Single Chain Forms of the Enhancer Binding Protein PspF Provide Insights into Geometric Requirements for Gene Activation

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Genetic information in the DNA is accessed by the molecular machine RNA polymerase following a highly conserved process, invariably involving the transition between double-stranded and single-stranded DNA states. In the case of the bacterial enhancer-dependent RNA polymerase (which is essential for adaptive responses and bacterial pathogenesis), the DNA melting event depends on specialized hexameric AAA⁺ ATPase activators. Involvement of such factors in transcription was demonstrated 25 years ago, but why these activators need to be hexameric, whether all the subunits operate identically, what is the contribution of each of the six subunits within the hexamer (structural, functional, or both), and how many active subunits are required for transcription activation remain open questions.

Using engineered single-chain polypeptides covalently linking two or three subunits of the activator (allowing the subunit distribution within a hexamer to be fixed), we now show that (i) individual subunits have differential contributions to the activities of the oligomer and (ii) only a fraction of the subunits within the hexameric ATPase is directly required for gene activation. We establish that nucleotide-dependent coordination across three subunits of the hexameric bacterial enhancer binding proteins (bEBPs) is necessary for engagement and remodeling of the closed complex (RPc). Outcomes revealed features of bEBP, distinguishing their mode of action from fully processive AAA⁺ proteins or from simple bimodal switches. We now propose that the hexamer functions with asymmetric organization, potentially involving a split planar (open ring) or spiral character. The formation of the ATPase active site requires the assembly of a higher order oligomeric state protein complex (usually hexamer) (2–7). Some hexameric AAA⁺ ATPases have evolved from homo- (e.g. the helicase MCM in prokaryotes) to hetero-hexamers (e.g. MCM2–7 in eukaryotes) comprising up to six different proteins, strongly suggesting differential roles of each subunit for hexamer activity (8–12).

The majority of AAA⁺ proteins are active as homo-oligomers, but knowledge of the contribution of individual subunits to the hexamers activity is incomplete or often absent. At several levels of analysis, the six subunits are virtually identical, and their precise organization and functionality in the hexamer is extremely difficult to probe. A challenging approach is to fix the organization of the hexamer by linking the six subunits together. Chemical cross-linking is commonly used to track the number of subunits present in the oligomer but does not provide a good tool to address the geometrical distribution of subunits for functionality. Two additional approaches have been developed recently: (i) mixing experiments based on the reconstitution of hetero-oligomers containing different variants (13–15) and (ii) a single-chain approach based on the engineering of a polypeptide covalently linking two or more subunits of the oligomer, allowing the differentiation of subunit organization in the final oligomer (16).

In this study, we combined both approaches and used a model AAA⁺ activator protein specialized in σ54-dependent transcription activation, the bacterial enhancer binding protein (bEBP)³ PspF (17, 18). After hexamerization, bEBPs use the mechanochemical energy derived from ATP hydrolysis to remodel the transcriptionally inactive RNA polymerase closed complex (RPc) to the transcriptionally proficient open complex (RPo) (6, 19–24).

Use of the nucleotide analog ADP-AlF (25–28) has highlighted the role of the conserved PspF “GAFTGA motif” (contained in exposed loop I) in a direct interaction with the single σ54 factor found in RPc, prior to ATP hydrolysis (6, 29, 30). The intrinsic organization of the bEBP hexamer (proposed as a ring with six similar subunits) raises questions of how many GAFTGA motifs are required to (i) interact with RPc, and (ii) activate transcription.

While using ATP hydrolysis to remodel very different substrates (via specific substrate interaction motifs), PspF is part of

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AAA⁺ proteins (ATPases associated with various cellular activities) are involved in multiple cellular processes in all kingdoms of life. As complex molecular machines, they use nucleotide binding and hydrolysis to remodel their substrate (1–3).

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the same clade as the AAA+ protease ClpX (4), which has recently been studied using single-chain polypeptides (16, 31, 32). However, in contrast to the current ClpX model, which is based on a stochastic ATPase activity and the requirement of only one initial interaction with the substrate (16), our previous studies on PspF strongly suggest a different mechanism of action, based on cooperative ATPase activity with more than one subunit involved in the initial substrate interaction (13, 27, 29).

Here, using a single-chain polypeptide approach, we reveal novel features of the initial PspF-σ4 interaction that suggest asymmetry and possibly ring discontinuity together with the roles of individual subunits within the hexamer in terms of substrate remodeling activity. We show that not all of the subunits within the hexamer are needed to actively engage the target (σ4 within the RPo) for substrate remodeling (RPo) formation. These newly revealed features of bEBP functioning now uniquely distinguish their action from fully processive AAA+ proteins, where all subunits contact the target for remodeling, and from simple bimodal switches where a single subunit alternates between an active ATP-bound state and an inactive ADP-bound state sufficient for its action.

EXPERIMENTAL PROCEDURES

Plasmids—Single-chain linked pspF1–275 were constructed by PCR and cloned into pET28b+. Single-chain monomers, dimers, trimers, and hexamers (supplemental Table IS) contained an N-terminal His6 tag cleaved during protein purification. Constructs were verified by DNA sequencing.

Protein Purification—Unlinked PspF1–275 and linked trimers were overproduced as described in Ref. 29. Linked dimers were overproduced using LB inoculated with a day culture (6 h) (2% v/v) and grown at 30 °C for 12 h. Proteins were purified by slightly modifying the standard protocol (29). Briefly, after centrifugation, cells were resuspended in buffer A (25 mM NaPO4 buffer, pH 7.0, 500 mM NaCl, and 5% (v/v) glycerol) and broken by sonication. The supernatant was loaded onto HiTrap™ Chelating HP column (GE Healthcare) precharged with nickel and purified as described (29). The His6 tag was removed by thrombin cleavage for 3 h at 23 °C. The protein was finally dialyzed overnight at 4 °C against storage buffer (20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM DTT, 0.1 mM EDTA, and 10% (v/v) glycerol) and frozen at −80 °C. σ4 was purified as described (40). Escherichia coli core RNAP enzyme was purchased from Cambio.

ATPase Activity—Steady-state ATPase assays were used to monitor PspF1–275 or PspF1–275-linked protein ATPase activity. The ATPase activity assays were performed at 37 °C in the presence of an NADH-coupled regeneration system (41) in a 100-μl final volume, in buffer containing the final concentrations of 25 mM Tris-HCl, pH 8.0, 100 mM KCl, 10 mM MgCl2, 1 mM DTT, 1 mM NADH, 10 mM phosphoenolpyruvate, 10 units/ml pyruvate kinase, 20 units/ml lactate dehydrogenase, ATP (from 0 to 50 μM), and PspF1–275 or PspF-linked proteins (from 0 to 5 μM hexamer equivalent).

Gel Filtration through Superdex 200—Proteins (at the specified 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 MgCl2, ± 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 ± nucleotide (13). 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 carbonyl anhydrase (29 kDa). All experiments were repeated at least four times, and the elution profiles obtained were similar.

σ4 Interaction Assay—Gel mobility shift assays were conducted to detect protein-protein or protein-DNA complexes (26). 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 DTT, 4 mM ADP, ± NaF (5 mM) ± radiolabeled σ4 (1 μM). Where indicated, PspF1–275 or PspF-linked proteins (2 μM hexamer equivalent) ± AlCl3 (0.4 mM) were added for a further 15 min at 37 °C. Complexes were analyzed on a native 4.5% polyacrylamide gel. Radiolabeled σ4 was detected by a phosphorimaging device (Fuji Bas-5000) and analyzed using the Aida software.

RPo Formation Assay—Full-length or short primed RNA 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 DTT, 4 mM dATP, 0.1 μM core RNA polymerase enzyme, 0.4 μM σ4, 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 PspF1–275 or PspF-linked proteins and incubated for varying times at 37 °C. Full-length transcription (from the supercoiled Sinorhizobium meliloti 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 [α-32P]UTP and incubated for a further 10 min. The reaction was stopped by addition of loading buffer and analyzed on 6% denaturing 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 [α-32P]GTP and incubated for a further 10 min. The reaction was stopped by addition of loading buffer and analyzed on a 20% denaturing gel. Radiolabeled RNA products were measured by a phosphorimaging device (Fuji Bas-5000) and analyzed using the Aida software.

Mathematical Modeling—During the mixing experiment, we used different assumptions to attribute the activity corresponding to the hetero-hexamer formed from two different trimers following the law of binomial distribution. When equimolar concentrations of A and B trimers were mixed, we obtained the following distribution.

\[ A + B \overset{1}{\Rightarrow} \frac{1}{4} AA + \frac{1}{4} AB + \frac{1}{4} BA + \frac{1}{4} BB \]  
(Eq. 1)

If we assume that \( AB = BA \), we can simplify the previous equation and calculate the activity of the AB hexamer.

\[ A + B \overset{1}{\Rightarrow} \frac{1}{4} AA + \frac{1}{2} AB + \frac{1}{4} BB \]  
(Eq. 2)

We determined the activity of AB hexamer using the following equations.
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activity_{obs}(A + B) = \frac{1}{4} \text{activity}(AA) + \frac{1}{2} \text{activity}(AB)
+ \frac{1}{4} \text{activity}(BB) \quad (\text{Eq. 3})

activity_{obs}(A + B) = \frac{1}{4} \text{activity}(AA) - \frac{1}{4} \text{activity}(BB)
= \frac{1}{2} \text{activity}(AB) \quad (\text{Eq. 4})

activity(AB) = 2 \times \left(\text{activity}_{obs}(A + B) - \frac{1}{4} \text{activity}_{obs}(AA)
- \frac{1}{4} \text{activity}_{obs}(BB)\right) \quad (\text{Eq. 5})

Activity_{obs}(AA), activity_{obs}(BB), and activity_{obs}(A + B) were determined experimentally.

RESULTS

Construction of Covalently Linked PspF_{1–275} Subunits—We engineered a gene to encode a single-chain polypeptide covalently linking PspF_{1–275} subunits. Unfortunately, the N-terminal and C-terminal region of PspF_{1–275} are not defined structurally in the crystal structure available, preventing us from precisely estimating a linking distance (Fig. 1A) (27, 33). Using a systematic approach testing different sizes and compositions of linkers, we identified a glycine-rich sequence (-GSL GGGGS GGGGS GGGGS AAAEF-) suitable for forming wild-type PspF covalently linked dimers and trimers (WW and WWW, respectively; Fig. 1B). The predicted position of the linker precludes introduction of a steric clash with the substrate interacting GAFTGA motif, located on “top” of the hexamer (Fig. 1, A and B).

As AAA+ proteins have the common requirement to form higher-order oligomers (usually hexamers) to hydrolyze ATP and remodel their substrate, we tested whether the presence of the linker in WW and WWW was affecting these activities. Using either W, WW, or WWW, we observed similar hexamer formation (Fig. 1C) and ATPase activities (Fig. 1F and Table 1), suggesting that the presence of the linker allows the formation of catalytically competent hexamers. We then investigated whether the engineered proteins were able to interact with \sigma^{54} (using an ADP-AlF trapping reaction) and activate RP_o formation (in vitro transcription) (26). We observed that the presence of the linker affects, to some extent, formation of the stable ADP-AlF-dependent PspF_{1–275} hexamer-\sigma^{54} complex (Fig. 1D; W, 100%; WW, 80%; and WWW, 30%). The amount of RP_o formed (Fig. 1E) strictly correlates with the amount of PspF_{1–275} hexamer-\sigma^{54} formed (Fig. 1D), demonstrating that the presence of the linker affects formation of the initial complex between PspF_{1–275} and RP_o, but not its subsequent remodeling. For simplicity, we compared the activity of each construct (monomer, dimer, and trimer) to the activity observed with each of the corresponding W-linked constructs (W, WW, or WWW) as 100%.

From these results, we infer that the ATPase and substrate interaction activities of the PspF_{1–275} hexamer require different oligomer organizations, potentially arising from different levels of sensitivity to subunit movements. In addition, these experiments reveal an unexpected differentiation between common properties of AAA+ proteins (oligomerization and ATPase activity) and specific PspF_{1–275} properties (\sigma^{54} binding interactions). To simplify the comparison between the different constructs, we subsequently expressed the activity associated with each single-chain peptide with respect to the wild-type version containing the same number of linked subunits.

Organization of PspF Hexamer—Having established that the single-chain forms of the dimer and trimer are active, we investigated whether all the subunits of the hexamer were required for \sigma^{54} binding interactions and RP_o formation. Substituting threonine in the GAFTGA motif (T86A, referred herein as T, Fig. 2A) greatly reduces \sigma^{54} binding interactions without impacting on other properties of the PspF_{1–275} hexamer (ATPase activity and oligomerization) (30). We engineered single-chain polypeptides linking native (W) and defective (for \sigma^{54} interaction) (T) subunits and showed that ATPase activity and hexamer formation of these peptides were similar (Table 1 and supplemental Table IS and Fig. 1S); demonstrating that introducing one, two, or three variant subunits (T) into the single-chain peptide does not significantly affect hexamer organization nor the formation of the ATPase catalytic site between adjacent subunits.

We next examined whether alternate \sigma^{54} binding interacting competent (W) and defective (T) subunits can support \sigma^{54}-dependent hexamer activities. Using WT or TW-linked dimers (allowing formation of WT/WT/WT or TW/TW/TW hexamers, respectively), we observed reduced hexamer-\sigma^{54} binding interactions (~30% of the WW/WW/WW hexamer; Fig. 2B, lanes 9–16). These results establish directly, for the first time, that more than one W subunit is required to support full formation of a stable PspF_{1–275} hexamer-\sigma^{54} complex. Furthermore, we tested whether the residual \sigma^{54} interaction was productive for RP_o formation. The remodeling activities were more reduced than predicted by simple extrapolation from the \sigma^{54} binding results, where each binding interaction is assumed to support a remodeling event. Instead, we observed 10% of RP_o formation in contrast with the 25 and 20% of \sigma^{54} binding interactions scored for WT and TW hexamers, respectively (Fig. 2C, lanes 4–6). As expected, we also observed that the TT/TT/TT hexamer completely failed to stably interact with \sigma^{54} and hence activate RP_o formation, consistent with results obtained for the T/T/T/T/T/T hexamer (Fig. 2, B and C).

We conclude that alternate arrangements of competent and defective subunits in the hexamer are not optimal for \sigma^{54} binding and even less so for RP_o formation. These results suggest the requirement of more than one competent subunit for these activities and imply that RP_o formation involves a different "set" of subunits than those needed for the initial \sigma^{54} interaction (based on higher levels of \sigma^{54} binding than RP_o formation). Clearly, the introduction of alternate defective subunits in the hexamer affects \sigma^{54} interactions and uncouples ATPase activity from substrate remodeling.
A Symmetric Hexamer Organization?

We next addressed whether two adjacent W subunits were required for binding interactions and RP\textsubscript{o} formation activities. Using single-chain trimers, WWT or TWW (to form dimers of trimers WWT/WWT or TWW/TWW), we observed similar levels of PspF hexamer complex formed as with WWW (80 and 75%, respectively, of WWW/WWW; Fig. 2B, lanes 23–28). Clearly, two adjacent W subunits are more favorable for binding interactions than alternate competent and defective subunits (see “WT, TW” studies above). Strikingly, the level of RP\textsubscript{o} formation observed with the WWT and TWW hexamers was different (80 and 40%, respectively, compared with WWW), demonstrating that the WWT and TWW hexamers are not fully equivalent (Fig. 2C, lanes 9 and 10). From these results, we infer that RP\textsubscript{o} formation is favored by a loop I-mediated contact to \( \sigma^{54} \), distinct from the one needed for the initial \( \sigma^{54} \) interaction.

### TABLE 1

**Kinetic constants for ATP hydrolysis for PspF\textsubscript{1–275} constructs**

| Protein | \( V_{\text{max}} \) \( \text{min}^{-1} \) | \( K_{\text{m}} \) \( \mu\text{M} \) |
|---------|-----------------|--------|
| W/W/W/W/W/W | 39.51 ± 4.62 | 250 |
| T/T/T/T/T/T | 40.19 ± 4.22 | 714 |

**Dimer-linked**

| Protein | \( V_{\text{max}} \) \( \text{min}^{-1} \) | \( K_{\text{m}} \) \( \mu\text{M} \) |
|---------|-----------------|--------|
| W/W/W/W/W/W | 40.60 ± 3.90 | 333 |
| T/T/T/T/T/T | 43.22 ± 4.15 | 303 |
| T/W/T/W/T/W | 46.04 ± 5.76 | 285 |
| T/T/T/T/T/T | 48.94 ± 3.33 | 344 |

**Trimer-linked**

| Protein | \( V_{\text{max}} \) \( \text{min}^{-1} \) | \( K_{\text{m}} \) \( \mu\text{M} \) |
|---------|-----------------|--------|
| W/W/W/W/W/W | 33.33 ± 2.59 | 181 |
| W/T/W/T/W/T | 28.46 ± 3.99 | 166 |
| T/W/T/W/T/W | 33.16 ± 1.52 | 166 |
| T/T/T/T/T/T | 28.93 ± 4.14 | 163 |
| T/W/T/W/T/W | 28.70 ± 3.63 | 173 |
| T/T/T/T/T/T | 29.12 ± 1.24 | 156 |
and operational with WWT/WWT but not with TWW/TWW hexamers.

We next tested whether two opposing W subunits in the hexamer were able to support $\sigma^{54}$ binding and RPo formation. Using WTT or TWT trimers, we observed a drastic decrease in the amount of complex formed with $\sigma^{54}$ (20% and none, respectively, compared with WWW; Fig. 2B, lanes 29–34). The amount of RPo formation was similar to $\sigma^{54}$ binding (18% and none, respectively), further demonstrating that the relative locations of the W subunits in the hexamer are critical for its activities and that all the subunits of the hexamer are not functionally equivalent (Fig. 2C, lanes 11 and 12).

Minimal Requirement for $\sigma^{54}$ Interaction—Based on the likelihood of an asymmetric hexamer arrangement and a physical discontinuity in the hexamer, we asked whether increasing the number of defective subunits in the hexamer has a linearly dependent output effect (or not) on $\sigma^{54}$ binding and RPo formation activities. We used a mixing approach to reconstitute different hexamers harboring from zero to six defective subunits (Fig. 3). Mixing experiments have been successfully used to investigate PspF (13) and other AAA+ proteins activities, including ClpX, ClpB, and MCM (14, 31, 34). As PspF hexamer formation is dependent on protein concentration (29), we measured mixing efficiency by gel filtration (supplemental Fig. 3S) and established that mixing between two different linked trimers was occurring. We assumed that the mixing of trimer A and trimer B follows a binomial distribution (see “Experimental Procedures,” Mathematical Modeling). First, we modeled the theoretical values expected if the mixing between the two different types of trimers (A and B) was not occurring, using the activity values observed in the case of the homo-hexamers (supplemental Fig. 4S). Clearly, the observed $\sigma^{54}$ binding and RPo formation activities of the hetero-hexamers are distinct from the activities expected if mixing was not occurring; establishing the formation of hetero-hexamers in solution (Fig. 3A). In addition, we observed that the $\sigma^{54}$ binding and RPo formation activities follow different trends, suggesting different requirements.
for substrate interaction and remodeling. We then compared the contribution of the predicted hetero-hexamer subpopulation to the observed total activity (Fig. 3B). Interestingly, we find that the \( \sigma^{54} \) binding activity of the hetero-hexamer is following a linear trend dependent on the number of adjacent W subunits up to just two adjacent W. Indeed, we observed that with only one W subunit, the hexamer could not detectably stably bind \( \sigma^{54} \), demonstrating that one W subunit is not sufficient to establish a stable interaction (Fig. 3, A–B). Taken together, these results confirm that the minimal configuration to form a stable complex between PspF and \( \sigma^{54} \) is to have at least two adjacent W subunits in the hexamer.

**RP\(_o\) Formation Requires Activity of More Subunits Than Needed for \( \sigma^{54} \) Binding**—We next performed mixing experiments to monitor the level of RP\(_o\) formation dependent on the hexamer arrangement (Fig. 3, A and B). Strikingly, the level of RP\(_o\) formed with each mixed species is very different to the level of stable complex formation with \( \sigma^{54} \) (Fig. 3, A and B).

When one subunit is defective, the remaining five other W subunits are sufficient to exhibit full activity of the hetero-hexamer (Fig. 3B). Clearly less than six (W) subunits are necessary for substrate remodeling activity. Introduction of two or three adjacent defective (T) subunits drastically decreases the RP\(_o\) formation efficiency of the hetero-hexamer, whereas four to six adjacent defective (T) subunits cause full loss of RP\(_o\) formation activity. From these results, we conclude that the RP\(_o\) formation activity requires at least one W subunit more than needed for \( \sigma^{54} \) binding.

**Asymmetric Hexamer Organization of PspF**—Using the mixing approach, we then investigated the impact of the location of the defective and active subunits within the hetero-hexamer on its activities (Fig. 4 and supplemental Fig. 5S).

One defective (T) subunit within the hexamer (Figs. 3 and Fig. 4, lanes 2 and 3) slightly affects \( \sigma^{54} \) binding but not RP\(_o\) formation activity of the hetero-hexamer, demonstrating that less than six subunits are sufficient for full RP\(_o\) formation. Introduction of two defective subunits (Fig. 4, lanes 4–8) has a more drastic effect on the RP\(_o\) formation activity than on \( \sigma^{54} \) binding. Furthermore, the location of the defective (T) subunits differentially affects the final activity of the hexamer (Fig. 4, compare lanes 7 and 8). This result is inconsistent with a model where all the subunits constituting the hexamer are functionally identical. Introduction of three defectives (T) subunits within the hexamer (Fig. 4, lanes 9–13) only modestly affects \( \sigma^{54} \) binding but drastically reduces the RP\(_o\) formation activity of the hetero-hexamer, except for WTT/TWW (Fig. 4, lane 10). Again, this observation is not consistent with the model proposing a planar hexamer where all the subunits are equivalent (Fig. 4, compare lanes 9 and 10). Introduction of four defective (T) subunits (Fig. 4, lanes 14–18) drastically reduces \( \sigma^{54} \) binding and inhibits RP\(_o\) formation activity. Introduction of five or six defective subunits (Fig. 4, lanes 19–21) completely inhibits \( \sigma^{54} \) binding or RP\(_o\) formation activities, demonstrating that a single W subunit within the hexamer is unable to support substrate binding or remodeling activities.

Taken together, these results demonstrate that the minimal requirement for a stable \( \sigma^{54} \) binding interaction is clearly different to that for RP\(_o\) formation; the latter is clearly more sensitive to the location of the W subunits. We thus propose that more than the two subunits needed for \( \sigma^{54} \) binding interaction are required for RP\(_o\) formation.

**DISCUSSION**

We now show that for the same number of PspF active (W) and defective (T) subunits, their relative positions within the hexamer are critical for substrate binding and remodeling output activities. We provide evidence that the PspF hexamer functions asymmetrically, inferring that all the subunits of the
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Hexamer cannot be identical in their contribution to final activity. We establish that the minimal subunit organization required for substrate interaction is different from the one needed for substrate remodeling. Monitoring both activities revealed that the engineered hexamer was more effective for substrate interaction than for substrate remodeling. This implies that the ability of the hexamer to accommodate nonoptimal organizations is higher (and so less stringent) for the target binding interactions than for remodeling activities. Clearly, the binding interactions detected are not always productive for Rp₀ formation. We propose that the PspF hexamer minimally requires two adjacent subunits for substrate binding, but substrate remodeling uses the σ²⁴ contacting contribution of at least one additional subunit. Furthermore, we demonstrate that more than one hexamer organization can support substrate interaction and remodeling. Indeed, we were able to observe the same level of activity for different configurations of the hexamer.

By investigating the impact of the relative location of the defective subunit within the hexamer, we revealed that for a given number of W and T subunits, the level of activity can differ depending on the particular hexamer organization (Fig. 4). We confirmed the minimal requirement of two adjacent W subunits for optimal σ²⁴ binding. These results clearly demonstrate that (i) there are several arrangements of the hexamer supporting σ²⁴ binding, which can be subdivided into two types, productive and unproductive for Rp₀ formation, and (ii) a range of possible interactions between the PspF hexamer and σ²⁴ can occur. Clearly, the position of the “competent” subunits dictates the final hexamer activities. Indeed, for the same number of defective subunits and the apparently similar internal organization within the hexamer, we observed different activities (Fig. 4, e.g. compare lanes 7 and 8 or 9 and 10). Current models proposing that the activator functions as a planar hexameric ring where all of the subunits are virtually identical needs to be revised to be consistent with the experimental data. We now propose that the operational hexamer is not planar but spiral (with one interface drastically different) or “split” (with two different opposite interfaces).

**Geometrical Requirement for AAA+ ATPase Hexamer Activity, Substrate Interaction versus Activation**—As illustrated by recent structural and functional studies on the hexameric ATPases Rho, MCM, and ClpX, current views favor an asymmetrical organization of the hexamer for functionality (10, 14, 35–39). This asymmetrical organization can be dependent on differential nucleotide binding as well as an intrinsic property of the apo form of the protein. In support of potential asymmetric functioning of wild-type ClpX, an active dimer of linked trimers containing nonequivalent ClpX AAA⁺ domains was crystalized yielding staggered AAA⁺ subunits in nucleotide-free and nucleotide-bound states. Clearly, an asymmetry can be tolerated, although its origins in a homo-hexamer are less well understood. For PspF, the subunits constituting the hexamer are not equivalent for each of its activities, inconsistent with a model based on a strictly planar hexameric ring where individual subunits are very similar or identical. We have demonstrated that the subunits constituting the hexamer have different impacts on its various activities depending on their relative...
location within the hexamer. These results imply an asymmetry in the hexamer imposed prior to substrate interaction. To rationalize this observation, we propose that PspF does not function as a strictly planar hexameric closed ring (as suggested previously) but tends toward a configuration with an opened hexameric spiral character with several distinct subunit interface conformations. In this case, each subunit can be distinct in relation to their interfaces and their relative place within the hexamer (subunits 1–6).

PspF structural results obtained previously are not in disagreement with this new proposal (of a spiral-like or split hexamer) because the only structural data obtained are a crystal structure of a monomer (modeled after as a hexamer) and a cryo-EM map of the PspF hexamer in complex with $\sigma^{54}$ in the presence of ADP-AlF (a nucleotide analog that effectively traps the complex potentially by changing the interface between subunits within the PspF hexamer), which locks the PspF-$\sigma^{54}$ interaction in a stable state. The specific substrate for PspF is Region I of $\sigma^{54}$ (in context of the RPc) (30). Rappas et al. (27) established that the PspF hexamer interacts with only one $\sigma^{54}$ factor, which is itself asymmetric. We have now shown that at least two subunits of the hexamer are required to bind $\sigma^{54}$, clearly implying that more than one PspF L1 loop GAFTGA motifs contact $\sigma^{54}$. (Potentially both interaction sites lie within region I.) The asymmetrical organization of PspF may be a key determinant in this interaction with $\sigma^{54}$. In support of this, we have shown that PspF ATPase activity is neither stochastic nor synchronized in the hexamer but is partially sequential (29). Importantly, the asymmetric nature of the hexamer could support the directionality of the ATPase activity in correlation with a dynamic binding interaction with $\sigma^{54}$ using a subset of L1 loops identified in this study (Fig. 5).

FIGURE 5. Model of transcription activation by an asymmetric bEBP hexamer. Based on the results obtained in this study, we propose the following model differentiating between substrate binding and remodeling activities of the bEBP. PspF subunits colored orange are not able to interact with $\sigma^{54}$ (T86A) but hydrolyze ATP. RPc represents the RNA polymerase-promoter closed complex, RP represents the RNA polymerase-promoter intermediate complex, and RPo represents the RNA polymerase-promoter open complex. Arrows represent subunit organizations leading to substrate remodeling with productive organizations in white and nonproductive organization in black.
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A Rationale for an Asymmetric Hexamer Organization of PspF—We speculate that the initial binding of PspF to its specific upstream enhancer sites on the DNA promoter will set the asymmetrical organization. A DNA bending event (brought about by IHF binding) brings PspF and RPc in close proximity, allowing the asymmetric PspF hexamer to interact with $\sigma^{54}$ in the RPc, but in a restricted topology, favoring an active ATP-dependent RPc formation (Fig. 5). In this scheme, the imposed asymmetry of the PspF hexamer prior to interacting with $\sigma^{54}$ could greatly reduce unproductive interactions that might arise due to nonoptimal coupling between substrate binding and ATP hydrolysis in the hexamer. Similar asymmetric encounters are likely to operate among other AAA+ and self-associated ATPases to effectively couple their ATP use to substrate remodeling.

Establishing that only a subset of L1 loops are used to bind $\sigma^{54}$ and to convert RPc to RPp provides clear evidence against bEBPs functioning through a processive mechanism in which all subunits of the hexamer are at some point contacting RPc (or an intermediate, RPp) for RPp formation. It follows that only a subset of ATPase sites need to be used to appropriately configure the L1 loops for contacting RPc and for its remodeling. These newly revealed features of bEBPs functioning now disinguish their mode of action from fully processive AAA+ proteins, where all subunits make contact with the target for remodeling, and from simple bimodal switches where a single subunit alternates between an active ATP-bound state and an inactive ADP-bound state that is sufficient for its action. We propose that bEBPs use an elaborated bimodal switching requiring coordination of switching across three subunits for engagement and remodeling of RPc. Implicit in this model is the asymmetric functioning of the bEBPs, revealed in these studies.

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