σ^{54} -dependent transcription activator phage shock protein F of *Escherichia coli*: a fragmentation approach to identify sequences that contribute to self-association

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Proteins that belong to the AAA (ATPases associated with various cellular activities) superfamily of mechanochemical enzymes are versatile and control a wide array of cellular functions. Many AAA proteins share the common property of self-association into oligomeric structures and use nucleotide binding and hydrolysis to regulate their biological output. The *Escherichia coli* transcription activator PspF (phage shock protein F) is a member of the σ^{54} -dependent transcriptional activators that belong to the AAA protein family. Nucleotide interactions condition the functional state of PspF, enabling it to self-associate and interact with its target, the σ^{54} -RNAP (RNA polymerase) closed complex. The self-association determinants within the AAA domain of σ^{54} -dependent activators remain poorly characterized. In the present study, we have used a fragment of the AAA domain of PspF as a probe to study the nucleotide-conditioned self-association of PspF. Results show that the PspF fragment acts *in trans* to

inhibit specifically self-association of PspF. The PspF fragment prevented efficient binding of nucleotides to PspF, consistent with the observation that the site for nucleotide interactions within an oligomer of AAA proteins is created between two protomers. Using proximity-based footprinting and cross-linking techniques, we demonstrate that the sequences represented in this fragment are close to one protomer-protomer interface within a PspF oligomer. As the sequences represented in this PspF fragment also contain a highly conserved motif that interacts with the σ^{54} -RNAP closed complex, we suggest that PspF may be organized to link nucleotide interactions and self-association to σ^{54} -RNAP binding and transcription activation.

Key words: AAA protein, FeBABE, oligomerization, σ^{54} , transcription activator.

INTRODUCTION

Members of the AAA (ATPase associated with various cellular activities) superfamily of proteins are found in all forms of life [1,2]. In bacteria, they contribute to a wide array of activities including cell division, cell differentiation and transcriptional activation [1–3]. Despite the diversity of their functional roles, AAA proteins share the ability to self-associate (usually to form hexamers) and to use the energy derived from NTP hydrolysis for biological output. One class of AAA proteins are the bacterial transcriptional activators of the σ^{54} -RNAP (σ^{54} -containing RNA polymerase) [3,4].

Open promoter complex formation by the σ^{54} -RNAP strictly depends on its activators [3,4]. These AAA activators bind to enhancer sequences and contact the promoter-bound σ^{54} -RNAP by looping out the intervening DNA [4]. Activators couple the energy derived from NTP hydrolysis to conformational changes in σ^{54} -RNAP and the promoter DNA so as to overcome an inhibitory interaction between σ^{54} and a DNA fork junction structure at the –12 promoter position (where DNA melting originates) [5–8]. Thus transcription activation at enhancer-dependent promoters involves several nucleotide-conditioned and activator-driven remodelling events of a nucleoprotein interface between σ^{54} -RNAP and promoter DNA [6–9].

Control of self-association is one way in which the NTPase activity of AAA proteins is regulated. For σ^{54} -RNAP-dependent activator proteins NtrC, DctD, DmpR and XylR oligomerization is controlled by a regulatory N-terminal domain [10–12]. *Escherichia coli* PspF (phage shock protein F) lacks an N-terminal

regulatory domain, and its activity is controlled by the PspA protein *in trans* [13,14]. PspF activates expression of the *psp* operon (*pspA-E*), products of which, in *E. coli*, are involved in adaptive responses to various cellular stresses [13,15] and, in *Yersinia enterocolitica*, are required for pathogenicity [16].

In AAA proteins, the active site for NTP binding and hydrolysis is contributed by two subunits, and so NTP binding and hydrolysis probably depend on and potentially determine the oligomeric state of the protein. Previously, we showed that PspF forms a stable oligomer in the presence of ADP · AlF_x, a transition state analogue of ATP at the point of hydrolysis [17]. This oligomer is a hexamer (J. Schumacher, X. Zhang, S. E. Jones, P. Bordes and M. Buck, unpublished work). The ADP · AlF_x-dependent PspF oligomer, in contrast with PspF in the absence of ADP · AlF_x, is capable of forming a stable complex with σ^{54} -RNAP, suggesting that NTP interactions lead to different functional states of PspF. These states may be coupled with stages of transcription activation through a PspF domain association reaction [3,17]. An important question that surrounds the functioning of AAA proteins and σ^{54} -RNAP-dependent activators concerns how NTP binding and hydrolysis lead to the conformational changes in AAA proteins that are required for activities such as self-association and substrate interactions.

To date, the information on self-association determinants within the AAA domain of σ^{54} -dependent activators remains sparse. To determine the sequences within the AAA domain of PspF that contribute to self-association, we constructed fragments of the AAA domain of PspF and sought to identify biochemically active sequences that interfere *in trans* with self-association-dependent

Abbreviations used: AAA, ATPase associated with various cellular activities; FeBABE, Fe (*p*-bromoacetamidobenzyl)-EDTA; MBP, maltose-binding protein; NTCB, 2-nitro-5-thiocyanobenzoic acid; PspF, phage shock protein F; RNAP, RNA polymerase.

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Figure 1 Amino acid sequence and SDS/PAGE analysis of PspF MBP fragments

(A) Schematic diagram showing (to scale) the two-domain organization of *E. coli* PspF. The C-terminal domain (residues 296–325; highlighted in black) contains a helix–turn–helix DNA-binding domain. The N-terminal AAA domain (residues 1–275) is directly responsible for ATP hydrolysis and transcriptional activation. This domain is highly conserved amongst σ^{54} activators and the seven conserved regions (C1–C7) [26] are indicated in white. The Walker A and B motifs involved in NTP binding and hydrolysis are indicated. The amino acid sequences of the PspF fragments F1 (residues 69–134), F2 (residues 69–102), F3 (residues 89–134) and F4 (residues 69–93) are shown. Boxed are C3 (containing the signature motif GAFTGA of σ^{54} activators), C4 and C5 regions. (B) Coomassie Blue stained SDS/PAGE (10% gel) showing the PspF fragments purified as MBP–fusion proteins.

functions of intact PspF. Using this fragmentation approach, we localized recently a surface in PspF that formed a stable complex with promoter-bound σ^{54} in the absence of any nucleotides [5]. In the present study, we report the identification of an AAA domain fragment of PspF that functions to inhibit the nucleotide-dependent self-association function of the AAA domain of PspF. Proximity-based protein footprinting and cross-linking techniques were employed to demonstrate that the sequences represented in this fragment of PspF localize near a promoter–promoter interface within the ADP·AlF₄-dependent oligomer of PspF. These results are consistent with the recent crystal structure of the σ^{54} -dependent activator NtrC1 from *Aquifex aeolicus* [19] and also indicate that some self-association determinants of PspF are close to a σ^{54} -binding surface within the PspF AAA domain. Thus it appears that the PspF surfaces that are involved in self-association and stable σ^{54} -binding are functionally inter-linked and regulated by the state of the bound nucleotide.

EXPERIMENTAL

Plasmids and strains

The plasmid encoding PspF residues 1–275 was the template to amplify DNA encoding PspF fragments F1–F4 using appropriate primers for PspF F1 (residues 69–134), PspF F2 (residues 69–102), PspF F3 (residues 89–134) and PspF F4 (residues 69–93) (Figure 1). PCR fragments were inserted into *Nco*I–*Hind*III-digested pMBP::parallel1 [8]. Plasmids pPB2, pPB3, pPB4 and pPB5 encode PspF fragments F1–F4 respectively in-frame with the *E. coli* *malE* gene, encoding the MBP (maltose-binding protein).

The pPB1 plasmid was used as the template for construction of single cysteine mutant Cys⁶⁵ of PspF_{1–275} (Cys⁶⁵-PspF_{1–275}) using the Quik Change mutagenesis kit (Stratagene) by replacing Cys¹⁴⁶ and Cys¹⁹⁸ with alanine. Briefly, mutated DNA was synthesized by *Pfu* DNA polymerase in a reaction containing pPB1 and a large molar excess of complementary mutagenic oligonucleotides. After 30 cycles of heating at 95 °C for 1 min, 50 °C for 1 min and

68 °C for 12 min, the reactions were treated with *Dpn*I to remove the parental DNA. The resulting reaction was used to transform *E. coli* MC1061 cells. Mutant clones were verified by DNA sequencing.

E. coli strain JM109 containing the σ^{54} -dependent transcriptional *pspAp-lacZ* fusion carried by the tetracycline-resistant plasmid pSJ1 [20], a derivative of pMR25 (a gift from Dr M. R. K. Alley, Anacor Pharmaceuticals, Palo Alto, CA, U.S.A.), was used to measure PspF_{1–275} activity *in vivo*. pPB1 derivatives containing the mutant forms of *pspF*_{1–275} were *Xba*I–*Hind*III-digested and the mutant sequences were cloned into the chloramphenicol-resistant plasmid pACT3 so as to express the wild-type or mutant PspF_{1–275} proteins under the control of a *tac* promoter.

Proteins

MBP-fused PspF fragments were purified as described in [8]. PspF_{1–275} and Cys⁶⁵-PspF_{1–275} were purified as described in [13] and σ^{54} (residues 1–477) was purified as described in [8]. For ³²P-end labelling, an HMK (heart-muscle kinase)-tagged PspF_{ΔHTH} (residues 1–292) was purified from pSRW-HMK-PspF_{ΔHTH} essentially as described in [21]. *E. coli* core RNAP was purchased from Epicentre Technologies (Madison, WI, U.S.A.). The proteins used in this study are listed in Figure 2(A).

Oligomerization assays

Assays to measure oligomerization of PspF were essentially performed as described previously [17]. Briefly, 0.4 μM of [³²P]PspF_{ΔHTH} was incubated at 30 °C for 5 min in STA buffer [25 mM Tris/acetate (pH 8.0)/8 mM magnesium acetate/100 mM KCl/1 mM dithiothreitol/3.5% (w/v) poly(ethylene glycol)-6000] with 0.2 mM ADP, 5.0 mM NaF and PspF fragments or σ^{54} as required. After addition of 0.2 mM AlCl₃, the reactions were incubated for a further 10 min and directly loaded on to a native gel. The native gels were run at 25 V/cm for 80 min in 25 mM Tris and 200 mM glycine buffer (pH 8.6). Protein complexes were detected by PhosphorImager (Fuji Bas-1500; Fuji Photo Film,

Figure 2 Proteins and promoter probes

(A) Proteins and (B) *S. meliloti nifH* promoter probes used in this study. (B) The consensus GG and GC nucleotides of the σ^{54} binding sites are indicated in bold. Boxed are the mismatched regions at positions -11 and -12 relative to the transcription start site (+1). Characters in grey indicate mutant sequences introduced to create the heteroduplex segments.

Tokyo, Japan) analysis. α -Lactalbumin (50 ng) was added to reactions containing 32 P-end-labelled proteins.

Native gel mobility-shift assays

Promoter DNA probes (Figure 2B) for the DNA-based assays were conducted essentially as described in [8]. All protein-binding reactions were performed in STA buffer using 16 nM DNA. When needed, σ^{54} or σ^{54} -RNAP holoenzyme was present at 0.5 μ M and 0.1 μ M (reconstituted with 1:4 molar ratio of core RNAP to σ^{54}) respectively and nucleotides at 2 mM. For assays with PspF MBP-F2, PspF₁₋₂₇₅ was preincubated in STA buffer with various amounts (as indicated in the Figures) of PspF MBP-F2, before adding the mixture to activator-dependent assays. The native gels were run at 60 V for 90 min. Protein-DNA complexes were detected by PhosphorImager analysis.

ATP-binding and hydrolysis assays

ATP-binding assays by UV cross-linking were performed as described previously [5]. Briefly, 5 μ M PspF₁₋₂₇₅ was incubated with 0.75 μ M [γ - 32 P]ATP (3000 Ci/mmol) $\pm$ 3 mM ATP in a final volume of 20 μ l reaction buffer A [35 mM Tris/acetate (pH 8.0)/70 mM potassium acetate/5 mM magnesium acetate/19 mM ammonium acetate/0.7 mM dithiothreitol]. For assays with PspF MBP-F2, 2 μ M protein was present in 50 μ l reaction volume. Samples were illuminated by a UV lamp (254 nm, UVG-54; UVP, San Gabriel, CA, U.S.A.) at 3 cm distance (15 min, 0 $^{\circ}$ C). Proteins were precipitated with 10% (w/v) trichloroacetic acid, washed with acetone and analysed by SDS/PAGE (12.5% gel). The gel was stained with Coomassie Blue, dried and then was visualized by PhosphorImaging.

ATPase assay was performed in reaction buffer A with 2 μ M PspF₁₋₂₇₅ as described previously [13]. Released P_i was separated from ATP using TLC as described in [22] and measured by PhosphorImaging.

In vitro transcription assays

The template for the *in vitro* transcription assays was either the supercoiled plasmid pSLE1 containing the *E. coli pspA* promoter or pFC50-m12 carrying the *glnHp2*-m12 promoter [13,21]. Activator-dependent transcription assays were performed essentially as described previously [5]. Reactions were performed at 37 $^{\circ}$ C and contained 100 nM holoenzyme (reconstituted with 1:5 molar ratio of core RNAP to σ^{54}). For activation, 800 nM PspF₁₋₂₇₅ or NtrC was added and for phosphorylation 10 mM carbamoyl phosphate was used. The indicated amounts of wild-type and mutant PspF MBP-F2 were preincubated with 800 nM PspF₁₋₂₇₅ or NtrC before adding to the reaction. After activation, the reactions were incubated for 20 min to allow open-complex formation. To allow synthesis of transcripts, 100 μ g/ml heparin, a cocktail of rNTPs and 12.5 μ Ci [α - 32 P]UTP (800 Ci/mmol; Amersham Biosciences, Chalfont St Giles, Bucks., U.K.) were added for a further 10 min. The reactions were stopped with 4 μ l of formamide loading buffer and heated to 95 $^{\circ}$ C, and half the sample was directly run on a 6% (w/v) denaturing sequencing gel. Dried gels were visualized and quantified using a PhosphorImager.

Conjugation of single cysteine Cys⁶⁵-PspF₁₋₂₇₅ mutant with FeBABA [Fe (p-bromoacetamidobenzyl)-EDTA]

Purified Cys⁶⁵-PspF₁₋₂₇₅ protein was dialysed overnight at 4 $^{\circ}$ C against conjugation buffer [10 mM Mops (pH 8.0)/0.2 M NaCl/5% (v/v) glycerol/2 mM EDTA]. Conjugation reactions were initiated by adding a 20-fold molar excess of FeBABA (600 μ M; Dojindo Chemicals, Kumamoto, Japan) to Cys⁶⁵-PspF₁₋₂₇₅ (100 μ M) at pH 8.0. After 1 h incubation at 37 $^{\circ}$ C, excess FeBABA was removed by overnight dialysis against conjugation buffer at 4 $^{\circ}$ C. The concentration of free cysteine thiol groups on the conjugated and unconjugated Cys⁶⁵-PspF₁₋₂₇₅ was determined fluorometrically by the CPM {N-[4-(7-diethylamino-4-methylcoumarin-3-yl)phenyl]maleimide} test and the conjugation yield was calculated as described previously [23].

Inter-protomer cleavage by FeBABA-conjugated Cys⁶⁵-PspF₁₋₂₇₅

The hetero-oligomers of the central domain of PspF were prepared by incubating [32 P]PspF_{90TH} and Cys⁶⁵-PspF₁₋₂₇₅ with a molar ratio of 1:5 (1 and 5 μ M respectively) or 1:10 (1 and 10 μ M respectively) at 30 $^{\circ}$ C for 5 min in STA buffer containing NaF and ADP before adding AlCl₃. After 10 min at 30 $^{\circ}$ C, cleavage reactions were initiated by the rapid sequential addition of freshly prepared ascorbate (10 mM final) and hydrogen peroxide (10 mM final) and were allowed to proceed for a further 10 min at 30 $^{\circ}$ C. Reactions were stopped with 2 $\times$ SDS sample buffer. The cleaved fragments were separated on a 16.5% Tris/tricine gel. The gels were dried and visualized using a PhosphorImager.

For the assignment of the cleavage sites, [32 P]PspF_{90TH} was cleaved at cysteine residues using the sequence-specific chemical reagent NTCB (2-nitro-5-thiocyanobenzoic acid) as described previously [24]. Briefly, [32 P]PspF_{90TH} was buffer-exchanged into unfolding buffer (0.1 M Mops, pH 8.5/8 M urea) and incubated for 10 min at 37 $^{\circ}$ C. Cleavage was initiated by adding a 5-fold molar excess of NTCB (in 0.1 M Mops buffer) over total thiol groups. After overnight incubation at 37 $^{\circ}$ C, the cleavage reactions were stopped with 2 $\times$ SDS sample buffer.

RESULTS

Design, expression and purification of PspF fragments

A fragmentation analysis of PspF was used to identify potential sequences in the AAA domain of PspF that might contribute to

Figure 3 Inhibition of the ADP · AIF_x-dependent oligomerization of PspF_{ΔHTH} by PspF MBP-F2 fragment

(A) Effect of PspF MBP-fragments on ADP · AIF_x dependent oligomerization of [³²P]PspF_{ΔHTH} when preincubated with [³²P]PspF_{ΔHTH} before adding ADP · AIF_x. The ratio of [³²P]PspF_{ΔHTH} to PspF MBP fragments was 1:1 (lanes 3, 5, 7 and 9) and 1:2 (lanes 4, 6, 8 and 10). (B) Effect of adding increasing amounts (200–800 nM) of PspF MBP-F2 (lanes 3–5) to oligomerized [³²P]PspF_{ΔHTH} (400 nM). (C) Effect of increasing amounts (100, 200, 400 and 800 nM) of PspF MBP-F2 and corresponding volumes of its storage buffer on ADP · AIF_x-dependent oligomerization of [³²P]PspF_{ΔHTH} (400 nM). (D) Graph showing the quantitative analysis of the inhibitory effect of [³²P]PspF_{ΔHTH} oligomerization by PspF MBP-F2 (grey bars) or by storage buffer of the PspF MBP-fragments as a control (black bars). Results were normalized to the value obtained for [³²P]PspF_{ΔHTH} oligomerization (C, lane 2) which was set at 100 %. The error bars indicate that the values obtained from at least two data sets are within ± 10 %.

self-association [5]. The fragments of PspF were chosen on the basis of known self-association defective mutants in the highly conserved region C3 of several σ^{54} -activator proteins (Figure 1A) [17,25,26]. The partial sequences of PspF were constructed and overproduced as fusions to the MBP (Figure 1B) [5]. The fragments variably include (i) the C3 region (fragments PspF MBP-F1, PspF MBP-F2 and PspF MBP-F4), which includes the GAFTGA motif implicated in energy coupling and/or direct interaction with the σ^{54} -RNAP [3,5,27]; (ii) the C4 region, where the Walker B motif associated with NTP binding is located (fragments PspF MBP-F1 and PspF MBP-F3); and (iii) the C5 region of unknown function (fragments PspF MBP-F1 and PspF MBP-F3). The identity and purity of the PspF MBP fragments were verified by SDS/PAGE (Figure 1B). The biochemical activity of the fragments was then determined in a number of assays designed to evaluate their contribution to the self-association of PspF.

PspF MBP-F2 inhibits NTP-dependent self-association of PspF

PspF₁₋₂₇₅ stably self-associates to form a hexamer in the presence of ADP · AIF_x ([17]; J. Schumacher, X. Zhang, S. E. Jones, P. Bordes and M. Buck, unpublished work). We initially screened for the ability of the four PspF MBP fragments to self-associate in the presence of ADP · AIF_x by native PAGE. No stable PspF MBP-fragment oligomers were detected (results not shown). To test whether we might have localized sequences involved in self-association by fragmenting PspF, we screened the four PspF MBP fragments for specific inhibition of ADP · AIF_x-dependent self-association of PspF_{ΔHTH} (residues 1–292). We reasoned that an inhibitory activity would mean that PspF-interacting sequences are present within the fragment, and that these sequences acted

in trans to inhibit ADP · AIF_x-dependent oligomerization of PspF_{ΔHTH}. Our analysis does not distinguish whether or not such self-interacting determinants in PspF are readily available in the absence of NTP or are ‘unmasked’ by NTP binding and hydrolysis.

As shown in Figure 3, when [³²P]PspF_{ΔHTH} was preincubated with increasing amounts of PspF MBP-F2 and then ADP · AIF_x was added, no detectable self-association of [³²P]PspF_{ΔHTH} occurred (Figure 3A, lanes 3 and 4). In contrast, no inhibition of self-association occurred when [³²P]PspF_{ΔHTH} was preincubated with any of the other PspF MBP fragments before the addition of ADP · AIF_x (Figure 3A, lanes 5–10), suggesting that PspF sequences involved in self-association may need to be present in a correct functional and structural context to cause this inhibition. For instance, for PspF MBP-F1, the large C-terminal extension could interact with N-terminal sequences so as to mask the surface(s) required for inhibition (as in PspF MBP-F2). The inhibitory activity of PspF MBP-F2 was dependent on the order of addition. Addition of PspF MBP-F2 after the occurrence of self-association of [³²P]PspF_{ΔHTH} (i.e. after the addition of ADP · AIF_x to the [³²P]PspF_{ΔHTH}) produced no detectable effect on the amount of self-associated [³²P]PspF_{ΔHTH} complexes formed (Figure 3B). Control assays with PspF MBP-F2 demonstrated that the inhibition of self-association of [³²P]PspF_{ΔHTH} was specific to the *in trans* action of PspF MBP-F2 and not due to buffer effects or to the MBP tag (Figures 3C and 3D; results not shown). It was efficient, since at a 1:1 molar ratio of PspF MBP-F2 to (monomer) < 50 % self-association occurred (when compared with self-association activity of [³²P]PspF_{ΔHTH} in the absence of PspF MBP-F2). The inhibitory activity of PspF MBP-F2 was also confirmed in assays using PspF₁₋₂₇₅ and protein staining to detect oligomers (results not shown).

Figure 4 Inhibition of PspF₁₋₂₇₅-dependent σ -isomerization by PspF MBP-F2

σ -Isomerization assays were conducted using 0.5 μ M σ^{54} , 2 mM dGTP and 16 nM 32 P-labelled DNA probe 1. Effect of PspF MBP fragments on (A) PspF₁₋₂₇₅- and (B) NtrC-dependent σ -isomerization when preincubated with PspF₁₋₂₇₅ or NtrC before adding dGTP, σ^{54} and DNA. The ratio of activator (PspF₁₋₂₇₅ or NtrC) to PspF MBP fragments was 1:1 (0.4 μ M). The isomerized complex is marked as ss σ^{54} -probe 1. The relative amount of ss σ^{54} -probe 1 is indicated below the lanes with respect to the value obtained in the absence of PspF MBP fragment set as 1. (C) Amino acid sequences of PspF MBP-F2 variants. For each fragment, the substituted amino acid is indicated by underlined bold letters.

Overall, these results suggest that PspF MBP-F2 harbours important determinants for stable NTP-dependent self-association of PspF (as captured by ADP·AlF_x), which can act *in trans* to prevent nucleotide-dependent oligomerization of intact PspF.

PspF MBP-F2 specifically inhibits NTP-dependent remodelling of a stable binary σ^{54} -DNA complex by PspF₁₋₂₇₅

Activators of the σ^{54} -RNAP need to form higher-order oligomers to couple effectively the energy derived from NTP hydrolysis to open-complex formation [17,28]. We tested whether PspF MBP-F2 was able to inhibit a PspF-dependent activity that reflects a step in open-complex formation but does not require the core RNAP. We measured isomerization of σ^{54} bound to *Sinorhizobium meliloti* heteroduplex promoter probe 1 (Figure 2B) that contains a two base-pair mismatch at the -12 and -11 positions next to the conserved GC region of σ^{54} -dependent promoters, representing the nucleation of DNA melting within the closed complex [7,29]. In the absence of activator, σ^{54} binds to probe 1 to form a binary complex (Figure 4A, lane 2) [8]. Addition of PspF₁₋₂₇₅ and hydrolysable nucleotide (such as dGTP) to the reaction results in the isomerization of the binary σ^{54} -probe 1 complex and formation of the 'supershifted' binary σ^{54} -probe 1 complex (ss σ^{54} -probe 1) (Figure 4A, lane 3). Preincubation of PspF MBP-F2 with PspF₁₋₂₇₅ resulted in approx. 96% inhibition of ss σ^{54} -probe 1 complex formation (Figure 4A, lane 4). Inhibition of ss σ^{54} -probe 1 complex formation was specific to the PspF MBP-F2. None of the other PspF MBP fragments tested had a detectable effect on PspF₁₋₂₇₅-dependent ss σ^{54} -probe 1 formation (Figure 4A, lane 9; results not shown), further highlighting the view that residues of

PspF MBP-F2 have to be in a specific structural context to cause this inhibition.

To assess whether inhibition by PspF MBP-F2 was specific to PspF, the σ^{54} -dependent activator protein NtrC was substituted by PspF₁₋₂₇₅ in the same assay (Figure 4B). Results show that PspF MBP-F2 had no detectable effect on NtrC-dependent ss σ^{54} -probe 1 complex formation (Figure 4B, lane 4). Since NtrC did not compete with PspF₁₋₂₇₅ for binding to σ^{54} (which was limiting in the test reaction) in the ADP·AlF_x-dependent assay (results not shown; [17]), the failure to see inhibition of NtrC activity by PspF MBP-F2 could not be due to NtrC having a higher affinity for σ^{54} when compared with PspF₁₋₂₇₅. Thus the negative effect of PspF MBP-F2 on PspF is probably due to a specific interaction between PspF MBP-F2 and PspF₁₋₂₇₅.

The integrity of the C3 region GAFTGA motif is required for the inhibitory action of PspF MBP-F2

Interestingly, PspF MBP-F2 and the other three PspF MBP fragments each have markedly different biochemical activities. The difference is particularly striking with PspF MBP-F4, which forms a stable ternary complex with the σ^{54} -probe 1 complex [5]. In contrast with PspF MBP-F2, PspF MBP-F4 failed to act *in trans* to inhibit the ability of PspF_{ΔHTH} or PspF₁₋₂₇₅ to self-associate in the ADP·AlF_x-dependent assay (cf. lanes 3 and 4 with lanes 9 and 10 in Figure 3A). Nevertheless, both the fragments contain the highly conserved C3 sequences suggested to play an important role in coupling NTP binding and hydrolysis to biological output in σ^{54} -RNAP-dependent activators [27,30]. The C3 region of PspF clearly has an activity associated with self-association and σ^{54} binding [5,17]. Each activity involves nucleotide interactions, and at least one appears to require nucleotide-dependent repositioning or some unmasking of an interacting surface [5]. A six-amino-acid motif (GAFTGA; residues 83–88) within the C3 region is strongly implicated in interactions with σ^{54} , and Thr⁸⁶ residue in the GAFTGA motif is required for PspF MBP-F4 binding to the binary σ^{54} -probe 1 complex [5,25,30]. PspF₁₋₂₇₅ harbouring the T86A (Thr⁸⁶ → Ala) and T86S mutations was defective for ADP·AlF_x-dependent self-association (Table 1). The ADP·AlF_x-dependent self-association of the T86S mutant was stabilized by σ^{54} (Table 1) [17]. First, we introduced the T86A and T86S mutations in PspF MBP-F2 to analyse their contribution in the inhibitory activity of PspF MBP-F2 on the self-association of PspF_{ΔHTH} and PspF₁₋₂₇₅ (Figure 4C). Secondly, we reasoned that because PspF MBP-F2 differs from PspF MBP-F4 by a C-terminal nine-amino-acid extension (amino acids 94–102; Figure 1A), it is probable that this extension contains some of the inhibition determinants. Therefore we substituted two conserved amino acid residues (Arg⁹⁵ and Phe⁹⁶) within this extension by alanine residues in PspF MBP-F2 (Figure 4C). Forms of PspF₁₋₂₇₅ harbouring either the R95A or F96A mutation each failed in transcription activation *in vivo* and *in vitro*, thus highlighting the importance of these conserved residues for PspF function (Table 1). The PspF₁₋₂₇₅ mutants R95A and F96A bind ATP normally, but fail to hydrolyse ATP and form the ADP·AlF_x-dependent oligomer (Table 1), and might thus be defective in conformational signalling needed for aspects of self-association.

PspF MBP-F2 fragments harbouring the T86A, T86S, R95A or F96A mutations were overexpressed and purified. Initially, we confirmed by SDS/PAGE and native PAGE that the mutant PspF MBP-F2 fragments were intact (Figure 1B; results not shown). We tested the ability of the PspF MBP-F2 mutants to act *in trans* to inhibit PspF₁₋₂₇₅ activity in the ss σ^{54} -probe 1 complex formation assay (see above). Each mutant form of PspF MBP-F2 failed to inhibit. In contrast with the reaction containing the wild-type PspF

Table 1 Properties of mutant forms of PspF₁₋₂₇₅

Amino acid substitution in PspF ₁₋₂₇₅	Transcription activation		Nucleotide interactions			
	<i>In vivo</i> (Miller units)*	<i>In vitro</i> (arbitrary units)†	ADP · AIF _x -dependent oligomerization of PspF‡	ADP · AIF _x -dependent binding of PspF to σ^{54} §	ATP binding	ATP hydrolysis (turnover · min ⁻¹)¶
None	878 (100%)	223 (100%)	+	+	+	13.5
T86A	12 (1.4%)	< 0.1	—	—	+	23.2
T86S	786 (89.5%)	187 (84%)	—	+	+	20.9
R95A	15 (1.7%)	1.34 (0.6%)	—	—	+	< 0.1
F96A	13 (1.5%)	< 0.1	—	—	+	< 0.1

* Expression of β -galactosidase from *pspAp-lacZ* fusions with mutant PspF₁₋₂₇₅ proteins in *E. coli*. PspF₁₋₂₇₅ proteins were overexpressed from pACT3-pspF₁₋₂₇₅ derivative plasmids. β -Galactosidase values are averaged from triplicate determinations with two different isolates of each strain. Standard error was < 10 %. Numbers in parentheses indicate the percentage of activity with respect to the wild-type PspF₁₋₂₇₅ activity. The level of β -galactosidase activity in the absence of pACT3 derivative plasmid is 8.

† *pspAp*-specific transcription as described in the Experimental section, in the presence of 4 μ M PspF₁₋₂₇₅. The amount of transcript is in arbitrary units and was quantified by Phosphorimager analysis. Numbers in parentheses indicate the percentage of activity with respect to the wild-type PspF₁₋₂₇₅.

‡ Ability of the PspF₁₋₂₇₅ mutants to oligomerize in the presence of ADP · AIF_x [17]. +, wild-type levels of oligomerization; —, no oligomerization detected.

§ Ability of σ^{54} to form a stable complex with the oligomerized PspF₁₋₂₇₅ [17].

|| ATP-binding activities of the variant forms of PspF₁₋₂₇₅ were measured by UV 254 nm cross-linking of [α -³²P]ATP with 5 μ M purified protein as described in the Experimental section and detected by Phosphorimager analysis. +, wild-type levels of ATP binding.

¶ ATPase activities of the variant forms of PspF₁₋₂₇₅ were measured with 2 μ M purified protein after 1 h reaction as described in the Experimental section. Hydrolysis was measured as the release of ³²P_i, which was detected by TLC and quantified by Phosphorimager analysis.

Table 2 Effects of mutant forms of PspF MBP-F2 proteins on oligomerization-dependent activities of PspF₁₋₂₇₅ *in vitro*

Values indicate the relative activities of PspF₁₋₂₇₅ in the presence of the wild-type PspF MBP-F2 or PspF MBP-F2 with the indicated mutation (1:1 molar ratio of PspF MBP-F2 to PspF₁₋₂₇₅). The activator-dependent activity in the absence of PspF MBP-F2 is set to 1. Values were average from a set of at least two experiments with S.D. = $\pm$ 10 %.

Amino acid substitution in PspF MBP-F2	ADP · AIF _x -dependent oligomerization of PspF ₁₋₂₇₅	ADP · AIF _x -dependent binding of PspF ₁₋₂₇₅ to σ^{54}	PspF ₁₋₂₇₅ -dependent isomerization of σ^{54} -promoter probe 2 complex in the presence of ATP γ S*	PspF ₁₋₂₇₅ -dependent acquisition of heparin stability by σ^{54} -RNAP on promoter probe 3†
None	0.20	0.15	0.47	0.51
T86A	0.88	0.38	0.75	0.80
T86S	0.60	0.43	1	0.90
R95A	0.30	0.35	1	0.81
F96A	0.27	0.33	0.79	0.78

* A nucleotide-dependent remodelling of σ^{54} by PspF₁₋₂₇₅, which results in the acquisition of binding activity between σ^{54} and probe 2 [9].

† A nucleotide-dependent remodelling of the σ^{54} -RNAP-probe 3 complex by PspF₁₋₂₇₅, which results in the acquisition of heparin stability by σ^{54} -RNAP on probe 3 [43].

MBP-F2, when the PspF MBP-F2 mutants were preincubated with PspF₁₋₂₇₅ before its addition to the σ^{54} -promoter probe 1 complex, formation of the σ^{54} -probe 1 complex was detected (Figure 4A, lanes 5–8). We conclude that amino acids Thr⁸⁶, Arg⁹⁵ and Phe⁹⁶ are important for the inhibitory activity of PspF MBP-F2 and by extension to self-association of intact PspF. In addition, this result further supports the view that PspF MBP-F2 acts specifically *in trans* to prevent self-association of PspF₁₋₂₇₅.

We explored further the inhibitory activity of PspF MBP-F2 on PspF₁₋₂₇₅ activity and the requirement for the integrity of residues Thr⁸⁶, Arg⁹⁵ and Phe⁹⁶ by applying PspF MBP-F2 variants to other assays, each of which was dependent at some step of the oligomeric states of PspF₁₋₂₇₅. The assays were (i) ADP · AIF_x-dependent self-association, (ii) ADP · AIF_x-dependent σ^{54} binding, (iii) probe 2 binding by σ^{54} in the presence of ATP γ S and (iv) open-complex formation by σ^{54} -RNAP (Table 2) [9,17]. The results in Table 2 show that the PspF MBP-F2 harbouring the T86A and T86S mutants were considerably decreased in their ability to act *in trans* to inhibit PspF₁₋₂₇₅ activity in several assays. In the context of the ADP · AIF_x-dependent self-association assay, PspF MBP-F2 fragments harbouring the R95A and F96A mutations retained the ability to inhibit self-association of PspF₁₋₂₇₅. However, in PspF₁₋₂₇₅ self-association-dependent assays involving σ^{54} and σ^{54} -RNAP, no inhibition by PspF MBP-F2 harbouring the R95A and F96A mutations was seen (Table 2).

These results suggest that PspF MBP-F2 R95A and F96A mutants may have a reduced affinity for binding to PspF₁₋₂₇₅ compared with the wild-type PspF MBP-F2 fragment. Therefore stabilization of PspF₁₋₂₇₅ oligomer formation occurring with σ^{54} , σ^{54} -RNAP or promoter complexes could allow some escape of inhibition from PspF MBP-F2 R95A and F96A mutants. Overall, the results summarized in Table 2 clearly demonstrate that PspF MBP-F2 inhibits self-association-dependent activities of PspF₁₋₂₇₅ *in vitro* and that Thr⁸⁶, Arg⁹⁵ and Phe⁹⁶ residues have different contributions at different steps of activator function.

PspF MBP-F2 inhibits activator-dependent transcription by the σ^{54} -RNAP *in vitro*

In the next set of assays, we tested whether PspF MBP-F2 inhibited PspF-dependent transcription initiation by the σ^{54} -RNAP from the *E. coli pspA* promoter in a single round *in vitro* transcription assay [13]. The basic assay consisted of forming closed σ^{54} -RNAP complexes on the *pspA* promoter in the presence of ATP and then stimulating open-complex formation by adding PspF₁₋₂₇₅. Subsequent elongation from the stable open complex requires the addition of rNTPs and heparin. Heparin is added to destroy unstable closed complexes and prevent re-initiation, thereby allowing single-round transcription. As shown in Figure 5(A), when PspF MBP-F2 was preincubated with

Figure 5 Effect of PspF MBP-F2 on activator-dependent *in vitro* transcription by the σ^{54} -RNAP

The effect of PspF MBP-F2 using (A) the activator protein PspF₁₋₂₇₅ and the *E. coli pspA* (*pspAp*) promoter and (B) the activator protein NtrC and the *E. coli glnHp2* promoter. In (A, B) the relative transcription activity (from three different data sets) with respect to wild-type transcription activity by σ^{54} -RNAP in the absence of PspF MBP fragments was within a 10 % error range.

increasing amounts of PspF₁₋₂₇₅, before adding the activator to the σ^{54} -RNAP closed promoter complex, RNA synthesis was hardly detectable from the *pspA* promoter (cf. lanes 1 and 3). Additional transcription assays on the σ^{54} -RNAP-dependent *S. meliloti nifH* and *E. coli glnHp2* promoters gave similar results (results not shown), further confirming the bona fide nature of inhibition by PspF MBP-F2. The order of addition experiments in which PspF MBP-F2 was added after open-complex formation had occurred and control assays in which an activator-independent form of σ^{54} -RNAP was used established that PspF MBP-F2 primarily and specifically targets the activation steps and not any other stages of the transcription cycle (results not shown). Further transcription assays showed that when PspF MBP-F2 was preincubated with the σ^{54} activator NtrC, it did not inhibit the ability of NtrC to activate transcription from the *E. coli glnHp2* promoter (Figure 5B). We tested the ability of the PspF MBP-F2 with the T86A, T86S, R95A and F96A mutations to act *in trans* to inhibit PspF₁₋₂₇₅ activity in the *in vitro* transcription assay. Consistent with the previous results (Table 2), a significant amount of RNA synthesis was detected from the *pspA* promoter with the PspF MBP-F2 mutants, in contrast with the reactions containing wild-type PspF MBP-F2 (compare lanes 4–7 with lane 3 in Figure 5A). Thus, the inhibition of RNA synthesis in the activator-dependent transcription reactions was probably due to PspF MBP-F2 specifically preventing nucleotide-dependent self-association of PspF₁₋₂₇₅, which is important for productive interactions with the σ^{54} -RNAP closed complex [17].

Effect of PspF MBP-F2 on ATP binding and hydrolysis by PspF₁₋₂₇₅

Since the site for NTP binding and hydrolysis is created between two protomers of PspF, we considered the possibility that the PspF MBP-F2 could also inhibit the ATP binding and hydrolysis activity of PspF₁₋₂₇₅. To investigate this, we looked at the effects of PspF MBP-F2 on the ATPase activity of PspF. Interestingly, ATP hydrolysis by PspF (measured as the release of 32 P from [α - 32 P]ATP) was not inhibited by PspF MBP-F2 when assayed at 0.4 μ M PspF and 4 mM ATP (results not shown). However, at lower concentrations of ATP (0.4 mM), a 50 % inhibition of PspF₁₋₂₇₅ and full-length PspF ATPase activity was detected (results not shown). Consistent with this, binding of ATP to PspF₁₋₂₇₅ was reduced in the presence of PspF MBP-F2. As shown in Figure 6, approx. 60 % reduction in the binding activity of ATP to PspF₁₋₂₇₅ was detected in the UV-cross-linking assay (cf. lanes 1 and 2). This reduction in ATP binding to PspF₁₋₂₇₅ was not

Figure 6 Effect of PspF MBP-F2 on UV-cross-linking of [α - 32 P]ATP to PspF₁₋₂₇₅

The relative [α - 32 P]ATP to PspF₁₋₂₇₅ was estimated by the Phosphorimaging reading compared with the amounts of protein in the reactions and are in arbitrary units, with respect to the value obtained in the absence of PspF fragment set as 1. The ATP-binding activity (from three different data sets) was within a 10 % error range.

detected in the presence of PspF MBP-F2 harbouring the T86A or the T86S mutation (Figure 6, lanes 3 and 4). Interestingly, the PspF MBP-F2 harbouring the R95A and F96A mutations did reduce ATP binding to PspF₁₋₂₇₅ by approx. 50 %. This result is in agreement with the inability (for T86A and T86S mutations) and ability (for R95A and F96A mutations) of PspF MBP-F2 mutants to inhibit ADP · AIF_x self-association of PspF₁₋₂₇₅ (see above; Table 2). Control assays showed that PspF MBP-F2 did not bind ATP (results not shown). Overall, the results are consistent with the view that PspF MBP-F2 specifically interferes with the self-association of PspF₁₋₂₇₅ and, by inference, causes a change in the ATP-binding site.

Sequences represented in PspF MBP-F2 are proximal to the protomer-protomer interface within an ADP · AIF_x-dependent oligomer of PspF₁₋₂₇₅

We used a proximity-based footprinting method to help determine whether sequences in PspF MBP-F2 might physically localize at the protomer-protomer interface within a nucleotide (ADP · AIF_x)-dependent oligomer of PspF₁₋₂₇₅. To do so, PspF₁₋₂₇₅ was converted into a proximity-based protein cleavage reagent through the conjugation of a reactive Fe²⁺ chelate using the thiol-specific reagent FeBABE [23]. FeBABE-mediated protein cleavage is initiated by the addition of ascorbate and hydrogen peroxide to generate hydroxyl radicals, which will cleave polypeptide chains in close proximity [approx. 12 + 3 Å (1 Å = 0.1 nm) diffusion distance of the hydroxyl radicals] [23]. To modify site-specifically

Figure 7 Inter-protomer cleavage by FeBABE-modified Cys⁶⁵-PspF₁₋₂₇₅

(A) Symbolic illustration of hetero-oligomer formation between Cys⁶⁵-PspF₁₋₂₇₅ and [³²P]PspF_{ΔHTH} in the presence of ADP·AlF_x. (B) ADP·AlF_x-dependent hetero-oligomerization reactions were performed by preincubating [³²P]PspF_{ΔHTH} with Cys⁶⁵-PspF₁₋₂₇₅-FeBABE (lanes 2, 3, 7 and 8) or Cys⁶⁵-PspF₁₋₂₇₅ (lanes 4, 5, 9 and 10). The amount of [³²P]PspF_{ΔHTH} was kept constant (1 μM) and the black triangles above the gel indicate increasing amounts of Cys⁶⁵-PspF₁₋₂₇₅ or Cys⁶⁵-PspF₁₋₂₇₅-FeBABE [at the molar ratio 1:5 (lanes 2, 4, 7 and 9) and 1:10 (lanes 3, 5, 8 and 10)]. The hetero-oligomers were treated with H₂O₂ and ascorbate and the cleavage products were separated on a 16.5% Tris/tricine SDS gel. Lane N contains the marker generated by treating [³²P]PspF_{ΔHTH} with NTCB. *, cleavage products specifically generated in the presence of Cys⁶⁵-PspF₁₋₂₇₅-FeBABE conjugate and ADP·AlF_x.

PspF₁₋₂₇₅ with FeBABE at a location proximal to the sequences represented by PspF MBP-F2, we constructed a single-cysteine variant of PspF₁₋₂₇₅ (Cys⁶⁵-PspF₁₋₂₇₅) by substituting two native cysteine residues at positions 146 and 198 with alanine residues and thereby leaving a single native cysteine residue at position 65 (recall that PspF MBP-F2 consists of residues 69–102). *In vitro* transcription assays showed that Cys⁶⁵-PspF₁₋₂₇₅ exhibited 86% of wild-type activity (results not shown), suggesting that alanine substitution of two native cysteine residues did not significantly impair PspF₁₋₂₇₅ activity. Cys⁶⁵-PspF₁₋₂₇₅ was derivatized with the FeBABE reagent under native conditions and the conjugation yield was determined fluorometrically by the CPM test from the fluorescence difference between the conjugated and unconjugated proteins [23]. The FeBABE conjugation yield for Cys⁶⁵-PspF₁₋₂₇₅ was estimated to be 60%. We tested the effects of FeBABE conjugation on Cys⁶⁵-PspF₁₋₂₇₅ activity using the *in vitro* transcription assay. After correction for the presence of unconjugated Cys⁶⁵-PspF₁₋₂₇₅, FeBABE-conjugated Cys⁶⁵-PspF₁₋₂₇₅ showed 47% transcription activity relative to the wild-type PspF₁₋₂₇₅ (results not shown).

An ADP·AlF_x-dependent heteromeric oligomer was formed using [³²P]PspF_{ΔHTH} and FeBABE-conjugated Cys⁶⁵-PspF₁₋₂₇₅ at a molar ratio of 1:5 and 1:10. Native-PAGE analysis of the reaction product confirmed ADP·AlF_x-dependent hetero-oligomer formation (results not shown), presumably from two different dimers, one with a ³²P-label and the other with the FeBABE reagent. We then sought evidence for inter-dimer cleavage of [³²P]PspF_{ΔHTH} by FeBABE-conjugated Cys⁶⁵-PspF₁₋₂₇₅. We envisaged that if the FeBABE at position 65 in one protomer (within a dimer) is within approx. 12 Å proximity to another protomer (within a dimer) within the ADP·AlF_x-dependent hetero-oligomer, then addition of ascorbate and hydrogen peroxide would result in inter-dimer protein cleavage (Figure 7A). This expectation was fulfilled. As shown in Figure 7(B), we detected clear FeBABE-mediated cleavage of [³²P]PspF_{ΔHTH}. Strikingly, the cleavage occurred proximal to Cys⁶⁵ residue in [³²P]PspF_{ΔHTH} and only in the presence of ADP·AlF_x, suggesting that position 65 and by extension sequences represented in PspF MBP-F2 are localized proximal to a protomer–protomer interface within an ADP·AlF_x-dependent oligomer of PspF. Since the FeBABE methodology reports interactions between two different types of dimers within an oligomer of PspF that forms with ADP·AlF_x,

the results suggest that the ADP·AlF_x oligomer is formed from an assembly of dimers.

We also conducted protein–protein-cross-linking experiments using a form of PspF₁₋₂₇₅ modified with a thiol-specific and proximity-based photoactivable cross-linking reagent *p*-azidophenacyl bromide [31]. Subsequent UV irradiation of the *p*-azidophenacyl bromide-modified Cys⁶⁵-PspF₁₋₂₇₅ and SDS/PAGE analysis (in the absence of NTP) resulted in the formation of a stable dimer of Cys⁶⁵-PspF₁₋₂₇₅ (results not shown). Such a stable dimer formation was not detected with unmodified Cys⁶⁵-PspF₁₋₂₇₅ (results not shown). Overall, the FeBABE-mediated cleavage data and the UV-cross-linking data strongly indicate that PspF MBP-F2 binds near a protomer–protomer interface to cause inhibition of PspF₁₋₂₇₅ activity (see Figure 7 and the Discussion section).

DISCUSSION

In bacteria, the interaction of transcriptional activators with the σ-subunit of the RNAP is important for efficient and regulated transcription initiation. At enhancer-independent promoters, σ⁷⁰-RNAP is recruited to the promoter by the transcription activators [32]. Enhancer-dependent promoters, which depend on the σ⁵⁴-RNAP, are utilized and regulated in a manner reminiscent of eukaryotic transcription regulation mechanisms. Eukaryotic transcription activators, in addition to mediating general transcription factor recruitment, also induce disruption of the basal transcription complex in an energy-dependent manner to stimulate open-complex formation and to facilitate promoter escape by RNAP [33].

Activators of σ⁵⁴-RNAP are members of the AAA protein family and utilize the energy derived from hydrolysing NTPs to disrupt a molecular block within the σ⁵⁴-RNAP closed complex to stimulate the rate of open-complex formation [4,6,7,28]. The NTP-dependent functioning of σ⁵⁴-dependent activators and AAA protein in general is believed to involve movements in the protein that reflect sensing the state of the bound NTP, and in particular, the presence of the γ-phosphate of the NTP that is to be hydrolysed. The AAA domain (residues 1–275) of PspF not only includes all key surfaces responsible for binding and hydrolysing nucleotides and the sensing of the bound nucleotide, but also includes the sequences required for self-association (this

work) and σ^{54} -RNAP binding [5]. In the present study, we have used a fragment of PspF (residues 69–102) to identify at least one set of sequences within the AAA domain of PspF required for self-association.

PspF surfaces involved in self-association

Self-association is a common property of AAA proteins and is of significance for NTP binding and hydrolysis, since strong evidence shows that the catalytic site for NTP binding and hydrolysis is created at the protomer–protomer interface [34,35]. In particular, an arrangement with an arginine residue from one protomer is suggested to be used to stabilize the accumulation of negative charge of the transition state of NTP hydrolysis [2]. The use of the fragmentation approach to study PspF function has led to the identification of a fragment of PspF (PspF MBP-F2), which functions *in trans* to inhibit specifically a nucleotide (ADP · AlF₃)-dependent self-association of intact PspF. The inhibition occurred at a 1:1 ratio of PspF MBP-F2 to intact PspF. The fragment specifically prevented efficient binding of nucleotide to PspF, thus, clearly arguing that the PspF MBP-F2 binding sequences on PspF are in communication with the nucleotide-binding surfaces. Consequently, several activator-dependent activities of σ^{54} or σ^{54} -RNAP are inhibited by PspF MBP-F2. The inhibitory effects of PspF MBP-F2 are consistent with the idea that protomer–protomer contacts can regulate nucleotide interactions, and vice versa, probably to establish alternate functional states of PspF. Alanine substitutions at conserved residues within PspF MBP-F2 decreased its inhibitory properties, suggesting that the mutated residues significantly contribute to an interface between two subunits of PspF. Consistent with this, mutations within the sequences represented in PspF MBP-F2 also influenced the self-association of intact PspF (this work and [25]). Interestingly, one such residue (Thr⁸⁶) is located within the GAFTGA motif that is involved in making direct interactions with σ^{54} -RNAP, suggesting a link between self-association and σ^{54} binding in PspF.

Self-association and σ^{54} -binding surfaces are co-localized in PspF

PspF MBP-F4 specifically interacts with a binary σ^{54} -probe 1 complex in a stable manner in the absence of any nucleotides [5]. In contrast, PspF MBP-F2 did not form a stable complex with probe 1-bound σ^{54} [5]. In the present study, we report that PspF MBP-F4 could not act *in trans* to inhibit ADP · AlF₃-dependent self-association of intact PspF, in contrast with PspF MBP-F2. PspF MBP-F2 did not inhibit PspF MBP-F4 binding to binary σ^{54} -probe 1 complex (P. Bordes and M. Buck, unpublished work), consistent with the idea that PspF MBP-F2 specifically inhibits the self-association-dependent activities of PspF. PspF MBP-F2 differs from PspF MBP-F4 by containing a C-terminal nine-amino-acid extension (Figure 1A). Thus we suggest that this nine-amino-acid extension may mask σ^{54} -interacting surfaces in PspF MBP-F2. Strikingly, an alanine substitution at residue Thr⁸⁶ in the GAFTGA motif of PspF MBP-F2 and F4 decreases their respective biochemical activities (i.e. inhibiting self-association and σ^{54} -binding respectively). The σ^{54} -RNAP interacting surface in the C3 region may therefore be in communication with or proximal to, a protomer–protomer interface. Results from tethered footprinting and cross-linking techniques (Figure 7; results not shown) are consistent with the idea of proximity. Strikingly, examination of the recently published crystal structure of the σ^{54} activator NtrC1 from *A. aeolicus* [19] fully supports our deduced proximity relationship for PspF. In the crystallized ADP-bound heptameric ring structure of the σ^{54} -dependent activator NtrC1 from *A. aeolicus*, residues corresponding to position Cys⁶⁵ of the

E. coli PspF are within 19 Å of each other [19]. Notably, our FeBABE data indicate that, in PspF, Cys⁶⁵ residues are within 12 Å to each other in the ADP · AlF₃-dependent oligomer. Thus the possibility that the sequences presented by PspF MBP-F2 bind to PspF surfaces distal from a subunit–subunit interface so as to stop simply structural transitions necessary for self-association seems unlikely on structural grounds.

Nucleotide interactions condition PspF activity

The inhibition of ADP · AlF₃-dependent self-association, σ -isomerization and open-complex formation suggests that the MBP-F2 fragment may specifically interfere with the formation of a nucleotide-dependent functional state of PspF associated with σ^{54} -RNAP interactions. Consistent with this, PspF MBP-F2 inhibited ATP binding to PspF₁₋₂₇₅, whereas the T86A and T86S mutant forms of PspF MBP-F2 did not. The probable co-localization of PspF sequences that bind σ^{54} and the sequences that are needed for ADP · AlF₃-dependent self-association (this work) suggest that there is a close physical coupling between NTP-conditioned conformational changes in the C3 region that lead to stable binding of σ^{54} [5].

We propose that after nucleotide binding and at the point of hydrolysis, the conserved C3 region of PspF, which harbours sequences important for σ^{54} -RNAP binding and self-association, is changed so that self-association can occur. A model of PspF (derived from the structures of the AAA proteins p97 and HslU) [5] and the crystal structure of NtrC1 [19] indicate that part of the C3 GAFTGA sequence is a loop structure, which could be relocated on nucleotide interactions. The co-localization of σ^{54} -binding surfaces and self-association sequences suggests that σ^{54} binding and self-association in σ^{54} -RNAP-dependent activators are inter-dependent processes, which are co-ordinated by the state of the bound nucleotide (this work and [5]).

Overview

Nucleotide interactions are necessary for biological output by many AAA proteins. We have employed a fragmentation approach to gain insights into the structural organization and nucleotide-conditioned activity of the σ^{54} -RNAP-dependent activator PspF. Our study implies that the σ^{54} -RNAP binding and self-association determinants of PspF are probably co-localized within a highly conserved region (the C3 region), which is predicted to be subject to movements during NTP binding and hydrolysis [3,5,19]. Membership of the AAA protein family by PspF is based on a low level of sequence similarity and identity, and structural and functional similarities [3]. The inter-protomer cutting by the FeBABE-derivatized PspF (Figure 7) provides a strong body of evidence, consistent with model building [5], to help localize a protomer–protomer interface in PspF. Nucleotide-conditioned conformational changes have also been seen in many ATP-dependent molecular machines, such as chaperonin, myosin, kinesin and F1-ATPases [36–39]. Two conformations [one when ADP was bound and another when p[NH]ppA (adenosine 5'-[β,γ -imido]triphosphate) was bound] of the AAA protein RuvB were also observed in crystal structures [40]. Similarly, multiple conformations (nucleotide-free, ADP and p[NH]ppA-bound) were observed in the AAA protein HslU [34,41]. Strikingly, and as predicted for PspF, in the *E. coli* AAA protease HslU that functions together with HslV, the HslV-binding surface is located in a hydrophobic pocket at the HslU–HslU interface within the AAA domain. During ATP hydrolysis, the HslV-binding surfaces distend to form the functional HslUV complex [42]. Thus the fragmentation analysis of the PspF described here presents a

widely applicable approach to identify self-association surfaces and explore how nucleotide binding and subsequent hydrolysis conditions the biological output in AAA proteins.

This work was supported by a Biotechnology and Biological Sciences Research Council grant to M.B. We thank Patricia Burrows and Jörg Schumacher for comments on the manuscript.

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Received 24 September 2003/3 December 2003; accepted 5 December 2003

Published as BJ Immediate Publication 5 December 2003, DOI 10.1042/BJ20031464