An Intramolecular Route for Coupling ATPase Activity in AAA⁺ Proteins for Transcription Activation*\(^5\)

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AAA⁺ proteins (ATPases associated with various cellular activities) contribute to many cellular processes and typically function as higher order oligomers permitting the coordination of nucleotide hydrolysis for functional output, which leads to substrate remodeling. The precise mechanisms that enable the relay of nucleotide hydrolysis to their specific functional outputs are largely unknown. Here we use PspF, a specialized AAA⁺ protein required for enhancer-dependent transcription activation in *Escherichia coli*, as a model system to address this question. We demonstrate that a conserved asparagine is involved in internal organization of the oligomeric ring, regulation of ATPase activity by “trans” factors, and optimizing substrate remodeling. We provide evidence that the spatial relationship between the asparagine residue and the Walker B motif is one key element in the conformational signaling pathway that leads to substrate remodeling. Such functional organization most likely applies to other AAA⁺ proteins, including Ltag (simian virus 40), Rep40 (Adeno-associated virus-2), and p97 (*Mus musculus*) in which the asparagine to Walker B motif relationship is conserved.

AAA⁺ proteins (ATPases associated with various cellular activities) are present in all kingdoms of life and play important roles in numerous cellular activities, including proteolysis, protein folding, membrane trafficking, cytoskeletal regulation, organelle biogenesis, DNA replication, and DNA transcription. AAA⁺ ATPases invariably contain Walker A and B motifs that define them as P-loop ATPases and a conserved sequence termed the second region of homology and function as higher order oligomers, which remodel their substrates in reactions that consume ATP (1–7). In many cases, AAA⁺ domains assemble into hexameric rings that change their conformation during the ATPase cycle. This nucleotide-dependent conformational change may, for example, apply mechanical tension to bound proteins or nucleic acids and thereby allow AAA⁺ proteins to remodel their substrate. The energy-dependent nature of their activities and their organization as ring assemblies raises important issues about how they function as molecular machines to engage with, and remodel, their targets. A common area of functionality that is poorly understood concerns how nucleotide binding and hydrolysis is relayed within the machines to engage with, and remodel, their targets. Importantly, AAA⁺ proteins represent a large class of mechano-chemical enzymes that have evolved many ways of using a fundamentally similar conformational change in different biological settings (8). Indeed, these proteins often become specialized by the insertion of specific motifs within the minimal AAA⁺ core.

One well studied example of this specialization is represented by the family of bacterial enhancer-binding proteins (bEBPs)\(^3\) required for \(σ^{54}\)-dependent transcription activation (9). In contrast to \(σ^{70}\)-dependent transcription, which is constitutively active, \(σ^{54}\)-dependent transcription requires specific activators (the bEBPs) that couple ATP hydrolysis to isomerization of the initial transcriptionally inactive closed complex (CC), to a transcriptionally proficient open complex (OC) (10–15). \(σ^{54}\)-dependent transcription activation is functionally analogous to eukaryotic RNAP II, which requires energy derived from ATP hydrolysis provided by TFIIH (16, 17). The bEBPs, which include the well studied activators DctD, DmpR, NifA, NtrC, NtrC1, PspF, and XylR, are characterized by an insertion: the L1 loop containing the “GAFTGA motif,” which is required for specific interaction with the \(σ^{54}\) N-terminal regulatory domain, \(σ^{54}\) region 1 (2, 4, 9, 18–20). These bEBPs are members of a sub-class of AAA⁺ proteins known as the sensor I \(β\)-hairpin super-clade and include the helicases RuvB, Ltag, and MCM as well as proteases HsLU, ClpX, and Lon (21).

In this study, we use the bEBP model, PspF (phage shock protein F), from *Escherichia coli*, which is composed of: (i) a catalytic AAA⁺ domain sufficient to activate \(σ^{54}\)-dependent transcription in vivo and in vitro (PspF\(_{1–275}\), see Fig. 1A), and (ii) a C-terminal helix-turn-helix domain, which binds the upstream activator sequence of the pspA and pspG specific promoters. In addition, PspF activity is negatively regulated by PspA (22–24).
Recently, we demonstrated that substitution of the highly conserved Walker B glutamate residue (Glu-108 in PspF) allowed ATP-dependent stable complex formation between PspF and $\sigma^{54}$ (or $\sigma^{54}_E$) (25). Using a functional approach, we established roles of the Walker B Glu-108 residue in nucleotide-dependent interactions between the GAFTGA motif and $\sigma^{54}$. Our functional data, in combination with crystal structures of PspF$_{1-275}$ soaked with different nucleotides, suggest that residue Glu-108 relays ATP hydrolysis to remodel the $\sigma^{54}$-DNA CC. Analysis of the different nucleotide-bound structures of PspF$_{1-275}$ demonstrated that a tight interaction between Walker B residues Glu-108 and Asn-64 occurs in the ATP-bound state, proposed to facilitate the exposure of the GAFTGA motif (25). ATP hydrolysis was suggested to disrupt the E108-N64 interaction, resulting in repositioning of the GAFTGA motif (26). Despite these observations and mutagenesis studies, the precise signaling pathway regulating nucleotide hydrolysis-dependent events to OC formation remains unknown.

Sequence alignments of bEBPs show that the Walker B motif-interacting asparagine (Asn-64 in PspF) is strictly conserved, suggesting this residue may play an important role in bEBP activities. This asparagine is not present in all AAA$^+$ proteins, however structural alignment of PspF with other AAA$^+$ proteins demonstrates conservation of the asparagine (corresponding to Asn-64) in several proteins (Fig. 1, B and C). Interestingly, all the AAA$^+$ proteins possess this conserved asparagine (Fig. 1, B and C, in red), also maintain the distance between this residue and the Walker B residues (Fig. 1, B and C, in green). Indeed, we note that in the case of Rep40 where the asparagine (Fig. 1, B and C, in orange) is not aligned structurally, the Walker B residues (Fig. 1, B and C, in purple) are also not aligned thereby maintaining a similar asparagine-Walker B distance as observed for other AAA$^+$ proteins. These observations suggest that communication between the asparagine and Walker B residues and the distance between them could be important for protein functionality.

Understanding the communication mechanism between residues of the same or adjacent subunits of AAA$^+$ proteins is important for understanding their global mechanisms of action. Determining how the positioning of the "functional motif" (GAFTGA in bEBPs), responsible for interacting with its target ($\sigma^{54}$), is regulated can provide insight into how AAA$^+$ proteins use ATP binding and hydrolysis, and evolve to become specialized. In this study, we investigated the contribution of residue Asn-64 to nucleotide-dependent outputs of PspF, which we used as a model system, and its role in relaying nucleotide hydrolysis to remodel the $\sigma^{54}$-DNA CC.

We provide evidence for the direct contribution of residue Asn-64 in the catalytic ATPase activity and hexameric organization of PspF and demonstrate a clear role for Asn-64 in the efficient relay of ATPase activity to substrate remodeling during OC formation. In addition, we show that the negative regulation imposed by PsPA on PspF ATPase activity (but not PsPA binding) is dependent on Asn-64, confirming its central role in PspF functionality. Finally, we demonstrate Asn-64 variants are affected in a stage of the transcription activation process that follows $\sigma^{54}$ isomerization. We show that functionalities dependent upon Asn-64, which primarily involves interactions...
between PspF and σ^54, are also sensitive to core RNAP enzyme and to promoter DNA conformation.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—Plasmid pPB1 encodes *E. coli* PspF<sub>1–275</sub> with an N-terminal 6-His tag in pET22b^+~(27)~. Variants of PspF<sub>1–275</sub> were generated from plasmid pPB1 mutagenized to yield pPB1-N64A (AAC → GCC), pPB1-N64D (AAC → GAA), pPB1-N64Q (AAC → AGC), and pPB1-N64S (AAC → GAC). Constructs were verified by DNA sequencing.

**Protein Purification**—PspF<sub>1–275</sub> proteins were purified as described~(28)~. σ^54 was purified as described in a previous study~(10)~. His-PspA was purified as described previously~(29)~.

**Filter Nucleotide Binding Assay**—Nucleotide binding assays were performed in 25-μl final volume containing: 20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 15 mM MgCl<sub>2</sub>, and 10 μM PspF<sub>1–275</sub> variants. The mix was preincubated at 4°C for 10 min, and the reaction was started by adding 7.5 μl of an ATP solution containing 0.3 μCi/μl [α-32P]ATP (300 Ci/mmol) or 0.3 μCi/μl [γ-32P]ATP (3000 Ci/mmol) and incubated for 10 min at 4°C. Binding reactions were then filtered through a Protran nitrocellulose 0.45-μm filter (Whatman) placed on a slot blot 48-well system (Hoefler, Inc.), and a vacuum was briefly applied (10 s) to remove the liquid. After sample application, the membrane was immediately washed with 1 ml of washing buffer (20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 15 mM MgCl<sub>2</sub>) at 4°C. Radioactivity retained in the membrane was measured by using a phosphorimaging device (Fuji Bas-1500) and analyzed using the Aida software. All experiments were carried out at least five times, and fluctuations of binding values were up to 30% of WT values.

**ATPase Activity**—The 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 5 μM PspF<sub>1–275</sub> (1 μM PspF<sub>1–275</sub> ± 5.2 μM His-PspA). The mix was preincubated at 37°C for 10 min, and the reaction was started by adding 3 μl of an ATP solution containing 0.6 μCi/μl [α-32P]ATP (3000 Ci/mmol) plus 0.1 mM ATP and incubated for varying times at 37°C. Reactions were quenched by addition of 5 volumes of 2 M formic acid. The [α-32P]ADP was separated from ATP by TLC, and radiolabeled ADP and ATP were measured by phosphorimaging and analyzed using the Aida software. Activity is expressed as a percentage of PspF<sub>1–275</sub> WT turnover value. All experiments were carried out in triplicate (at least), and fluctuations of turnover values were maximally 10%.

**Gel Filtration through Superdex 200**—PspF<sub>1–275</sub> WT and N64 (at different concentrations) were incubated for 5 min at 4°C in buffer containing 20 mM Tris-HCl (pH 8.0), 50 mM NaCl, and 15 mM MgCl<sub>2</sub> ± 0.5 mM ATP or ADP where indicated. 50-μl samples were then injected onto a Superdex 200 column (10 × 300 mm, 24 ml, GE Healthcare) and equilibrated with the sample buffer with or without nucleotide. Chromatography was performed at 4°C at a flow rate of 0.5 ml/min, and columns were calibrated with globular proteins: apoferritin (443 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa). All experiments were repeated at least four times, and the elution profiles obtained were similar.

**β-Galactosidase Assays**—Cells were grown overnight at 37°C in LB broth containing the appropriate antibiotic and then diluted 100-fold (initial A<sub>600</sub> ~ 0.025) into 5 ml of LB. Following incubation to A<sub>600</sub> ~ 0.30, cultures were induced with different concentrations of arabinose for 1 h (as indicated), further grown to mid-exponential phase (A<sub>600</sub> ~ 0.5–0.6) and then assayed for β-galactosidase activity as described before~(30)~. Enzyme activities (in Miller units) represent the means ± S.D. of the triplicate average values from at least two independent cultures.

**Affinity Chromatography with Immobilized PspA**—Affinity chromatography was performed at 4°C in Micro Biospin® Bio-Rad columns packed with 50 μl of nickel-nitritoltriacetic acid-agarose (Qiagen). Solutions were passed through the columns by centrifugation at 5 × g for 30 s. The columns were equilibrated with buffer A (20 mM Tris-HCl (pH 8.0), 50 mM NaCl, and 15 mM MgCl<sub>2</sub>) and loaded with 500 μl of 6 μM His-PspA. The columns were washed with buffer A (1 ml), and purified PspF<sub>1–275</sub> WT or W56A or N64 (400 μl at 3.6 μl) was allowed to flow through the column. Unbound proteins were removed by washing with 5 × 100 μl of buffer A plus 40 mM imidazole. His-tagged PspA was eluted with 2 × 100 μl buffer A plus 500 mM imidazole. 100-μl fractions were collected, and 20 μl was analyzed by 12% SDS-PAGE. Proteins were detected by Coomassie Blue staining.

**Native Gel Mobility Shift Assays**—Gel mobility shift assays were conducted to detect protein-protein or protein-DNA complexes. Assays were performed in a 10-μl final volume containing: 10 mM Tris acetate (pH 8.0), 50 mM potassium acetate, 8 mM magnesium acetate, 0.1 mM dithiothreitol, 4 mM ADP, NaF (5 mM) ± σ^54 (1 μM) ± core RNAP (0.15 μM) ± 0.2 μM HEX-labeled DNA probe. Where required, PspF<sub>1–275</sub> WT or N64 (5 μM) ± AlCl<sub>3</sub> (0.4 mM) were added for a further 10 min at 37°C. Complexes were analyzed on a native 4.5% polyacrylamide gel. Proteins were detected by Coomassie Blue staining and fluorescent HEX-DNA was measured by phosphorimaging and analyzed using the Aida software.

**In Vitro Full-length or Abortive Transcription Assays**—Full-length or abortive transcription assays were performed in a 10-μl volume containing: 10 mM Tris acetate (pH 8.0), 50 mM potassium acetate, 8 mM magnesium acetate, 0.1 mM dithiothreitol, 4 mM dATP, 0.1 μM core RNAP enzyme, 0.4 μM σ^54, and 20 mM promoter DNA. The mix was preincubated at 37°C for 5 min, and the reaction was started by addition of 5 μM of PspF<sub>1–275</sub> WT or N64, and incubated for varying times at 37°C. Full-length transcription (from the supercoiled *Sinorhizobium melloti* nifH promoter) was initiated by adding a mix containing 100 μg/ml heparin, 1 mM ATP, CTP, GTP, 0.05 mM UTP, and 3 μCi of [α-32P]UTP for a further 10 min. The reaction was stopped by addition of loading buffer and analyzed on 6% sequencing gels. Synthesis of the abortive transcript (UpGGG) was initiated by addition of heparin (100 μg/ml), the dinucleotide UpG (0.5 mM), GTP (0.01 mM), and 4 μCi of [α-32P]GTP for a further 10 min. The reaction was quenched by addition of loading buffer and analyzed on a 20% denaturing gel. Radio-
Asn-64 Contributes to Nucleotide Binding, Hydrolysis, and Self-association—We first determined whether the PsF1–275 Asn-64 variants (N64v) maintained their ability to bind and hydrolyze nucleotides (Fig. 2, A and B). The Asn-64 substitutions tested resulted in either an apparent increase (N64A, N64Q, and N64S) or decrease (N64D) in ATP binding, compared with PsF1–275 WT (WT). Having demonstrated that all the N64v were able to bind ATP (Fig. 2A), we next determined whether they were affected in their capacity to hydrolyze ATP (Fig. 2B). We observed that N64S ATPase activity was not affected, whereas the other N64v tested were all deficient for ATPase activity (N64S (100%)) > N64A (36%) > N64Q (12%) > N64D (0%)). We conclude that Asn-64 contributes to, but is not essential for, nucleotide binding and ATPase activity.

We have previously shown that the ATPase activity of PsF1–275 is directly related to its oligomeric state (a hexamer being the most active form) and that PsF1–275 oligomerization is strongly stimulated in the presence of nucleotides (ATP or ADP) (28). Defects in oligomer formation are therefore predicted to negatively affect hydrolysis due to a loss in cooperativity between subunits. Because Asn-64 does not appear to be absolutely required for ATPase activity, we sought to determine whether a lack of ATPase activity was due to an effect on catalytic site formation comprising “in cis” and “in trans” residues, thereby potentially a global change in hexamer organization (28, 33). To investigate the effect of N64v on PsF1–275 oligomerization we performed gel filtration experiments and observed that in the absence of nucleotide, the elution profiles obtained with the N64v all differ from WT (Fig. 2C). When N64v hexamers formed, they had the same elution position as WT, suggesting no large scale changes in structure, as seen in some other PsF1–275 variants (28). We divided the N64v into three different classes: (i) constitutive hexamer formation (N64S), (ii) reduced hexamer formation (N64Q and N64A), and (iii) defective hexamer formation (N64D). In the presence of nucleotide (ATP or ADP), as with WT, a stimulatory effect on hexamerization was observed with N64A and N64Q (data not shown). However, N64D was still unable to form a hexamer. Overall, the results demonstrate that Asn-64 is important in ensuring the optimal oligomerization of PsF and therefore contributes to forming the active site of the protein (see “Discussion”).

Formation of a Stable PsF1–275-Ex54 Complex Is Dependent on N64—We then addressed the question whether the N64v can form biologically relevant complexes with (E)τ54. Stable in vitro interactions between PsF1–275 and its natural target
capable of activating transcription using an *in vitro* transcription assay. Having confirmed under the chosen assay conditions that ATP hydrolysis by PspF 1–275 was required to activate Eo 54 transcription (data not shown) we performed *in vitro* transcription assays with the N64v. To determine the rate of transcription, we incubated the Eo 54-DNA (CC) and WT or N64v with dATP for varying activation times. As shown in Fig. 3B, all N64v, are negatively affected in the initial rate of transcript formation (compared with WT at 5 min activation time, N64A (66%), N64D (not detected), N64Q (14%) and N64S (34%)). Yet, after 30 min activation time N64A produces significantly more transcript than WT (N64A (135%), N64D (not detected), N64Q (55%) and N64S (38%)), and after 60 min activation time N64Q reaches WT activity levels (N64A (170%), N64D (not detected), N64Q (180%) and N64S (50%)). In contrast to N64A and N64Q, N64S is clearly affected in the efficiency of transcription and not just slowed down, because after 60-min activation time the amount of transcript obtained remains substantially lower than WT. As expected from strong defects in self-association and ATPase, N64D did not activate transcription.

Because direct binding interactions between PspF 1–275 and (E)α 54 occur, we investigated whether the reduction in transcripts formed by Eo 54 with N64v was a consequence of either a defect in “activation” (OC formation) or promoter “escape” (transition to the elongating complex). To address this question, we performed abortive transcription assays to monitor OC formation. A defect in promoter escape should be accompanied by an accumulation of more abortive than full-length transcripts. To reduce the experimental error, we performed the abortive assays with the same reaction mix used for the full-length transcription assays. After activation, the sample was divided into two equal parts and supplemented with the appropriate nucleotide mix (see “Experimental Procedures”). For all the proteins tested, we observed similar amounts of abortive transcript as full-length transcript (Fig. 3B).

We conclude that the defect in the initial transcription rates observed with the N64v, is not due to a deficiency in promoter escape but due to a fault in using ATP hydrolysis to drive OC formation. Notably, N64A and N64Q appear to be slower in OC formation (transcript levels similar to WT after longer activation times following OC accumulation), whereas N64S is slower and less efficient (because after 60-min activation time, the
amount of transcript formed is substantially lower than WT). Although N64S forms more stable complexes with (E)σ54 than N64Q, it is more defective in OC formation, suggesting a problem in using target binding for remodeling Eσ54.

N64, Are Less Active Than PspF1–275 WT in Vivo—The in vivo activities of the N64, were then assayed to validate the results of the in vitro transcription experiments. In vivo assays were conducted using a strain lacking PspF and pspA (the negative effector of PspF, see below), in which we measured the amounts of β-galactosidase made by a single chromosomal lacZ gene copy under the control of pspAp using pBAD18C plasmids harboring pspF1–275 WT or pspF1–275 N64, genes. At maximal PspF1–275 induction levels transcription activities, compared with WT, were N64S (40%) > N64A (34%) with N64Q and N64D not detected, yet all N64, had similar levels of protein production (protein accumulation, see supplemental Fig. S2).

PspA Interacts with the N64, but Does Not Inhibit Their ATPase Activity—Because PspF ATPase activity is negatively regulated by PspA and Asn-64 contributes to PspF ATPase, we measured the sensitivity of N64, to negative regulation by PspA using an in vitro PspF ATPase assay in the presence of purified PspA. In the presence of PspA the ATPase activity of WT is ~70% inhibited; however, with the N64, no significant decrease of ATPase was observed (Fig. 4A). Importantly, direct binding interactions between PspA and WT or N64, were observed, but not with the negative control PspF1–275 W56A, which is specifically defective in binding to PspA (Fig. 4B). Taken together these results suggest that the repressive regulatory interaction between PspA and PspF (via residue Trp-56) occurs through Asn-64 acting to reduce Rrep of ATPase activity of PspF (see “Discussion”).

The Asn-64 Side Chain Affects Productive Communication between PspF and the σ54-DNA Complex—We hypothesized that the lower rate of transcription observed, in vivo (supplemental Fig. S2) and in vitro, for N64S could be due to a major defect in relaying of ATP hydrolysis to remodeling of the CC. To distinguish between interactions with σ54 from Eσ54, we used a σ54 supershift assay in the absence of core RNAP. Cannon et al. (10) showed that σ54 forms a stable complex with a linear promoter DNA probe harboring a mismatch at positions −12 and −11 on the non-template strand (−12 −11/wt). In the presence of a hydrolysable nucleoside triphosphate (dATP), PspF1–275 WT can convert this binary σ54-DNA complex to an isomerized ssσ54-DNA complex (super shifted σ54-DNA complex), which does not contain PspF1–275 and migrates differently on a native gel (10, 25). In addition, this DNA (−12−11/wt) is active, albeit at a significantly lower level than that observed with homoduplex DNA (0/wt), for OC formation (data not shown). As shown in Fig. 5A, the N64, with the exception of N64D, were all able to form the ssσ54-DNA complex, although some differences were observed. N64A formed a similar amount of ssσ54-DNA complex as WT, but N64S and N64Q formed clearly less ssσ54-DNA complex. Interestingly, when using N64Q an additional band (C_A) was also observed. Characterization of the C_A complex by UV cross-linking demonstrated the presence of N64Q, suggesting that C_A is a putative intermediate in the pathway to form the ssσ54-DNA complex (Fig. 6A, lanes 5 and 11). A similar complex was observed with E108D, suggesting an overlapping phenotype between N64, and E108, (25). Using another DNA probe (mismatched between −12 and −1 on the non-template strand; −12−1/wt) that supports ssσ54-DNA complex formation by E108, but not by N64, (25), N64Q forms the C_A complex, suggesting C_A is related to protein isomerization rather than DNA structure changes (supplemental Fig. S3).

In conclusion, with the exception of N64D, all the N64, tested supported formation of the ssσ54-DNA complex in an ATP hydrolysis-dependent manner on the −12−11/wt DNA, but not on pre-opened DNA (−12−1/wt). Interestingly, in the presence of N64Q a stable PspF1–275σ54-DNA complex (C_A) similar to that formed by E108D was observed, suggesting overlapping phenotypes.

PspF Activity Is Sensitive to the DNA Opening Step—We further explored the basis for the differences observed in the abilities of the N64, to use ATP hydrolysis to remodel the CC. For N64A, the amount of ssσ54-DNA complex formed is comparable to WT, although this variant is clearly affected in the rate of OC formation. For N64A the rate-limiting step in OC formation may not be σ54-DNA isomerization, but a core RNAP-dependent stage, potentially involving the conformation of the promoter DNA region melted within the OC. We tested this idea using abortive transcription assays with linear promoter DNA probes reflecting the closed DNA conformation (0/wt) or open DNA conformation (~−10−1/wt).

We first confirmed that the levels of abortive transcription from the linear (0/wt) and supercoiled nifH DNA were similar; demonstrating that the abortive initiation assays faithfully reflect the full-length transcription experiments (compare Figs. 3B and 5B). We then compared (Fig. 5, B and C) the activity of...
suggesting Asn-64 functions to effectively link $\sigma^{54}$ isomerization to DNA opening within the CC.

$\sigma^{54}$-DNA Interactions Are Modified by N64v—Because substituting Asn-64 alters the transcription activation efficiency of PspF$_{1-275}$ in a DNA template-dependent manner, it would seem likely that protein-DNA interactions made during remodeling of (E)$_{\sigma^{54}}$ would be different among the N64v. To investigate the nature of these protein-DNA interactions we employed a UV cross-linking experiment on the DNA probes (−12−11/yt and −12−1/yt) used in the $\sigma^{54}$ isomerization assays. The photoreactive DNA probes were constructed by conjugating a single, strategically placed phosphorothioate with APAB, between positions −7/−6 (−7), within the melted region in the E$_{\sigma^{54}}$ OC (single-stranded between positions −12 and −1) and between positions −1/1 (−1), the downstream edge of the transcription bubble and the transcription start site (15, 31).

Using the −12−11/yt DNA (Fig. 6A), the $\sigma^{54}$-DNA proximities at −7 N64v to WT for the initial rates of OC formation (5-min activation time) on closed (0/yt; WT > N64A > N64S > N64Q > N64D) or pre-opened DNA (−10−1/yt; N64A > WT > N64S > N64Q > N64D). In the presence of pre-opened DNA, we note a global increase in the amount of OC formed. Indeed, for N64A we observed ∼2-fold more OC than WT, suggesting that the asparagine side chain may negatively influence a step during OC formation that is dependent on DNA conformation. In addition, the amount of OC observed with N64S on pre-opened DNA is similar to WT (yet ∼3-fold lower on 0/yt DNA). Because we observed lower amounts of sso$^{54}$-DNA complex with N64S, it appears that the defect in transcription observed for N64S is most likely due to a deficiency in the isomerization of $\sigma^{54}$ (see “Discussion”). Interestingly, N64Q showed a linear increase in OC formation with time, independent of the DNA conformation used, whereas all the other N64v and WT reached a plateau or showed reduced levels of OC formation at later time points. N64Q appears to have traded fast initial rates of OC formation for a prolonged period of activation competency.

Overall in OC formation assays the N64v tested, compared with WT, exhibited very different sensitivities to pre-opened DNA, in initial rates and over prolonged time courses. The abilities among N64v to maintain OC were very different at later time points. Competency in $\sigma^{54}$ isomerization was not always accompanied by an equivalent competency in OC formation, and pre-opening DNA recovered some N64v, especially N64A, or −1 are similar in the presence of WT or N64v (except N64D), although the intensity of the cross-linked $\sigma^{54}$ species at position −7 is clearly stronger than those at −1. Cross-linking at position −7 (Fig. 6A) appears to reflect binding of $\sigma^{54}$ in initial and isomerized complexes and cross-linking at position −1 (Fig. 6A) reflecting $\sigma^{54}$ isomerization (Fig. 5A, lanes 1, 2, 4, and 5), suggesting that a range of Asn-64-dependent $\sigma^{54}$-DNA interactions are detected in these assays. Clearly a different set of interactions between $\sigma^{54}$, N64v, and DNA exists at, or close to, these positions (Fig. 6A, compare lanes 9−12 and 3−6). In addition, we note in the presence of N64Q a weak PspF$_{1-275}$ DNA band at position −1, similar to that observed with E108D, further suggesting an overlapping phenotype between Glu-108 and Asn-64 (supplemental Fig. S4).

To determine whether DNA conformation could affect interactions between $\sigma^{54}$ and DNA in the presence of N64v, a pre-opened DNA probe (−12−1/yt) was used. The cross-linking pattern of $\sigma^{54}$ at position −7 is clearly changed in the presence of the N64v, compared with WT (Fig. 6B, lanes 14−18). Significantly, cross-linked $\sigma^{54}$ at position −1 (Fig. 6B, lane 20) was comparable to WT in all the N64v, except N64D (Fig. 6B, lanes 20−24). We conclude that the interactions $\sigma^{54}$ makes with the single-stranded promoter DNA at position −7, but not at position −1, are altered in the presence of the N64v.

Asn-64 Mutations Affect the Core RNAP-DNA Interactions—We next determined whether N64 could alter the set of interactions that E$\sigma^{54}$ makes with DNA. When the −12−11/yt DNA complex with N64S, it appears that the defect in transcription observed for N64S is most likely due to a deficiency in the asparagine side chain may negatively influence a step during OC formation that is dependent on DNA conformation. In addition, the amount of OC observed with N64S on pre-opened DNA is similar to WT (yet ∼3-fold lower on 0/yt DNA). Because we observed lower amounts of sso$^{54}$-DNA complex with N64S, it appears that the defect in transcription observed for N64S is most likely due to a deficiency in the isomerization of $\sigma^{54}$ (see “Discussion”). Interestingly, N64Q showed a linear increase in OC formation with time, independent of the DNA conformation used, whereas all the other N64v and WT reached a plateau or showed reduced levels of OC formation at later time points. N64Q appears to have traded fast initial rates of OC formation for a prolonged period of activation competency.

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To determine whether DNA conformation could affect interactions between $\sigma^{54}$ and DNA in the presence of N64v, a pre-opened DNA probe (−12−1/yt) was used. The cross-linking pattern of $\sigma^{54}$ at position −7 is clearly changed in the presence of the N64v, compared with WT (Fig. 6B, lanes 14−18). Significantly, cross-linked $\sigma^{54}$ at position −1 (Fig. 6B, lane 20) was comparable to WT in all the N64v, except N64D (Fig. 6B, lanes 20−24). We conclude that the interactions $\sigma^{54}$ makes with the single-stranded promoter DNA at position −7, but not at position −1, are altered in the presence of the N64v.
Functional Pathway in AAA⁺ Proteins

DNA is conjugated at −7, the cross-linked σ54 species observed with WT (Fig. 6C, lane 26) is significantly increased in the presence of all N64v, including N64D (Fig. 6C, lanes 26–30). Clearly N64D can modify σ54-DNA interactions, although it cannot fully remodel the CC. Notably, a very weak cross-linked core RNAP band (corresponding to the β/β' subunits) was also observed with N64Q (identical to that of E108D, supplemental Fig. S4), suggesting that the organization of the N64Q-Eσ54-DNA and E108D-Eσ54-DNA complexes are similar. A similar cross-linking profile for WT and N64 was obtained when using the pre-opened −12−1/wt DNA (data not shown).

When the −12−11/wt DNA is conjugated at −1, the cross-linking profile observed with WT was clearly different to that obtained when using σ54 alone (Fig. 6A, lanes 7–12).

In the presence of core RNAP and WT, the intensity of the cross-linked σ54 clearly increases and an additional band, corresponding to cross-linked core RNAP is also apparent. In all the N64v tested except N64D, which does not support OC formation, the cross-linking profile obtained was similar to that obtained with WT (Fig. 6A, compare lanes 1 and 4). Similar cross-linking patterns were observed using the pre-opened DNA (data not shown).

Interestingly, when we used the cross-linking assay to examine the stable complexes formed between Eσ54 and PspF1-275 with the non-hydrolysable ATP transition-state analogue ADP-AlF₄⁻, we observed that in the presence of either N64Q or N64D the cross-linked PspF1-275 species was absent (Fig. 6D, lanes 44–45). This is not surprising for N64D, because this variant was unable to form a stable complex with Eσ54 (Fig. 3A). However, the differences observed with N64Q likely reflects an altered organization within the N64Q-Eσ54-DNA complex when ADP-AlF₄⁻ was used, because N64Q was weakly cross-linked in the σ54 isomerization reactions (Fig. 6A, lane 11). The results from the cross-linking assays suggest that N64A and N64S more closely resemble WT than N64D and N64Q, with N64Q having similar properties to E108D. Overlapping properties of Glu-108 and N64, suggest that they could each form part of the same nucleotide-dependent signaling pathways. A common basis for overlapping properties may reside in interactions Asn-64 makes with Walker B motif residues.

DISCUSSION

Determining the internal communication route operating between residues of AAA⁺ proteins is key to understanding how nucleotide-dependent outputs of AAA⁺ proteins are achieved. Structural alignments of AAA⁺ proteins with a range of cellular activities demonstrate the presence of an asparagine (Asn-64 in PspF) proximal to Walker B motif residues (Fig. 1, B and C), suggesting a common importance for this arrangement. In specialized AAA⁺ proteins (bEBPs) the asparagine is highly conserved suggesting the significance of this residue in the functionality of bEBPs. In this study, using the AAA⁺ domain...
of the model bEBP PspF, we show that Asn-64 contributes to the internal hexameric organization and regulation of the ATPase activity. Using different substitutions, we established the importance of this residue in substrate remodeling. Several steps from the initial interaction between PspF and the CC until OC formation are affected by N64v. Asn-64 has a critical role in regulating nucleotide-dependent contacts between PspF and its specific target, the CC, establishing the importance of this asparagine (Asn-64) in the optimal coupling of ATPase activity to OC formation.

**Conserved Asparagine Affects ATPase Activity and Self-association of an AAA⁺ Protein**—In this study, we showed that substituting Asn-64 causes changes in the self-association of PspF<sub>1–275</sub>. The gel filtration profiles suggest Asn-64 functions in the internal organization of the PspF<sub>1–275</sub> hexamer (Fig. 2C). We identified three different phenotypes associated with the specific Asn-64 substitutions: (i) an increase in hexamer formation as a function of PspF<sub>1–275</sub> concentration (N64S), (ii) a decrease in hexamer formation as a function of PspF<sub>1–275</sub> concentration (N64A and N64Q), and (iii) an absence of hexamerization (N64D). If we compare the different ATPase activities of the N64, and their elution profiles (Fig. 2, B and C), we note in the absence of oligomerization (N64D), ATP hydrolysis did not occur. Yet, an increase in hexamer formation (N64S) was not accompanied by an increase in ATPase activity (100% of WT level). In addition, despite similar elution profiles for N64A and N64Q, their ATPase activities are different. These results suggest a role for Asn-64 in the catalytic activity of PspF at the level of the detailed organization of the active site. This view is supported by structural data in which the position of an activating water molecule, used for the nucleophilic attack of the β-γ bond of ATP, and the Mg-ATP clearly suggests a possible involvement of Asn-64 in the ATPase activity of PspF (26).

**The Asparagine Side Chain Plays a Crucial Role in the Communication between the AAA⁺ Protein and Its Target**—We have identified distinct biochemical properties associated with the overall structure, concentration of PspF, and the associated loading of DNA into the RNAP during OC formation.

An increase in Asn-64 side chain length (N64Q) negatively affects concentration-dependent hexamer formation and greatly reduces the ATPase activity, binding interactions with (E)α<sup>54</sup> and isomerization of the ω<sup>54</sup>-DNA complex. In particular, N64Q affects the nucleotide-dependent contact with α<sup>54</sup>. Indeed, a new C<sub>α</sub> complex was observed in the isomerization experiments, clearly demonstrating that N64Q affects the process of ω<sup>54</sup>-DNA isomerization, thereby generating a new stable state similar to that observed with E108D (25). These results suggest that Glu-108 and Asn-64 may have interconnected functionalities. In agreement with this, the DNA cross-linking experiments, clearly demonstrating that N64Q affects the process of ω<sup>54</sup>-DNA isomerization, thereby generating a new stable state similar to that observed with E108D (25). These results suggest that Glu-108 and Asn-64 may have interconnected functionalities. In agreement with this, the DNA cross-linking results (Fig. 6) show a similar set of interactions between E<sub>α<sup>54</sup></sub> and DNA in the presence of either N64Q or E108D (supplemental Fig. S4).

Reducing the Asn-64 side chain length and altering the charge (N64S) favors PspF hexamerization, had no detectable effect on ATPase activity, but reduced the nucleotide-dependent interaction with (E)α<sup>54</sup> thereby reducing the amount of sso<sup>54</sup>-DNA and OC formed. Because the amount of OC formed in the presence of N64S was low and never reached WT levels from the closed DNA template, we infer that N64S is negatively affected in the coupling of ATPase activity to OC formation, at least at the level of changing the ω<sup>54</sup> organization for DNA opening. The latter view is supported by the recovery of initial rates of OC formation by N64S (near WT levels) on pre-opened DNA.

**Asparagine Couples PspA Binding to PspF ATPase-negative Regulation**—PspA, via PspF residue Trp-56, negatively regulates PspF ATPase activity through an as yet unidentified mechanism that does not involve reduced binding of ATP (29). Here we show that, although N64, can bind PspA, their ATPase

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activities are not significantly affected, suggesting that the negative regulation imposed by PspA on PspF ATPase activity may occur via Asn-64. We propose a functional pathway that links PspA binding (to PspF) with its negative effect on PspF ATPase activity (Fig. 7). Here Trp-56 senses an interaction with PspA and relays this binding event via β-sheet 2, to Asn-64, altering the position of the Asn-64 side chain, ultimately effecting the distance (and potentially the coordination of the water molecule) between N64-ATP and N64-E108. In N64S, the -OH side chain of Asn-64 can bind ATP ~4-fold better than WT, they have similar ATPase activity. These results further indicate the importance of the relative position of asparagine (Asn-64) to ATP and Walker B residues in the nucleotide binding pocket.

Asparagine-Walker B Distance and Optimal Coupling of ATPase Activity to Substrate Remodeling in an AAA + Protein—Previous researchers (26) have proposed a model based on structural studies of PspF1–275 crystals soaked with different nucleotides, in which they suggest that at the point of ATP hydrolysis a tight interaction between the side chains of residues Glu-108 and Asn-64 would stabilize exposure of the GAFTGA-containing L1 loop, thereby reinforcing the interaction between PspF and the DNA CC. Upon P i release, they proposed that the Glu-108 side chain rotates 90°, disrupting the Asn-64-Glu-108 interaction, causing rotation of helix 3 leading to a significant relocation of the GAFTGA-containing L1 loop into an unproductive, buried conformation. From functional data obtained on residues Glu-108 (25) and Asn-64 (this study), we revisited this model (Fig. 7). Significantly, we have established that in the absence of these residues’ side chains (E108A and N64A) PspF1–275 can still form a stable complex with σ 54 in the presence of ATP or ADP·AlF 4 , demonstrating that a stable interaction between PspF and σ 54 is clearly not strictly dependent on Glu-108-Asn-64 side chain interactions. Phenotypes of Asn-64 and Glu-108 substitutions, including alanine substitutions, suggest these residues are involved at different levels of a pathway coupling ATP hydrolysis to OC formation (Fig. 7). The pathway couples ATP hydrolysis to substrate remodeling by controlling the productive interaction between PspF and σ 54, changes in σ 54 allowing DNA loading into the RNAP during transcription activation (15). Determinants of the pathway emanating from the ATPase active site may well include Asp-107 and residues controlling the positioning of the central β-sheet of the AAA + domain (37).

Because structural alignments of AAA + proteins also point to conservation of the distance between the asparagine and Walker B residue, we suggest that such organization is important for other AAA + proteins. The positioning of these two residues (Asn-64-Walker B) may be required for their communication with each other and for creating a fully functional active site. The negative regulator of PspF (PspA) used this common property to control PspF activity. Indeed, PspA, via β-sheet 2, probably alters the position of Asn-64 affecting the optimal distance between Asn-64, ATP, and the Walker B residue.

In consequence, the ATPase activity of PspF is reduced and transcription activation is repressed. In the case of bEBPs, the communication between the asparagine and the Walker B motif residues contributes to controlling the positioning of the L1 loop (inserted in helix 3). In AAA + proteins that possess this asparagine but not the L1 loop insertion, we propose that the functional interactions between these two motifs could allow more global conformational changes in the oligomeric ring thereby regulating the functionality of the AAA + protein. Indeed, changes in the positions of residues proximal to the nucleotide pocket are relatively small when different nucleotide bound states are compared. The substrate remodeling must then depend upon amplification by the hexameric assembly of the small local nucleotide-dependent changes at the subunit level. The distance between Walker B residues and the asparagine studied here seems a good candidate for enabling such nucleotide dependent changes.

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