₅₄ activator. We speculate that ADP-promoted hexamer formation could have a mechanistic role and a positive effect on one or more functional PspF1–275 activities.

ATP and ADP Can Stimulate ATPase Activity of PspF1–275—Influences of nucleotide upon any intrinsic functional activities of PspF1–275 have not yet been reported. Nucleotide (ATP or ADP)-triggered hexamer formation of PspF1–275 (Fig. 2, C and D) and hydrolysis of ATP are essential for EBPs to activate transcription. We addressed the issue of the importance of hexamer formation for PspF1–275 ATPase activity and whether ADP-aided hexamer formation could also support ATP hydrolysis.

We performed ATPase activity assays using PspF1–275 in the presence of ATP. First, we reproduced the same concentration dependent sigmoidal activity curve with PspF1–275 (no His tag) as observed with His-PspF1–275 (Fig. 3A and data not shown). The six-histidine tag fused to PspF1–275 does not obviously lead to a change in kinetic properties of PspF1–275 ATPase activity.

FIGURE 2. PspF1–275 filtration through a Superdex 200 column. A, calibration of the Superdex 200 filtration column. Standard globular proteins were filtered at 4 °C. Samples containing different concentrations of PspF1–275 were chromatographed at 4 °C (B) or preincubated with 1 mM ATP and chromatographed in the presence of 1 mM ATP at 4 °C (C) or 1 mM ADP and chromatographed in the presence of 1 mM ATP at 4 °C (D). The scale bars give the scale of the ordinate axis; absorption units (AU) correspond to an A₂₈₀ of 1. WT, wild type.
Heterogeneous Nucleotide Occupancy in a AAA+ Activator

FIGURE 3. ATPase activity of PspF₁₋₂₇₅. ATPase assays of PspF₁₋₂₇₅ were performed with different concentrations of PspF₁₋₂₇₅ in the presence of different concentrations of ATP (A) or ADP (B) as indicated with a fixed concentration of ATP as substrate (1 μM). ATPase activity of PspF₁₋₂₇₅ was performed at 23 °C, and turnover was calculated when the radiolabeled ADP formed was around 20% of total nucleotide.

FIGURE 4. Inhibitory effect of ATP on the ATPase activity of PspF₁₋₂₇₅. ATPase assays of PspF₁₋₂₇₅ were performed at 23 °C with 2 μM PspF₁₋₂₇₅ in the presence of different concentrations of ATP, and turnover was calculated when the radiolabeled ADP formed was around 20% of total nucleotide.

ATPase activity suggests that nucleotide binding and not hydrolysis per se affects the equilibrium between the different conformational states of the protein and that it favors an active PspF₁₋₂₇₅ hexamer (competent for ATP hydrolysis).

High Concentrations of ATP Inhibit Transcriptional Activator Function of PspF₁₋₂₇₅—Heterogeneous ATP and ADP occupancy within a hexamer of PspF₁₋₂₇₅ leads to an increase in ATPase activity. To determine a precise effect of ATP concentrations on the ATPase activity of the hexamer (and not the stimulation of hexamerization), we performed ATPase assays in the presence of different ATP concentrations at a fixed PspF₁₋₂₇₅ concentration (2 μM). Initial rates of ATP hydrolysis increase linearly at ATP concentrations in the range of 0.001 to 0.1 mM (Fig. 4 and data not shown), implying that nucleotide binding has a positive effect on PspF₁₋₂₇₅ ATPase activity. Maximum turnover (Kcat) of 23 min⁻¹ (Fig. 3A and 4) is reached between 0.5 and 2 mM ATP (Fig. 4). At higher ATP concentrations, however, we observe a marked decrease of ATPase activity with little activity detectable above 4 mM ATP (Fig. 4). To exclude the possibility that ATP concentrations above 2 mM would irreversibly inhibit PspF₁₋₂₇₅ activity by altering its tertiary or quaternary structure, an ATPase reaction at 4 mM ATP was carried out and diluted 2-fold after 30 min. Maximal ATPase activity was recovered after dilution. Given the unexpected inhibition by ATP, we tested whether this loss of PspF₁₋₂₇₅ ATPase activity was reflected in ATP-independent open complex formation assays.

ATP- and PspF₁₋₂₇₅-dependent open complex formation can be measured by quantifying heparin stable Eo₅₄-open promoter complexes on native gels (45). In this experiment we can measure the amount of labeled DNA that forms an isomerized complex with Eo₅₄ after activation by the hydrolysis of ATP by PspF₁₋₂₇₅. At low ATP concentrations we detected the appearance of a heparin-resistant protein DNA complex; that is, the open complex (Fig. 5). When ATP concentrations are increased (above 2 mM), the amount of this complex decreases (Fig. 5), in complete agreement with the observed inhibition of the ATPase activity (Fig. 4). To ensure that the presence of different concentrations of nucleotide does not affect the binding of Eo₅₄ to the promoter DNA the same experiment was performed with different concentrations of ATP but in the absence of heparin (data not shown). In this case all the DNA is fully bound to Eo₅₄ (similar to Fig. 5, lane...
Taken together, the ITC results, gel filtration results, and ATPase assays strongly suggest that PspF\textsubscript{1–275} hexamers will contain both ATP and ADP simultaneously. This is in agreement with the observation that total nucleotide occupancy during ATP hydrolysis by PspF\textsubscript{1–275} is maximal when ATP and ADP are present, as suggested by UV cross-linking experiments with [$\alpha$-\textsuperscript{32}P]ATP (38).

Heterogeneous Nucleotide Present in PspF\textsubscript{1–275} Hexamer—

Stable complexes between EBP and $\sigma^{54}$, bound or not to promoter DNA, are observed in the presence of the non-hydrolyzable ATP transition state analogue ADP-AlF\textsubscript{3} (31). This in situ “trapping,” using ADP, NaF, and AlCl\textsubscript{3}, is experimentally a nucleotide hydrolysis-independent outcome, and the resulting complex represents a useful tool to study functional intermediates of the ATP hydrolysis driven open complex transition. We postulated that it should be possible to trap PspF\textsubscript{1–275} in a productive interaction with $\sigma^{54}$ or E$\sigma^{54}$ during ATP hydrolysis and to determine the nucleotides bound.

We incubated PspF\textsubscript{1–275} with $\sigma^{54}$ or E$\sigma^{54}$ with ATP before the addition of trapping reagents, assuming that the ADP produced during hydrolysis would become trapped with AlF\textsubscript{3}, in some binding pockets of the hexamer, whereas an other nucleotide binding pocket would potentially still be able to bind (and possibly hydrolyze) the remaining ATP present in the reaction mixture. Under ATP-hydrolyzing conditions, we detected a “trapped complex” of PspF\textsubscript{1–275} in the presence of either $\sigma^{54}$ or E$\sigma^{54}$, as formed with ADP-AlF\textsubscript{3}, as supplied nucleotide (Fig. 6A, lanes 2 and 5 and lanes 3 and 6, respectively). Two control experiments show that the trapped complexes depend on the hydrolysis product ADP and not ATP. First, the non-hydrolyzable ATP analogue AMPPNP in the presence of trap reactants (NaF and AlCl\textsubscript{3}) did not result in stable complex formation with $\sigma^{54}$ or E$\sigma^{54}$ (lanes 8–9). Second, the ATP hydrolysis but not ATP binding-deficient Walker B motif variant, PspF\textsubscript{1–275-D107A}, can still form (albeit reduced) trapped complexes in the presence of $\sigma^{54}$ or E$\sigma^{54}$ with ADP but not with ATP (Fig. 6B, lanes 2 and 3 and lanes 5 and 6, respectively). Taken together these results suggest that ADP-AlF\textsubscript{3} is generated in situ after ATP hydrolysis.

To detect which nucleotides are present in the “ATP-trapped” PspF\textsubscript{1–275}-$\sigma^{54}$ or PspF\textsubscript{1–275}-E$\sigma^{54}$ complexes, we carried out trapping reactions in the presence of either [$\alpha$-\textsuperscript{32}P]ATP or [$\gamma$-\textsuperscript{32}P]ATP followed by native PAGE (to resolved the trapped complexes) and visualization of radioactive signal in complexes (Fig. 7). In the presence of [$\gamma$-\textsuperscript{32}P]ATP, the [$\gamma$-\textsuperscript{32}P] radioactivity, which is most likely due to the presence of non-hydrolyzed ATP, is found in complexes formed with either $\sigma^{54}$ or E$\sigma^{54}$ (lanes 2 and 3). This suggests that heterogeneous nucleotide occupancy exists in PspF\textsubscript{1–275} when in complex with $\sigma^{54}$ and E$\sigma^{54}$, as [$\gamma$-\textsuperscript{32}P]ATP-PspF\textsubscript{1–275}-ADP-AlF\textsubscript{3}.

As a control, we conducted a trapping with [$\alpha$-\textsuperscript{32}P]ATP. In this case we observed a much stronger radioactive signal within $\sigma^{54}$- or E$\sigma^{54}$-dependent stable complexes compared with [$\gamma$-\textsuperscript{32}P]ATP (Fig. 7). The difference in signal could be explained by the accumulation of both [$\alpha$-\textsuperscript{32}P]ADP-AlF\textsubscript{3} and [$\alpha$-\textsuperscript{32}P]ATP. Furthermore the [$\gamma$-\textsuperscript{32}P]ATP signal decreases at longer reaction times presumably due to the complete hydrolysis of ATP, suggesting that
Heterogeneous Nucleotide Occupancy in a AAA+ Activator

FIGURE 7. Gel mobility shift assay for ADP-AlF₃-dependent complex formation with PspF₁₋₂₇₅ in the presence of radiolabeled nucleotide. Reactions contained PspF₁₋₂₇₅ (7.1 μM), NaF (5 mM), AlCl₃ (0.4 mM), and when indicated, ATP (0.3 μM), with or without RNA polymerase (pol) core enzyme (0.3 μM) and, as indicated, cold ATP (0.4 mM) plus γ-32P-labeled ATP or α-32P-labeled ATP. Assays were performed at 23 °C, and radioactivity was detected by phosphorimaging.

γ-32P is readily released (data not shown). In contrast, the radiolabel signal obtained when using [α-32P]ATP increases with time, presumably due to the accumulation of stable radiolabeled [α-32P]ADP-AlF₃. Control ATPase assays of PspF₁₋₂₇₅ in the presence of either ATP or ADP showed no detectable increase in ATPase activity. The presence of either ATP or ADP does not affect the steady state ATPase activity of PspF₁₋₂₇₅ and does not change the nucleotide occupancy in the trapped complex. Clearly, stable ADP-AlF₃-PspF₁₋₂₇₅-α-32P-dependent complexes can be formed with ATP and ADP-AlF₃, bound simultaneously in PspF₁₋₂₇₅ nucleotide binding sites.

DISCUSSION

For AAA+ proteins, nucleotide hydrolysis is the major event that promotes their specific biological activities, for example, transcription initiation (NtrC or PspF) or protein degradation (ClpX/P). In this work we show, that (i) nucleotides (ATP and ADP) also play a role in the conformational rearrangement of PspF₁₋₂₇₅ by promoting and stabilizing the functional hexamer form, (ii) the hexamer form of PspF₁₋₂₇₅ is the most active and is inferred to be the functional form, and (iii) ADP and ATP can be present at the same time in a PspF₁₋₂₇₅ hexamer and influence its catalytic properties.

ATP and ADP Promote Higher Order Oligomer Formation but Not Stable Interactions with α-32P—AAA+ proteins often assemble into oligomeric structures (1, 39), and in most cases hexamers are thought to represent the active conformation (for review, see Refs. 4 and 5). We have shown that in solution PspF₁₋₂₇₅ can form higher order oligomers in the absence of nucleotide and that ATP and, notably also, ADP promote hexamer formation of PspF₁₋₂₇₅. This indicates that nucleotide binding, but not necessarily hydrolysis, strongly increases self-association of PspF₁₋₂₇₅. During gel filtration experiments we estimated the dilution effect due to physical properties of the chromatography column and dynamic PspF₁₋₂₇₅ self-association/dissociation by comparing theoretical and experimental A₂₈₀ nm. An approximate 10-fold dilution probably occurs, although precise dilution coefficients vary somewhat depending on the elution volumes. When applying this estimate, the range of concentrations (0.9 to 7.1 μM) at which apparent intermediate subunit composition oligomers of PspF₁₋₂₇₅ elute during gel filtration overlaps with those at which we observe a PspF₁₋₂₇₅ concentration-dependent ATPase activity (0.2–2 μM). Differences can be explained by (i) the requirement of ATP during ATPase activity assays, which shifts the self-association equilibrium of PspF₁₋₂₇₅ toward the higher order oligomer species and (ii) the difference of sensitivity in each assay. Structures of AAA+ proteins, including PspF₁₋₂₇₅ showed that the catalytic site for hydrolysis lies at the interface between two subunits (8, 12, 24, 37, 40). We propose that in the case of PspF₁₋₂₇₅, the presence of nucleotide modulates this interface to promote and stabilize the formation of a functional hexamer.

A direct role of nucleotide in ATP-triggered hexamerization has been shown for a few AAA+ proteins, for example RuvB (39), and to our knowledge direct evidence illustrating ADP-induced hexamerization in solution has not been reported for any AAA+ protein. In a number of cases, AAA+ protein oligomer assembly is aided by domains that lie outside the AAA+ domain, and substrate binding by these domains can also promote hexamer assembly (2, 15, 26, 27, 46). A similar role was suggested for the helix-turn-helix domain of PspF (20). The tendency of PspF₁₋₂₇₅ to form hexameric assemblies independent of added nucleotides points to the propensity of the AAA+ domain to self-associate into hexameric rings in the full-length protein. Full-length His₆-PspF does not show the protein concentration-dependent increase of ATPase activity (i.e. maximal activity was observed for all tested concentrations of PspF (38)), suggesting that the helix-turn-helix domain contributes positively to oligomerization. However, nucleotide binding in the AAA+ domain of PspF is sufficient for hexamer formation and ATP hydrolysis. This is in agreement with the activity of this domain shown in vivo and in vitro (20).

We do not detect α-32P in the peak corresponding to the PspF₁₋₂₇₅ hexamer when gel filtrating PspF₁₋₂₇₅ mixed with α-32P in the presence of nucleotide (ATP or ADP), although PspF₁₋₂₇₅ can promote EΔ₅₄-promoter isomerization when in the presence of ATP alone. Also, we only detect a stable PspF₁₋₂₇₅-α-32P complex when we preincubate PspF₁₋₂₇₅ and α-32P in the presence of ADP-AlF₃. These results are consistent with results from native gel experiments where we did not detect a PspF₁₋₂₇₅-α-32P complex in the absence of ADP-AlF₃ (Ref. 31 and data not shown). We infer from these results that interactions between PspF₁₋₂₇₅ and α-32P are transient under ATP binding conditions. We expect PspF to exist mainly as hexameric form in vivo due to (i) the optimal nucleotide concentrations and (ii) the hexamer form of PspF being necessary for efficient promoter activation.

ADP Has a Functional Role in the PspF₁₋₂₇₅ Hexamer Activity—ADP-dependent higher order oligomerization has not been reported for any α-32P activator, and a possible functional role for this effect has not been established. Gel filtration experiments have shown that ADP stimulates and stabilizes the hexameric form of PspF₁₋₂₇₅. At PspF₁₋₂₇₅ Concentrations between 0.5 and 1.5 μM, we have shown an ADP-dependent increase of ATPase activity (as observed with ATP), providing strong evidence that ADP-induced hexamer formation results in an increase of ATPase activity and suggests that ADP and ATP
bind simultaneously to the same PspF_{1–275} hexamer. Importantly, in the presence of ADP, PspF_{1–275} ATP turnover rates are higher than in the absence of ADP and are independent of PspF_{1–275} hexamerization (Fig. 3B). Therefore, the ATPase activity stimulation due to ADP binding is probably due to structural changes, which might positively influence the ATP hydrolysis rate of some subunits within the same hexamer. This ADP-dependent increase in the ATPase activity could in principle be a consequence of ADP-induced structural changes that increase ATP binding to adjacent subunits. However, this possibility seems unlikely given that turnover rates are constant over time and occur under conditions where ADP is produced.

At higher ADP concentrations (Fig. 3, 1 mM ADP), where the maximum of binding sites are occupied (Fig. 1), ADP-induced hexamer formation still stimulates the ATPase activity of PspF_{1–275} over the hexamerization range of PspF_{1–275}. However, at higher PspF_{1–275} Concentrations this positive effect is outweighed by the negative effect of ADP, which competes with ATP for binding sites. Our nucleotide binding and hydrolysis data are in complete agreement with a functionally competent mixed nucleotide bound state of PspF_{1–275}. Taken together, these results show that ADP binding has two effects; (i) it favors and stabilizes hexamer formation, and (ii) it induces structural changes in hexameric PspF_{1–275} that increase the intrinsic ATP hydrolysis rates of other subunits within the hexamer.

We speculate that ADP-promoted hexamer formation and hexamer activity could be consistent with the in vivo physiology of the induction of the Psp regulon. Transcription activation of the Psp regulon genes by PspF is induced by various stresses that dissipate the proton-motive force (47), potentially resulting in changes in the cellular ATP/ADP ratio. The ability of ADP to activate PspF could be valuable during the Psp stress response. If the ATP concentration decreases in favor of ADP, the presence of ADP could yield active hexameric forms of PspF, which can then bind and hydrolyze the ATP to promote transcription activation.

Globally, the EBPs promoting σ^{54}-dependent transcription are frequently implicated in stress response pathways (16). We speculate that a rapid switch between an inactive to an active form could be important. ADP-dependent hexamerization could be a global selective advantage in stress response, where ADP could intrinsically activate an EBP. The activated form could be prepared for binding of, for example, ATP, which can produce (after hydrolysis) energy necessary for physiological output.

**Heterogeneous Nucleotide Occupancy**—At non-saturating ATP substrate concentrations (0.01–0.5 mM), ATP hydrolysis rates increase, in good agreement with the binding curve for ATP/PsP (Fig. 1A), with only moderate cooperativity for ATP binding (n = 1.33) and no obvious cooperativity for hydrolysis (Fig. 4 and data not shown). At optimal substrate concentrations (0.5–2 mM ATP), PspF_{1–275} reaches K_\text{cat}. Surprisingly, turnover rates drop drastically above 2 mM ATP, inconsistent with constant turnover rates at substrate saturation and at ATP concentrations that do not alter the stability of PspF_{1–275}. The estimated concentration of ATP in *E. coli* is 1–2 mM (48), suggesting that the inhibitory effect of ATP seen in *vitro* has no physiological role. However, this effect is important in understanding the ATPase cycle of PspF_{1–275}. It appears that saturation of the nucleotide binding sites by ATP in hexameric PspF_{1–275} inhibits hydrolysis. We infer that hexameric PspF_{1–275} has a catalytic optimum for hydrolysis at a substoichiometric ATP/PspF_{1–275} ratio. It is worth noting that in a PspF_{1–275} crystal soaked with a very high concentration (40 mM) of ATP or ADP, all the binding sites are occupied by the respective nucleotide (37). However, in solution this ATP concentration is inconsistent with PspF_{1–275} activity (total inhibition above 4 mM ATP), and we obtain maximum activity at physiological concentrations of ATP (about 1 mM). Importantly, this ATP concentration optimum is reproduced when examining the capacity to form Eσ^{54}-promoter open complexes, thereby directly linking optimal ATP concentrations with maximum biological output in *vitro*. This strongly suggests that a substoichiometric ATP/PspF_{1–275} ratio can exist when PspF_{1–275} is in complex with its substrate Eσ^{54} bound to its cognate promoter. We infer that the Eσ^{54} promoter DNA complex cannot or cannot entirely counteract suboptimal nucleotide binding in PspF_{1–275}.

**Concerted Versus Non-concerted ATPase Cycle**—To date several models have been proposed to help explain the different ATP hydrolysis mechanisms observed for various members of the AAA + protein family. Structural data have supported a homogenous nucleotide occupancy, which could provide evidence for a concerted ATP hydrolysis mechanism (13), and a heterogeneous and mixed nucleotide occupancy, which favors a non-concerted mechanism (stochastic, sequential, or rotational hydrolysis) (14). To experimentally distinguish between these models has been difficult, especially when hexamer formation is stochastically determined in a substrate- and/or nucleotide-dependent manner, as for σ^{54} activators. Martin *et al.* (49) have overcome this difficulty by covalently linking the σ^{54} bound to its cognate promoter and probing functionality of the resulting hexamer by using mutagenesis. They have elegantly shown that ClpX ATP hydrolysis is neither consistent with a concerted hydrolysis cycle (*i.e.* coordinated) nor with a strictly non-concerted mechanism. The authors proposed a stochastic hydrolysis order by which the best placed subunit within the hexamer would hydrolyze ATP to translocate ClpX substrates.

In the case of PspF_{1–275}, we have shown that a heterogeneous nucleotide occupancy in a hexamer (containing ADP and ATP) correlates with the maximal catalytic activity, whereas high concentrations of ATP inhibit turnover rates and biological output. These results suggest that in a hexameric PspF_{1–275} a positive relationship between bound ADP and ATP hydrolysis exists. We observe maximal hydrolysis rates at ADP and ATP concentrations that exist in bacteria, although we cannot readily draw conclusions about the precise availability and nucleotide chemistry in the cell that may involve additional factors. The simultaneous presence of ATP and ADP within ATP-AlF_{4}PspF_{1–275}σ^{54} complexes and the ADP-dependent stimulation of ATPase activity rule out concerted hydrolysis models in which (PspF_{1–275})_{6}−(ATP)_{6} or (PspF_{1–275})_{6}−(ADP)_{6} are formed during ATP hydrolysis events. Based on our results, we propose the following model for nucleotide binding and hydrolysis activity for a PspF_{1–275} hexamer (Fig. 8). In solution PspF_{1–275} exists in equilibrium between a dimer and a hexamer form (gel filtration data; Fig. 2), although we cannot discount low levels of other self-assemblies being present. Stochastic
binding of ATP (or ADP) to apo-PspF1-275 dimer to hexamer induces the formation of an active hexamer (gel filtration data Fig. 2, B and C). However, an excess of ATP inhibits the ATPase activity, probably due to the saturation of all the nucleotide binding sites (inhibition of ATPase activity; Fig. 4). Hence, we do not favor a synchronized mechanism of ATP hydrolysis where all the sites are simultaneously occupied by the same nucleotide (Fig. 8, iv), a view further supported by our data (Fig. 7) demonstrating heterogeneous nucleotide occupancy in the PspF1-275 hexamer bound to $\sigma^{34}$ and $E\sigma^{54}$. It seems unlikely that a stochastic mechanism for ATP hydrolysis exists for PspF1-275, since in this model all sites should be independent for hydrolysis (Fig. 8, i). In contrast we observe an inhibition of PspF1-275 ATPase activity at high ATP concentrations and a stimulation of this activity by ADP. Therefore we favor a rotational (Fig. 8, ii) or a sequential (Fig. 8, iii) model for ATP hydrolysis. However, we cannot yet differentiate between these two possibilities. Both models are based on cooperativity between subunits within the PspF1-275 hexamer for ATP hydrolysis, which would explain both the “ADP stimulation” and the “ATP inhibition.” Because PspF1-275 functions as a hexamer assembled from identical subunits, there is clear potential for directional communication between subunits and for additive subunit contributions. By analogy with ClpX, a probabilistic sequence of ATP hydrolysis in different subunits of the hexamer is possible for establishing an asymmetric mechanism that could give rise to an asymmetrical exposure of GAFTGA loops in contact with $\sigma^{34}$ (36).

It would be desirable to obtain high resolution crystal structures of mixed nucleotide bound states of PspF1-275 hexamers to advance our understanding of how the functional form of the hexamer is organized at the atomic level. The common property of all AAA+ proteins is to bind and hydrolyze nucleotides. The regulation of the functions of AAA+ proteins could be largely achieved by the control of their intrinsic nucleotide binding and hydrolysis activities. Understanding these processes could provide key insights into how the activity of AAA+ proteins is controlled in a physiological context. Based on heterogeneous nucleotide occupancy, we propose an alternative view about the regulation of intrinsic activity of AAA+ proteins by clearly differentiating binding and hydrolysis. The binding of ATP could be stochastic, but its hydrolysis could be a coordinated event.

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