Sensor I Threonine of the AAA+ ATPase Transcriptional Activator PspF Is Involved in Coupling Nucleotide Triphosphate Hydrolysis to the Restructuring of $\sigma^{54}$-RNA Polymerase

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Transcriptional initiation invariably involves the transition from a closed RNA polymerase (RNAP) promoter complex to a transcriptional competent open complex. Activators of the bacterial $\sigma^{70}$-RNAP are AAA+ proteins that couple ATP hydrolysis to restructure the $\sigma^{54}$-RNAP promoter complex. Structures of the $\sigma^{54}$ activator PspF AAA+ domain (PspF1–275) bound to $\sigma^{54}$ show two loop structures proximal to $\sigma^{54}$ as follows: the $\sigma^{54}$ contacting the GAFTGA loop 1 structure and loop 2 that classifies $\sigma^{54}$ activators as pre-sensor 1 $\beta$-hairpin AAA+ proteins. We report activities for PspF1–275 mutated in the AAA+ conserved sensor 1 threonine/asparagine motif (PspF1–275 T148A, PspF1–275 N149A, and PspF1–275 N149S) within the second region of homology. We show that sensor I asparagine plays a direct role in ATP hydrolysis. However, low hydrolysis rates are sufficient for functional output in vitro. In contrast, PspF1–275 T148A has severe defects at the distinct step of $\sigma^{54}$ promoter restructuring. This defect is not because of the failure of PspF1–275 T148A to stably engage with the closed $\sigma^{54}$ promoter, indicating (i) an important role in ATP hydrolysis-associated motions during energy coupling for remodeling and (ii) distinguishing PspF1–275 T148A from PspF1–275 variants involved in signaling to the GAFTGA loop 1, which fail to stably engage with the promoter. Activities of loop 2 PspF1–275 variants are similar to those of PspF1–275 T148A suggesting a functional signaling link between Thr148 and loop 2. In PspF1–275 this link relies on the conserved nucleotide state-dependent interaction between the Walker B residue Glu108 and Thr148. We propose that hydrolysis is relayed via Thr148 to loop 2 creating motions that provide mechanical force to the GAFTGA loop 1 that contacts $\sigma^{54}$.

Regulation of transcription enables cells to adapt and differentiate through coordination of protein synthesis. Two major mechanisms exist to control gene transcription as follows: one through recruitment to or preventing the RNA polymerase (RNAP) binding to promoter DNA and one through activation or inhibition of the RNA polymerase activity. Despite the variations in subunit numbers that constitute the basal transcription machinery, RNA polymerases are structurally and functionally highly conserved in all kingdoms of life (1). During the initiation of transcription, RNA polymerases invariably melt the promoter DNA and have to engage the single-stranded template DNA in the structurally conserved catalytic cleft (2).

The major variant bacterial $\sigma^{54}$-RNAP is regulated by an activating mechanism with some resemblance to the operation of eukaryotic RNAP II. In both cases, passing from the closed to the open RNAP promoter complex requires activator proteins that hydrolyze nucleoside triphosphate to drive open RNAP promoter complex formation (3). The $\sigma^{54}$-RNAP binds to specific promoter sites centered on positions −24 and −12 relative to the transcription start site and remains in a transcriptionally silent conformation. Open complexes of the $\sigma^{54}$-RNAP promoter complexes are thermodynamically labile, and activation relies on the productive coupling of nucleoside triphosphate hydrolysis (energy coupling) from $\sigma^{54}$ activators to the $\sigma^{54}$-RNAP promoter complex. The $\sigma^{54}$ activator-dependent initial events in open complex formation are mediated by structural changes between $\sigma^{54}$ and the promoter DNA, which can be melted from position −12 to −5 in a reaction that can occur independently from RNAP core determinants. The restructuring of promoter DNA by activated $\sigma^{54}$ has been termed $\sigma^{54}$ isomerization (4–6). Following the initial events and requiring the presence of the RNAP core, the promoter DNA opening extends to position +3 relative to the transcription start site (7).

Energy coupling primarily involves intimate interactions between the activator and the $\sigma^{54}$ promoter complex. $\sigma^{54}$ activators, also termed enhancer-binding proteins (EBP), belong to the versatile AAA+ protein (ATPases associated with various cellular activities) family of molecular machines (for review see Refs. 8–10). Members of EBPs include the well studied NtrC, PspF, DctD, XylR, NifA, and DmpR proteins (11). PspF is the phage shock protein F that activates transcription of psp genes involved in the phage shock response in Escherichia coli (12).

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4 The abbreviations used are: RNAP, RNA polymerase; EBP, enhancer-binding proteins; SRH, second region of homology; AMPNP, adenosine 5’- (β,γ-imino)triphosphate; PDB, Protein Data Bank; ATPγS, adenosine 5’-O-(thiotriphosphate).
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FIGURE 1. Schematic representation of the PspF AAA+ domain with conserved motifs and structural features. AAA+ motifs are as follows: conserved motifs of AAA+ proteins (W-A, Walker A; W-B, Walker B; SRH, second region of homology). EBP conserved are as follows: regions CL–C7 of homologies identified for the AAA+ domains of EBP (47). Motif/structures are as follows: EBP conserved structural features, including the P-loop, loop 1 (L1), Walker B, pre-sensor I β-hairpin insertion (pre-SI) forming loop 2 (L2), sensor I motif (SI) within the SRH, and sensor II motif (SII). PspF sequences within relevant motifs are shown.

13). The AAA+ domain (see Fig. 1 for AAA+ domain features) of the α54 activator PspF (PspF1–275) is necessary and sufficient to activate transcription of the α54–RNAP in vitro and in vivo (14, 15). The α54 activator signature sequence GAFTGA loop 1 directly binds to the region I of α54, which is involved in maintaining a closed α54–RNAP promoter complex (16, 17). Stable interactions between PspF1–275 and α54 form when PspF1–275 is bound to the nonhydrolyzable ATP transition state mimic ADP-AlF4 (16). This transition state complex was deemed a structural and functional intermediate complex that is “trapped” in a conformation en route to open complex formation (18). Structures of PspF1–275 in complex with α54 and PspF1–275 structures bound to different nucleotides, combined with biochemical properties of PspF1–275 variants, suggested a detailed molecular mechanism by which different nucleotide states would trigger motions of the GAFTGA motif within a PspF1–275 subunit (19, 20). Signaling between Walker motif B and the GAFTGA loop 1 involves tight interactions between the PspF Walker B residue Glu108 and Asn64, nucleotide-binding-dependent motions of linker regions 1 and 2, and a number of signaling residues that would position the GAFTGA loop 1 to contact α54. Motions of the GAFTGA loop 1 appear synchronized with motions of the adjacent contacting loop 2. The AAA+ sequence insertion that structurally forms loop 2 in EBP classifies this family as member of the pre-sensor I β-hairpin insertion superclade of AAA+ proteins (Fig. 1) (21). Loop 1 and loop 2 are positioned near the pore of the hexameric PspF1–275 assembly (19).

AAA+ proteins invariably contain the Walker A and Walker B motifs that structurally define them as P-loop ATPases. The second region of homology (overlapping with the previously described AAA minimum consensus sequence) or degenerate forms thereof is the major distinguishing primary sequence motif that distinguishes AAA+ proteins from other P-loop ATPases (22). The SRH contains the sensor I motif, often a threonine/asparagine pair, and an arginine residue known as the R-finger that functions as a trans-acting catalytic residue between adjacent subunits that form the catalytic site (8, 9). The R-finger provides a structural rationale for why oligomerization is required for efficient hydrolysis in AAA+ proteins. The first sensor residue of the SRH is predominantly a threonine, and the second one is often an asparagine, although threonines and methionines are also commonly found at this position. Well studied members of the AAA+ superfamily that share this motif include N-ethylmaleimide-sensitive fusion protein (TN), p97 (TN), RuvB (TT), ClpA (TT), FtsH (TN), and PspF (TN). It was suggested that the second residue of sensor I works in concert with the second acidic residue of the Walker B motif to coordinate a catalytic water molecule (23). Currently, the role of the first residue of sensor I is not clear.

We wished to identify specific roles for sensor I residues of PspF in the steps that are known to be required for open complex formation within the α54–RNAP promoter complex. We tested activities of sensor I variants (PspF1–275 T148A, PspF1–275 T148A, and PspF1–275 N149A) of the PspF AAA+ domain (PspF1–275) in vitro, including the following: ATP binding, nucleotide binding-dependent hexamer formation, nucleotide-dependent engagement with the α54 promoter complex, ATP hydrolysis, open complex formation, and transcription activation.

The sensor residue variants PspF1–275 T148A and PspF1–275 N149A show no apparent deficiencies in nucleotide binding or nucleotide-dependent hexamer formation. Albeit reduced ATP hydrolysis rates found for all sensor I variants, PspF1–275 T148A and PspF1–275 N149A, show only moderate deficiencies for activating transcription. The nucleotide binding and hydrolysis parameters of PspF1–275 T148A and PspF1–275 N149A suggest a direct catalytic role for Asn149. Despite similar ATP turnover rates of PspF1–275 T148A and PspF1–275 N149A, only PspF1–275 T148A is severely affected in transcriptional activity. We show that defects of PspF1–275 T148A are because of defects in productive coupling nucleoside triphosphate hydrolysis to a restructuring of the α54 promoter complex and is not because of the failure to stably engage with the closed α54 promoter complex, suggesting that Thr148 is critically important in the events of open complex formation subsequent to α54 promoter engagement and independent of the presence of the RNAP core. The α54 promoter binding by PspF1–275 T148A distinguishes this variant from other PspF1–275 variants proposed to alter the position the GAFTGA loop (20). This suggests that an additional important structural link between the nucleotide-binding site and the α54 proximal contacting sites exists.

Thr148 appears to enable hydrolysis-dependent changes of the second acidic Walker B residue (Glu108) to propagate to other SRH residues that could mediate movements to the PspF loop 2. In support of this communication route, activities of PspF1–275 variants that connect loop 2 with the adjacent GAFTGA loop are similar to those of PspF1–275 T148A. We propose that Thr148 structurally couples hydrolysis-dependent motions to movements of loop 2 that are needed to restructure the α54–RNAP closed complex via the loop 1 GAFTGA adaptor sequence.

EXPERIMENTAL PROCEDURES

Mutagenesis and Protein Purifications—Plasmid pPB1 (14) encoding E. coli PspF residues 1–275 (PspF1–275 with an N-terminal His6 tag in pET28b+) was mutagenized (QuikChange mutagenesis kit, Stratagene) to obtain the desired single amino acid substitutions in pspF, resulting in pPB1 (T148A), pPB1 (N149A), and pPB1 (N149S). All PspF1–275 proteins were purified in the same manner and as described previously (24). Briefly, E. coli B834 cells containing pPB1 plasmids were grown in 1 liter of LB (10 g of peptone, 5 g of yeast extract, and 5 g of...
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NaCl) until an A$_{600}$ of $\sim$0.5 and induced with 1 mM isopropyl 1-thio-$\beta$-D-galactopyranoside for 3 h at 25 °C. After sonication and following nickel column purification, including a 70 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM dithiothreitol, 0.1 mM EDTA, and 50% glycerol and stored at $-20$ °C.

**Gel Filtration Chromatography**—Gel filtration chromatography on Superdex 200 10/300 (Amersham Biosciences) was carried out exactly as described previously (25). Briefly, PspF$_{1-275}$ proteins were introduced at high (63 $\mu$M) and following nickel column purification, including a 70 mM Tris-HCl, pH 8.0, 50 mM NaCl, and 15 mM MgCl$_2$) supplemented to give final ATP or ADP concentrations of 0.5 mM. Filtration was performed at 4 °C at a flow rate of 0.5 ml/min. The column was calibrated with globular proteins as follows: apoferritin (443 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa). All experiments were repeated at least twice.

**ATP Binding and Hydrolysis**—ATP binding assays of PspF$_{1-275}$ proteins by UV cross-linking and ATPase assays were essentially as described (24). Briefly, 20-$\mu$L samples containing 3.4 $\mu$g of purified protein in reaction buffer A (35 mM Tris acetate, pH 8, 70 mM potassium acetate, 5 mM magnesium acetate, 19 mM ammonium acetate, 0.7 mM dithiothreitol) contained 3 $\mu$Ci of [32P]ATP (3000 Ci/mmol) ± 1 mM ATP. Samples were incubated (254 nm, UV lamp UVG; UVP Inc., CA) for 15 min on ice. PspF$_{1-275}$ was analyzed by 12% SDS-PAGE after staining with Coomassie Blue, and the gels were scanned and then dried, and radioactivity was determined by phosphorimaging (Fuji Bas-1500 with Tina 2.10g software). The amount of protein per band was estimated by light transmission scanning.

ATPase assays were in reaction buffer A (above) supplemented with MgCl$_2$ to give final Mg$^{2+}$ concentrations of 15 mM and incubated at 30 °C with different ATP concentrations containing 0.06 $\mu$Ci/$\mu$L [32P]ATP. Reactions were stopped by adding 5 volumes of 2 M formic acid. Released 32P$_{i}$ or [32P]ADP was separated from ATP by thin layer chromatography, and radiolabeled P$_{i}$, ADP, and ATP amounts were measured by phosphorimaging.

**Single Round in Vitro Transcription Assays**—Standardized in vitro transcription assays were as in Ref. 26. Reactions contained 100 nM $\sigma^{54}$-RNA polymerase, 5 $\mu$M PspF$_{1-275}$ proteins, and 10 nM supercoiled Sinorhizobium meliloti nifH promoter DNA (26).

PspF$_{1-275}$ Protein Binding to and Isomerization of the $\sigma^{54}$ Promoter Complex—$\sigma^{54}$ promoter complexes were performed for binding and isomerization assays with PspF$_{1-275}$ essentially as described (4). Briefly, the bottom strand of the nifH promoter oligomer ($\sim$0 to +28 of the S. meliloti nifH promoter) was labeled with a fluorescent HEX tag, purchased from Operon AG and named WVC3-HEX. Duplex formation between the bottom strand and nonlabeled top strand occurred by mixing 5 $\mu$L of Hex-labeled NifH bottom strand (200 nM) and 5 $\mu$L of (400 nM) nonlabeled top strand in 10 mM Tris-HCl, pH 8.0, and 1 mM MgCl$_2$. The mixture was then heated to 95 °C and allowed to anneal slowly while cooling over 2.5 h to minimize the formation of mismatch double-stranded DNA. For isomerization assays, the top strand carries a mismatch at positions −12/−11 that is thought to stabilize the melted $\sigma^{54}$-DNA structure (4). Final concentrations in binding to and isomerization of the $\sigma^{54}$ promoter complexes were 20 nM promoter DNA, 2.3 $\mu$M $\sigma^{54}$, and 3 $\mu$M PspF$_{1-275}$. Binding of PspF$_{1-275}$ proteins to $\sigma^{54}$ promoter DNA was assessed by in situ formation PspF$_{1-275}$-ADP-Al$_3$ in the presence of 0.4 mM ADP, 5 mM NaF, 0.4 mM AlCl$_3$ (16). Isomerization assays were performed in the presence of 0.5 mM dGTP.

**RESULTS**

**Sensor I Residues Are Not Required for Nucleotide Binding or Nucleotide-dependent Hexamer Formation**—The process of transcription activation depends on a number of distinct functionalities of the PspF AAA+ domain that are thought to occur in the following order: nucleotide binding, hexamer formation, contacting the $\sigma^{54}$ promoter complex, ATP hydrolysis coupled to restructuring of the RNAP promoter, and disengagement from the open RNAP promoter complex. Disruption at any stage in this process should prevent subsequent events and result in the failure to activate transcription. It is therefore important to test PspF$_{1-275}$ variants for each of those activities to attribute discrete functional roles in the multistep process of transcriptional activation.

We substituted the sensor I TN residues to Ala as well as the Asn to the polar Ser and purified the respective proteins to obtain PspF$_{1-275}$T148A, PspF$_{1-275}$N149A, and PspF$_{1-275}$N149S in the same manner as PspF$_{1-275}$. All proteins were soluble. We applied UV cross-linking of PspF$_{1-275}$ proteins to radiolabeled [32P]ATP to detect changes in nucleotide binding. PspF$_{1-275}$ and other EBP appear to have a high off rate for ATP, in agreement with the high $K_4$ values (34 $\mu$M) of PspF$_{1-275}$ for ATP in previous using isothermal calorimetry (25). The nonequilibrium UV cross-linking method allowed us to identify significant changes in binding affinities for PspF$_{1-275}$ Walker A and Walker B variants consistent with their established binding characteristics in other AAA+ proteins, including the EBP NtrC (24, 27, 28). We found that none of the sensor I variants of PspF$_{1-275}$ was severely affected in ATP binding (Fig. 2A).

We next tested the PspF$_{1-275}$S6 sensor I variants for their ability to form higher order oligomers in the absence and presence of nucleotide. PspF$_{1-275}$ was shown to form higher order oligomers, probably hexamers, in a strictly concentration-dependent fashion that correlates with ATPase activity (25). Defects in oligomer formation are therefore predicted to negatively affect hydrolysis because of loss of cooperativity in ATP hydrolysis between subunits. It is established that ATP and ADP binding induces structural changes within PspF$_{1-275}$ (20) and strongly promotes hexamer formation of PspF$_{1-275}$, indicating nucleotide binding induces structural changes to increase self-association (25). To test if PspF$_{1-275}$ sensor I variants are defective in higher oligomer formation in their apo-, ADP-, and ATP-bound forms, we carried out gel filtration chromatography experiments in the presence and absence of nucleotides. When ADP or ATP was present, we chose the lowest protein concentrations that could still be detected with satisfactory confidence to maximize the sensitivity for detecting...
possible defects in higher oligomer formation. Chromatographs of PspF1–275 T148A, PspF1–275 N149A, and PspF1–275 N149S (Fig. 2B and data not shown) showed no apparent defects in hexamerization in the absence of nucleotide and at high protein concentrations (64 μM), nor in the presence of ATP or ADP at low protein concentrations (10 μM). Apparent hexamer formation of PspF1–275 T148A, PspF1–275 N149A, and PspF1–275 N149S in the presence of 0.5 mM ADP or ATP during gel filtration indicates that these variants have no ADP or ATP binding-dependent oligomer formation defects. Apparent apo-PspF1–275 hexamers elute around 11.7 ml and apparent nucleotide-bound PspF1–275 hexamers at around 11.2 ml. Apparent dimers elute at around 15 ml. Structural and functional data argue that the difference in elution volumes between higher order oligomeric apo- and nucleotide-bound forms of PspF1–275 are because of nucleotide-induced conformational changes in the hexamer rather than to a different subunit composition (25). We conclude that substitutions of sensor I residues do not result in conformational changes at the subunit interface and that ADP or ATP binding-dependent conformational changes occur that are compatible with hexamer formation.

Sensor I Residues Are Required for High ATP Hydrolysis Rates—We determined the kinetic ATP hydrolysis parameters of mutant proteins at 30 °C (Table 1). Sensor I variants showed 6–15-fold decrease in ATP hydrolysis rates, and PspF1–275 N149A and PspF1–275 N149S have a significantly reduced $K_m$ value. A reduced $K_m$ value usually indicates an increase in nucleotide affinity and/or structural changes in

| Protein                  | $k_{cat}$ | $K_m$  |
|--------------------------|-----------|--------|
| PspF1–275 T148A          | 38.9 ± 2  | 455 ± 23|
| PspF1–275 N149A          | 3.2 ± 0.3 | 484 ± 24|
| PspF1–275 N149S          | 2.5 ± 0.3 | 179 ± 9 |
| PspF1–275 N149S          | 6.1 ± 0.4 | 224 ± 11|

$K_{cat}$ values were determined at 3 μM PspF1–275, 1 mM ATP at 30 °C. $K_m$ values are derived from Lineweaver-Burk plots between ATP concentrations from 0.01 to 2 μM.
the catalytic site that alter product formation rates. A 4-fold excess of ADP over ATP at saturating nucleotide concentrations (1 mM ATP and 4 mM ADP) inhibits PspF1–275 turnover in ADP binding competition experiments that fully agree with the reported binding constants of PspF1–275 for ATP/PS (34 μM) and ADP (118 μM) (24, 25). We found the same nucleotide ratio-dependent inhibition on ATP turnover for PspF1–275 N149A as for PspF1–275, indicating the reduced ATPase activity of the Asn149 to Ala substitution variant is not because of decreased ADP release kinetics (data not shown). We conclude that the side chain of Asn149 is involved in catalysis per se. This interpretation is further supported by the steady-state parameters kcat and Km of the polar substitution of Asn149 to S in PspF1–275.

Previous studies have shown that subsaturation of ADP concentrations (100 μM) stimulate the ATPase activity of PspF1–275, whereas above saturation concentrations of ATP (>4 mM) inhibit turnover, showing that ADP-bound PspF1–275 has a positive cooperative effect on hydrolysis within a heterogeneously occupied hexamer, whereas ATP bound PspF1–275 can act as a negative allosteric effector (25). Changes in ADP- or ATP-mediated allosteric effects under conditions where PspF1–275 T148A and PspF1–275 N149A form hexamers (above) could indicate a role in intersubunit signaling, which is dependent on the bound type of nucleotide. For PspF1–275 T148A and PspF1–275 N149A, ATPase activity was stimulated in the presence of subsaturation ADP concentrations and inhibited at >4 mM concentrations of ATP (data not shown). Therefore, no obvious role for sensor I residues in cooperative hydrolysis is evident.

Low ATP Turnover Rates of PspF1–275 N149A Are Not Detrimental for Activating Transcription—ATP hydrolysis by PspF1–275 is needed to restructure the stable σ34-RNAP promoter-closed complex to an open thermodynamically unfavorable conformation (4, 29). We determined whether the reduced turnover rates of PspF1–275 T148A and PspF1–275 N149A could still activate transcription in vitro, in single round transcription assays from the S. meliloti supercoiled nifH promoter (Fig. 3). PspF1–275 T148A and PspF1–275 N149A show defects in transcription activation, although PspF1–275 N149A only moderately (42 ± 4% activity compared with PspF1–275) compared with PspF1–275 T148A (10 ± 2%). These findings cannot be readily explained by differences in hydrolysis rates between PspF1–275 T148A and PspF1–275 N149A, especially because PspF1–275 N149A has a lower kcat than PspF1–275 T148A (Table 1). The amount of transcripts shown in Fig. 3 reflects the capacity of PspF1–275 proteins to use ATP hydrolysis to activate transcription of the σ34-RNAP.

RNAP Transcription Activation Defects Are Directly Associated with the Failure to Isomerize the σ34 Bound to Promoter DNA—We have described previously a number of PspF1–275 variants, PspF1–275 S75A, PspF1–275 E76A, PspF1–275 H80A, PspF1–275 R95A, PspF1–275 S131A, and PspF1–275 S132A, with defects in transcriptional activation that are independent from nucleotide binding and thus similar to the defects of PspF1–275 T148A (19, 20). The substituted residues of these variants form part of a structural conformational signaling pathway that extends from the nucleotide-binding site to the GAFTGA containing loop 1 (Ser77, Glu76, His80, and Arg89) or are part of loop 2 (Arg131 and Val132) that is adjacent to the GAFTGA loop 1 (see Fig. 6 for orientation). We proposed that the signaling to and the integrity of the GAFTGA loop 1 was critical for contacting σ54 and energy coupling. Structures (19) and models (10, 30) of the PspF1–275-σ54 complex suggested that the promoter DNA lies "sandwiched" between σ54 and the upper face of the PspF1–275 hexameric ring. Contact features of PspF subunits, notably the GAFTGA loop 1, the associated loop 2, and σ54, suggest that the GAFTGA loop 1 and loop 2 are proximal to promoter DNA. Proximity-based studies support this architecture, although direct PspF1–275 promoter DNA contacts have not been identified (31). To directly assess the capacity of PspF1–275 variants to restructure the σ54 promoter complex independently from a possible contribution of the RNAP core, we carried out direct σ54 promoter isomerization assays (4). The assay detects bona fide open σ54 promoter complexes after isomerization by and disengagement from PspF1–275 permitting distinction between activities directly associated with energy coupling to the σ54 promoter and those that may be RNAP core-dependent. Fig. 4 shows the isomerization activities of PspF1–275 T148A, PspF1–275 S131A, PspF1–275 S132A, PspF1–275 E76A, PspF1–275 R95A, PspF1–275 R131A, and PspF1–275 in the presence and absence of the hydrolyzable triphosphonucleotide dGTP. Using dGTP instead of ATP gives stronger signals (4). Only PspF1–275 N149A shows similar isomerization activity to PspF1–275 and PspF1–275 T148A, which shows some residual isomerization activity in agreement with our in vitro transcription results. All other transcriptionally inactive PspF1–275 variants tested also fail to isomerize the σ54 promoter. These results underline the major mechanistic importance of energy coupling-dependent isomerization of the σ54 promoter in initiating σ34-RNAP-dependent transcription.

FIGURE 3. Single round in vitro transcription assays from supercoiled nifH promoter with PspF1–275 T148A and PspF1–275 N149A. The nonlabeled lane comes from a PspF1–275 T148A variant of no relevance here. Proteins are as indicated. Defined mRNA transcripts are visualized and quantified by the incorporation of [α-32P]UMP followed by PAGE and phosphorimagery.
We infer an important role for Thr\textsuperscript{148} at this step in nucleotide hydrolysis-dependent open complex formation of the \(\sigma\textsuperscript{54}\)-RNAP promoter.

\(\sigma\textsuperscript{54}\) Promoter DNA Binding Activities Functionally Link PspF\textsubscript{1–275} T148A with Loop 2—Considering the likely spatial organization of PspF in complex with \(\sigma\textsuperscript{54}\) bound to promoter DNA (see above), we reasoned that the \(\sigma\textsuperscript{54}\) bound to promoter DNA would provide the best structural \textit{in vitro} mimic to study PspF\textsubscript{1–275} \(\sigma\textsuperscript{54}\) promoter complex interactions. ADP-AIF\textsubscript{+}-dependent structural changes within the PspF\textsubscript{1–275}-\(\sigma\textsuperscript{54}\)-RNAP promoter complex have been reported and represent a structural and functional transition conformation that is trapped \textit{en route} to open complex formation (5, 32). To test if the failure of PspF\textsubscript{1–275} variants to isomerize the \(\sigma\textsuperscript{54}\) promoter complex is a consequence of a reduced capacity to stably engage with the \(\sigma\textsuperscript{54}\) promoter complex, we incubated a preformed \(\sigma\textsuperscript{54}\)-nifH promoter probe complex with PspF\textsubscript{1–275} variants in the presence of ADP-AIF\textsubscript{+}, reagents. Fig. 5 shows activities of PspF\textsubscript{1–275} T148A, PspF\textsubscript{1–275} N149A, PspF\textsubscript{1–275} S75A, PspF\textsubscript{1–275} R95A, PspF\textsubscript{1–275} V132A, and PspF\textsubscript{1–275} R131A to stably engage with the \(\sigma\textsuperscript{54}\) promoter. PspF\textsubscript{1–275} N149A (Fig. 3), PspF\textsubscript{1–275} S75A, and PspF\textsubscript{1–275} R95A fail to engage with the \(\sigma\textsuperscript{54}\) promoter DNA (Fig. 3). This defect explains why PspF\textsubscript{1–275} S75A and PspF\textsubscript{1–275} R95A fail to isomerize the \(\sigma\textsuperscript{54}\) promoter complex, which relies on complex formation and coupling nucleoside triphosphate hydrolysis to the \(\sigma\textsuperscript{54}\) promoter complex. Activity data are in full agreement with the suggested function of Ser\textsuperscript{75}, Glu\textsuperscript{76}, His\textsuperscript{80}, and Arg\textsuperscript{95} to help position the GAFTGA loop 1 to stably contact the \(\sigma\textsuperscript{54}\) promoter. In marked contrast, PspF\textsubscript{1–275} N149A, PspF\textsubscript{1–275} S75A, and PspF\textsubscript{1–275} V132A represent a novel class of PspF\textsubscript{1–275} variants that in that they show full \(\sigma\textsuperscript{54}\) promoter binding activities but fail to isomerize the \(\sigma\textsuperscript{54}\) promoter DNA structure. These results indicate that severe isomerization and transcription activation defects of PspF\textsubscript{1–275} T148A and PspF\textsubscript{1–275} V132A are caused by defects subsequent to engaging with \(\sigma\textsuperscript{54}\) promoter DNA. PspF\textsubscript{1–275} R131A shows minor defects in contacting \(\sigma\textsuperscript{54}\) promoter DNA. The functional defects of PspF\textsubscript{1–275} T148A and the loop 2 variants PspF\textsubscript{1–275} R131A and PspF\textsubscript{1–275} V132A that are directly associated with energy coupling (\(\sigma\textsuperscript{54}\) promoter isomerization) and not with \(\sigma\textsuperscript{54}\) promoter DNA engagement suggest a structure/function link between Thr\textsuperscript{148} and loop 2 (see below). Importantly, this is a distinct link from the nucleotide binding pocket to the GAFTGA loop 1 signaling pathway.

DISCUSSION

More generally, we wished to establish the functional roles of the AAA+ conserved sensor I residues of AAA+ proteins. In particular, we studied sensor I residue variants of the AAA+ domain of the \(\sigma\textsuperscript{54}\) activator PspF that is necessary and sufficient to activate transcription \textit{in vivo} and \textit{in vitro}. A systematic analysis of various PspF\textsubscript{1–275} activities that are required for functional output allowed assignment of specific roles of sensor I residues during the nucleotide binding initiated process that results in transcriptional activation. The wealth of structural and functional data for PspF\textsubscript{1–275} and the availability of methods to study basal and full transcriptional activation properties helped to identify specific mechanistic functions of the PspF sensor I residues and by extension their roles in other AAA+ proteins.

The Asparagine of the PspF Sensor I Is Involved in ATP Hydrolysis per Se but Is Not Essential for Functional Output—We found that the conserved Asn residue of sensor I is required for high ATPase rates and affects the Michaelis-Menten kinetics. Gel filtration experiments with PspF\textsubscript{1–275} N149A (Fig. 2B) and PspF\textsubscript{1–275} N149S in the presence of ATP or ADP show that Asn\textsuperscript{149} is not essential for nucleotide binding-dependent hexamer formation, excluding the possibility that reduced hydrolysis rates are caused by oligomerization defects. ADP was not a stronger binding competitor for ATP in PspF\textsubscript{1–275} N149A compared with PspF\textsubscript{1–275} as judged by ATPase rates in the presence of excess ADP, suggesting that the reduced \(k\textsubscript{cat}\) for PspF\textsubscript{1–275} N149A is not because of slower ADP release. Substitutions of sensor I Asn residue of the FtsH protease (33), the
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p97/VCP homologue VAT (34), or the Lon protease (35) also resulted in strongly reduced hydrolysis rates in those proteins. Consistent with our findings with PspF1–275, a strong decrease in $k_{cat}$ and a 2–3-fold decrease in $K_m$ was reported for the NBD2 AAA+ domain of Hsp104 carrying an Asn to Ala substitution of the sensor I (36). Hsp104 is involved in thermotolerance, and decreased high temperature survival rates were observed for this sensor I mutant. In FtsH the corresponding mutation resulted in reduced but not abolished in vivo protease activity. In PspF, Asn149 is not essential for nucleotide-dependent interactions with $\sigma^{54}$, isomerization of the $\sigma^{54}$ promoter complex, or transcriptional activation of the $\sigma^{54}$-RNAP. We deduce that Asn149 of PspF is involved in ATP hydrolysis per se but is not essential for functional output in vitro. We propose that the side chain of Asn149 contributes to the catalytic site geometry that allows for the high turnover rates of PspF. Our functional assignment is in agreement with the previously suggested roles of the second sensor I residue in other AAA+ proteins (37, 38).

In contrast to PspF1–275 $^{N149A}$, PspF1–275 $^{T148A}$ fails to activate transcription. Potentially, its reduced ATPase activity could be insufficient for open complex formation. However, the intrinsic ATPase activity of PspF1–275 $^{N149A}$ is lower than that of PspF1–275 $^{T148A}$. Importantly, PspF1–275 $^{T148A}$ and PspF1–275 $^{N149A}$ appear not to be defective in apparent hexamer formation, either in the presence or absence of nucleotide, suggesting that their reduced hydrolysis rates are not a consequence of defects in subunit interactions that are required for cooperative hydrolysis. Integrity of the cooperativity is substantiated by the gain of activity in ATP hydrolysis rates in the presence of ADP and a decrease in hydrolysis rates at high ATP concentrations (>3 mM) for PspF1–275 $^{N149A}$ and PspF1–275 $^{T148A}$, suggesting no obvious allosteric defects between subunits for these variants (25). The observation that PspF1–275 $^{T148A}$ shows full $\sigma^{54}$ promoter complex binding affinity in the presence of the ATP hydrolysis transition state analogue ADP-AlF$_4^-$ further narrows down the function of Thr148. The defects of PspF1–275 $^{T148A}$ in activating transcription can be directly correlated with defects in isomerizing the $\sigma^{54}$-promoter complex, suggesting that Thr148 plays a crucial role in transcriptional initiation at the point of restructuring the $\sigma^{54}$ promoter complex that is independent of the presence of the RNAP core structure. Therefore, Thr148 is involved in events that precede later stages of RNAP promoter open complex formation that involve RNAP core determinants (5, 39). We conclude that Thr148 plays a mechanistic role in productively translating ATP hydrolysis-derived energetic motions into structural changes of the $\sigma^{54}$ promoter structure within the $\sigma^{54}$-RNAP promoter complex. PspF1–275 $^{T148A}$ shows similar defects to those observed for PspF1–275 $^{V132A}$ and PspF1–275 $^{R131A}$, which similarly fail in transcription activation and $\sigma^{54}$ promoter complex isomerization despite retaining good $\sigma^{54}$ promoter binding activity. The post-$\sigma^{54}$ promoter binding defect provides evidence for a structural link between Thr148 and loop 2. PspF1–275 $^{V132A}$ in loop 2 has no detectable ATPase activity, whereas PspF1–275 $^{R131A}$ retains 10% of ATPase activity compared with PspF1–275 (20). We do not know why loop 2 variants have reduced ATPase activities, but defects in ATP hydrolysis of loop 2 variant PspF1–275 $^{R131A}$ proteins support a structural link between loop 2 and the catalytic site. Despite ATPase rates of PspF1–275 $^{T148A}$ and PspF1–275 $^{R131A}$ that can otherwise support transcription activation in PspF1–275 $^{N149A}$, the defects in PspF1–275 $^{T148A}$ and PspF1–275 $^{R131A}$ indicate common functions. Furthermore, PspF1–275 $^{T148A}$ differs from output deficient residues involved in the signaling pathway (PspF1–275 $^{S75A}$, PspF1–275 $^{E76A}$, PspF1–275 $^{H80A}$, and PspF1–275 $^{R95A}$) from the catalytic site to the $\sigma^{54}$ contacting GAFTGA loop 1, where gross defects in binding to $\sigma^{54}$ promoter are evident.

Distinct Signaling Pathways Position Loop 2 and GAFTGA Loop 1 during Hydrolysis—The crystal structures of PspF1–275 bound to ATP and ADP indicate a structural link between the ATP hydrolysis-dependent conformational changes and Thr148 (Fig. 6). In the ATP-bound state, Walker B residue Glu108 clearly interacts with Asn64. The Glu108–Asn64 interaction is required to position the distant GAFTGA loop 1 for engagement with $\sigma^{54}$ via linker 1 and a number of signaling residues, including Ser75, Glu76, His92, and Arg95 (20) (Glu76 and His92 are shown in Fig. 6). In the ADP-bound state, Glu108 pivots away from Asn64 toward Thr148 and then interacts with Thr148 via a water molecule. The Thr148–interacting water molecule is present in all PspF1–275 structures (apo, ADP, ATP, and AMP-PNP (20)), suggesting that it is an integral and significant structural element of PspF. The Glu108–Thr148 connectivity can link the ADP-bound state to the SRH and thus via the SRH C-terminal helix to $\alpha$-helix 4 that forms the structural base of loop 2. More directly, the Thr148-associated water molecule could...
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structurally bridge between the Glu108 side chain and the Glu108 main chain. Main chain movements are important for linker 2 movements, which in turn positions α-helix 4. Although a detailed structural signaling pathway from Thr148 to loop 2 cannot be described with complete confidence with the available structural and functional data, our results strongly suggest that two distinct signaling pathways from the P-loop Glu108 exist. One signal toward the GAFTGA loop (via Asn64 and linker 1), and one signal to loop 2 (via Thr148 and possibly linker 2). Similarities of the linker 1 and linker 2 conformations in the ADP- and the ATP-bound structures between PsPF1–275 and the Ltag helicase (40) provided evidence for some conserved nucleotide hydrolysis-dependent dynamics between these two AAA+ proteins (20). The functional similarities of PsPF1–275, PsPF1–275, and PsPF1–275 support a structural linkage between Thr148 and loop 2. Furthermore, properties of PsPF1–275 and PsPF1–275 suggest that loop 2 does not directly contact σ54, because neither variant fails to engage with the σ54 promoter DNA complex. Nucleotide hydrolysis-induced changes in the ATP-binding pocket probably propagate toward loop 2. The loop 2 motion then drives the GAFTGA loop adaptor during the power stroke that remodels the σ54-RNAP promoter complex to an open, transcriptionally competent conformation, a coupling mechanism that critically involves Thr148.

The clear conservation of the sensor I motif suggests a generalizable role within the AAA+ family. Interactions between the first sensor I residue (1stSI, Thr148 in PsPF) with the second acidic residue of the Walker B motif (2ndWB) are probably common in the wider AAA+ family as judged by the available high resolution structures of AAA+ proteins. 1stSI-2ndWB direct interactions exist in p97 (41), PDB entry 1E32, sensor I motif TN), N-ethylmaleimide-sensitive fusion protein (42), PDB entry 1D2N, TN), ClpA (43), PDB entry 1R6B, TN), and judging from proximity also in the Ltag helicase (40), PDB entry 1SVO), which has unconventional sensor 1 (T/MMN) and Walker B motifs (ED instead of DE). 1stSI-2ndWB interactions exist in FtsH (44), 45), PDB entry 1DXZ, TN) and RuvB (PDB entry 2C9O, SN) and also involve a water molecule, very similarly positioned as the Thr148-associated water molecule in PsPF. The coupling role of the Thr148 of PsPF to loop 2 could also be of functional importance in other AAA+ proteins. Insertions at sites corresponding to the position of loop 2 of PsPF define a subclass of AAA+ proteins termed pre-sensor I β-hairpin (loop 2 equivalent) super-clade (21). Members of this super-clade also include the HslU, Clp, and Lon proteases, RuvB, the Ltag helicase, and the MCM helicases. The loop 2 equivalent insertions form hairpin structures in RuvB (46), sensor motif TT) and Ltag. In RuvB the β-hairpin is important for interactions with RuvA to cooperate in Holliday junction branch migration. In Ltag helicase, the β-hairpin appears directly involved in ATP-driven translocation of double-stranded DNA (40). Further functional studies that address the role of the conserved first residue of sensor I are needed to understand mechanistic similarities and differences of AAA+ proteins.

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