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Review

Coupling AAA protein function to regulated gene expression ☆

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A B S T R A C T

AAA proteins (ATPases Associated with various cellular Activities) are involved in almost all essential cellular processes ranging from DNA replication, transcription regulation to protein degradation. One class of AAA proteins has evolved to adapt to the specific task of coupling ATPase activity to activating transcription. These upstream promoter DNA bound AAA activator proteins contact their target substrate, the σ^{54}-RNA polymerase holoenzyme, through DNA looping, reminiscent of the eukaryotic enhance binding proteins. These specialised macromolecular machines remodel their substrates through ATP hydrolysis that ultimately leads to transcriptional activation. We will discuss how AAA proteins are specialised for this specific task. This article is part of a Special Issue entitled: AAA ATPases: structure and function.

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1. Introduction

In order to survive environmental changes, to replicate and to differentiate, the genetic information stored in DNA needs to be transcribed to allow protein production. In all kingdoms of life, transcription of DNA to yield RNA is catalysed by a highly conserved multi-subunit RNA polymerase enzyme (RNAP) [1–6]. The tight regulation of the initial steps in transcription is critical for the survival of the organism because the regulated gene transcription allows appropriate genes to be expressed depending on the growth condition or state of differentiation and to avoid wastage. Despite the lack of an extensive sequence similarity, the multisubunit RNAP molecular machines adopt a similar structure as a "crab claw" with the catalytic subunits forming the two pincers [1]. During the transcription, the RNAP using accessory factors binds to a specific DNA consensus sequence at the level of the promoter (P) forming a closed complex (RP_c). The RP_c isomerises, through multiple intermediate states that can be promoter and factor specific [7,8], to a final open complex state (RP_o), competent for transcription, where the double-stranded DNA has melted out and the transcription starting site (the +1 site) is at the active centre of RNAP.

In eukaryotes, RNA polymerase enzymes are specialised for the transcription of a set of genes. There are three classes of RNA polymerase: RNA Pol I which transcribes rRNA, RNA Pol II which transcribes mRNA as well as most of the small nuclear RNAs and microRNAs [9], and RNA Pol III which specifically transcribes tRNA as well as 5S rRNA and other small nuclear RNAs. In contrast, bacteria possess only one type of RNA polymerase core (RNAP with a subunit composition of $\alpha_2\beta\beta'\omega$) responsible for all the transcriptional events. This core RNA polymerase is complemented by a dissociable factor: the sigma factor which allows promoter recognition specificity. These sigma factors can be divided into two classes based on their sequence homology and mechanism of action: the σ^{70} and the σ^{54} classes. The σ^{70} class, which is most represented with multiple alternative σ factors belonging to this class, is involved in the regulation of the transcription of "housekeeping" genes and operons. In contrast, apart from in a few bacterial species such as *Brandyrhizobium japonicum* and *Rhodobacter sphaeroides,* two forms of σ^{54} very rarely coexist in the same bacteria [10–12]. The role of σ^{54}-dependent transcription was historically related to nitrogen metabolism and fixation (and so the gene coding σ^{54} is *rpoN*) but has now been reported in a great repertoire of functions, including carbon source utilisation, expression of alternative σ factors, pathogenicity, electron transport, metabolism, RNA modification, heat/phage shock responses [13], polar and lateral flagella biogenesis for swimming and swarming in *Vibrio parahaemolyticus*[14], biofilm formation and bioluminescence in *Vibrio fischeri*[15]. The σ^{54}-dependent transcription is also important for human pathogens such as *Borrelia burgdorferi*, the agent of Lyme disease, and *Vibrio cholera*, the most feared epidemic diarrheal disease [16,17]. σ^{54} can be found in approximately 60% of the bacterial genomes [18], from extreme thermophiles, enteric pathogens,

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spirochetes to green sulfur bacteria [19,20]. The expression of σ^{54} is typically constitutive and the protein level remains constant (approximately 110 molecules) throughout the exponential phase and stationary phase in *Escherichia coli*[21].

As the σ^{54} and σ^{70} types of transcriptional complex have different properties, they are also regulated in different ways. The constitutively active σ^{70}–RNAP, which recognises −35 (TTGACA) and −10 (TATAAT) consensus DNA sequences, is regulated by activators (or repressors) which enhance (or reduce) the RNAP interaction with the promoter. The regulation of σ^{70}–RNAP is therefore largely dependent on RNAP recruitment. In contrast, in the case of the constitutively silent σ^{54}–RNAP that recognises −24 (GG) and −12 (GC) consensus DNA sequences, the transcription initiation invariably requires specific activators, termed bacterial Enhancer Binding Proteins (bEBPs). These bEBPs (also called σ^{54}-activators) are AAA proteins that use ATP hydrolysis to remodel the initial Eσ^{54}-promoter DNA complex (RP_c), to a transcriptionally-proficient open complex (RP_o) (Fig. 1) [19,22–30]. The σ^{54}–RNAP is likely to be pre-bound to the promoter, forming the stable closed complex (RPc). Upon inducing signals to trigger formation of an active conformer of the activator protein, nucleotide dependent interactions with the RNAP closed complex are made and the stimulation of open complex formation kick-starts the transcription in a nucleotide dependent fashion. These activators are members of the sub-clade 6 of the AAA family [31]. Similar to other AAA ATPases, bEBPs are P-Loop ATPases that convert the chemical energy derived from nucleoside tri-phosphate (ATP) into biological output, most often via mechanical force [32,33]. The AAA ATPases are defined by the presence of a conserved region of about 200 amino acids (the AAA core), comprising an amino-terminal α/β sub-domain and a carboxyl-terminal α-helical sub-domain.

2. Domain organisation of bEBPs

The bEBPs are modular proteins that typically consist of three domains: an amino-terminal domain for regulation, a highly conserved central AAA domain for σ^{54} interaction and ATPase activity, and the carboxyl-terminal domain for DNA binding (Fig. 2A) [34–36]. Usually the "regulatory domain" will be the target of phosphorylation or small molecules that will modulate the activity of the AAA catalytic domain. The DNA binding domain recognises Upstream Activation Sequences (UAS) within the promoter ensuring promoter specificity [37–39]. Based on their domain organisation, bEBPs can be classified into five groups (Fig. 2A).

Groups I bEBPs contain a regulatory domain and are regulated through its phosphorylation. The majority of Group I bEBPs belong to the commonly found two-component systems. Upon signal sensing by a cognate sensor kinase, the phosphate is transferred from the donor domain of the sensor kinase to a specific aspartic residue present in the regulatory domain of the bEBP [40–42]. Other Group I bEBPs, such as Levan utilisation operon regulator (LevR), contain a phosphotransferase regulation domain (PRD) which is regulated by phosphorylation of a conserved histidine residue [26,43].

The Group II bEBPs are regulated by direct binding of small effector molecules, such as heme/flavin (to the per-arnt sim (PAS) domain), hydrocarbons (to the vinyl 4 reductase (V4R) domain), Xylene (XylR), Zinc (Zinc resistance-associated regulator (ZraR)), Nitric Oxide (NorR), acetoin (AcoR), propionate (PrpR) or gamma amino-butiric acid (GabR) [34,44–49]. For a comprehensive list and detailed information on the regulatory domains and the sensory molecules, please refer to Dixon and Studholme [34].

The Group III bEBPs are regulated by protein–protein interactions through the N-terminal domain such as the GAF (cGMP-specific and -stimulated phosphodiesterases) adenylate cyclases formate hydrogen-lyase transcriptional activator (FhlA) domain [50].

A number of bEBPs lack the regulatory domain and either consist of the central AAA+ domain and the DNA binding domain (Group IV such as Phage Shock protein F (PspF) and Hypersensitive response and pathogenicity (HrpR/S)) or just the central AAA+ domain (Group V—Flagelar gene regulator (FlgR) or chlamydial two-component system C (CtcC)) [51–54]. These group V bEBPs lacking a HTH domain activate transcription by interacting with the RPc of a very limited number of genes that need to be simultaneously expressed.

The ATPase domain of bEBPs contains seven conserved motifs called C1 to C7 within bEBPs [55]. These motifs include conserved features in all AAA proteins such as Walker A and Walker B motif which are involved in ATP binding and hydrolysis, respectively, as well as a Second Region of Homology (SRH) which contains the Arg-finger residues (Fig. 2B [32,33,56–58]. AAA ATPases usually function as higher-order oligomers (commonly hexamers) [58–65]. The oligomerisation allows the formation of the catalytic active site at the interface between adjacent subunits of the oligomer. The catalytic site is composed of Walker A and B motifs from one subunit and of *trans*-acting determinants from the adjacent subunit (e.g. the R-finger residues) [32,66].

3. Roles of the bEBPs-specific inserted motifs: loop1 and loop2

AAA proteins have several specific functional motifs inserted into the AAA+ core. These are responsible for substrate recognition, binding and remodelling. There are two major inserted motifs allowing

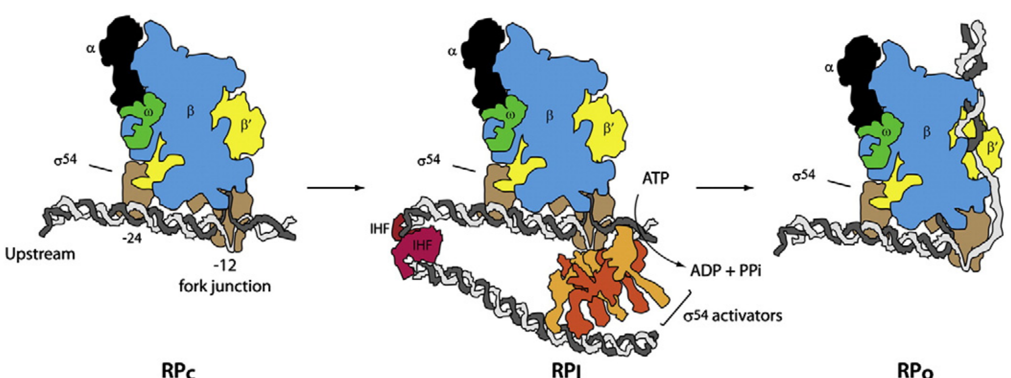

Fig. 1. Structural transitions during σ^{54}-dependent transcription initiation. At the initial stage, the RNAP–σ^{54} is bound to the DNA promoter sequence forming the close complex RPc. In this complex the DNA is partially melted at the level of the −12 position forming a repressive fork junction. After activation of the AAA+ activator by its specific stimuli, activators interact with the UAS (Upstream Activating Sequence) of the promoter located around 150 bp upstream relative to the +1 transcription starting site. Through DNA looping with the help of IHF (integration host factor, scarlet) the activators interact with its target RPc. Upon ATP hydrolysis, the RP_c is isomerised in RP_o where the DNA is opened in the region −10 to −1. The template single strand is then loaded by the polymerase in its active site and the transcription can occur. The holoenzyme (σ^{54}, brown; α, black; β, blue; β', yellow), promoter DNA (template strand, dark grey; non-template strand, light grey), transcription factor and σ^{54} activators (light and dark orange for alternating protomers) are shown at different stages along the transcription pathway.

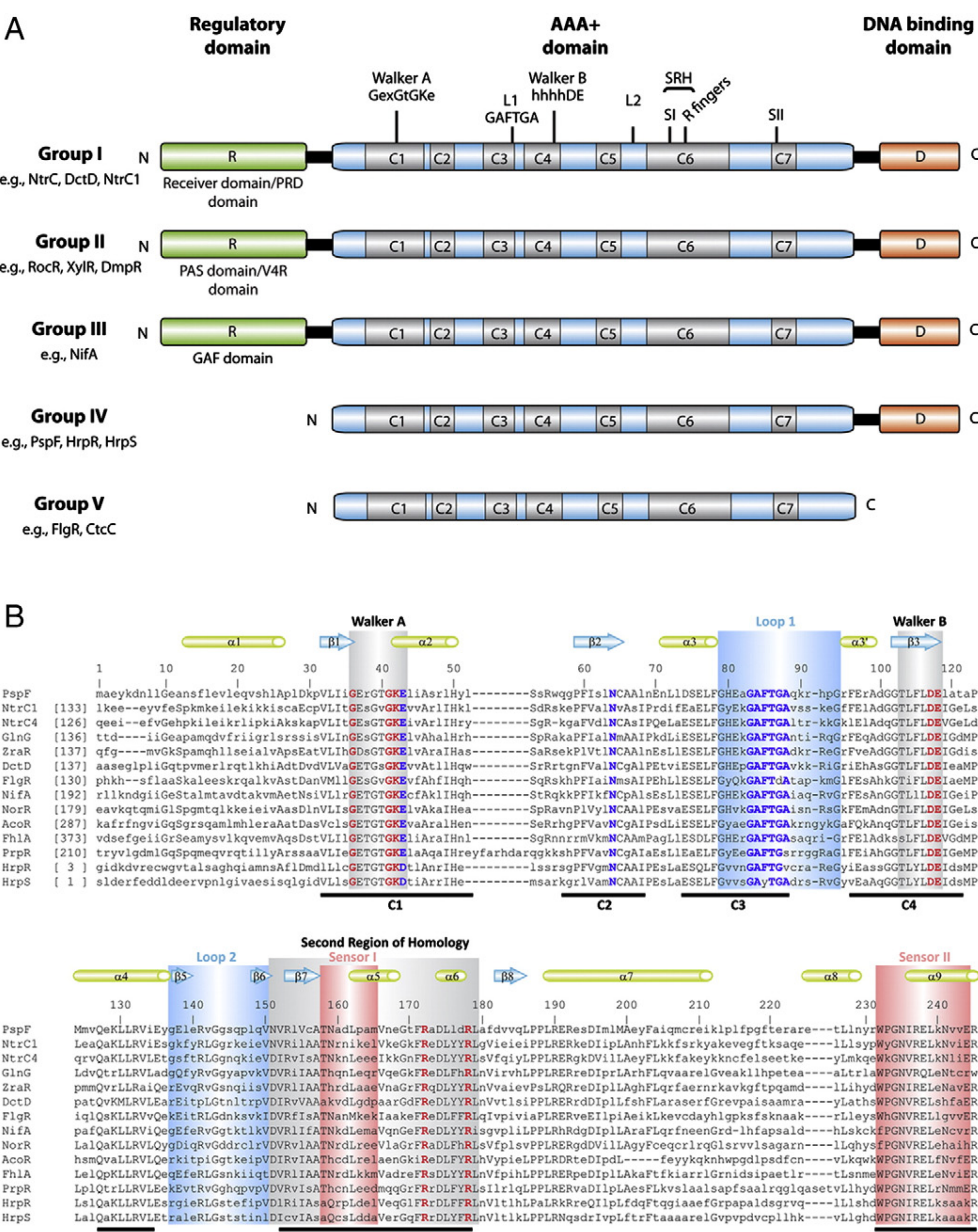

Fig. 2. Domain organisation of bEBPs and sequence alignment of the AAA domain. A) The receiver domain (R), the central AAA + domain, and the DNA binding domain (D) are presented from N-terminus to C-terminus. Within the AAA + domain, regions of high conservation (C1-C7) [55], Walker A (GxxxxGK, where x is any residue), Loop1 (L1, containing the conserved 'GAFTGA' motif), Walker B (hhhhDE, where h is any hydrophobic residue), Loop2 (L2), Second region of homology (SRH, containing sensor I (SI) and the putative Arg-fingers), and sensor II (SII) are indicated. Red characters represent the signature residues of AAA + family and in blue the signature residues specific to bEBPs. Sequences used: PspF (*Escherichia coli*, gi:1209663), NtrC1 (*Aquifex aeolicus*, gi:39654269), NtrC4 (*Aquifex aeolicus*, gi:15605734), GlnG (*Escherichia coli*, EG10385), ZraR (*Escherichia coli*, GI:20140981), DctD (*Rhizobium meliloti*, gi:17380399), FlgR (*Helicobacter pylori F32*, gi:317180418), NifA (*Sinorhizobium meliloti 1021*, gi:193073065), NorR (*Escherichia coli*, gi:29428733), AcoR (*Bacillus subtilis*, gi:81637511), FhlA (*Escherichia coli*, gi:1789087), PrpR (*Escherichia coli*, gi:1786524), HrpR (*Pseudomonas syringae pv. tomato*, gi:3228542) and HrpS (*Pseudomonas syringae pv. tomato*, gi:3228543).

efficient transcription activation activity: loop 1 (inserted in α-helix 3) and loop 2 (or Pre-Sensor I, inserted in α-helix 4) (Fig. 2B). These loops are exposed at the same surface of the protein and their orientations are regulated by the bound nucleotide state. It is interesting to note that, in contrast to AAA helicases or AAA proteases that use their central pore to bind and translocate their substrates in a processive fashion, the bEBP AAA activators do not use their central pore to remodel their substrate. Instead, the bEBPs use Loop1 to directly contact and so remodel their asymmetric substrate, the RPc. The molecular mechanism that couples nucleotide binding with substrate remodelling in transcription activation will therefore be somewhat different to those found in the AAA helicase or AAA protease, especially within the hexameric ring even though some global coupling between nucleotide binding and substrate remodelling is likely to be conserved due to the high degree of sequence and structural conservation.

The exposed Loop1 contains the conserved "GAFTGA" motif that is the signature motif defining bEBPs. Bordes et al. [67] have established that this motif is directly involved in the interaction with region I of σ^{54} present in the closed complex (RP_c). Studies on several bEBPs such as NtrC, PspF, NorR or HrpR/S have shown the importance of the threonine residue (T86 in PspF) in establishing the critical interactions with the closed complex for transcription activation. More recently this "GAFTGA" motif has been studied by systematic site-

directed mutagenesis and Zhang et al. [68] have shown that the phenylalanine residue (F85 in PspF) participates in sensing of the DNA state in the promoter DNA complex by the activator. Structural and functional studies on PspF have shown that nucleotide state in the subunit controls the position and nature of Loop1 [69–73]. Based on structural data, Rappas et al. have proposed that the Loop1 could adopt at least two distinct conformations: a fully extended state above the hexameric plane and so exposing the GAFTGA motif in the presence of ATP or the absence of nucleotide, or a more buried conformation towards the central pore in the presence of ADP [72,73]. The role of the Loop1 is therefore to expose the GAFTGA motif (to allow the activator to interact with σ^{54}) in a nucleotide dependent manner.

The role of the second exposed loop, Loop2 (or Pre-Sensor I insertion—Pre-SIi), is less clear. From structural data, Rappas et al. [72,73] proposed that Loop2 could help coordinate the Loop1 orientation in a nucleotide dependent manner. A detailed biochemical study by mutating all the residues constituting this loop in PspF (131 to 139) allowed Burrows et al. [74] to propose at least three major roles of the Loop2 on the AAA domain activity: (i) in co-ordinating the Loop1 position, (ii) in σ^{54} interaction and RPc remodelling activities through the tip of Loop2, and (iii) in modulating the ATPase activity through the more variable N-terminal region. The authors proposed that most of the Loop2 related activities were mediated via Loop1 and relayed through a conserved molecular switch located in the catalytic centre of the ATPase. Structural data on NtrC1 have also reported evidence for different conformations of Loop1 and Loop2 dependent on the nucleotide bound state of the protein [73,75]. More recently, Joly and Buck [76] have biochemically characterised E81, R91, E97 in Loop1 and R131 in Loop2, revealing a sophisticated signalling pathway based on salt-bridge switching within the same monomer of the hexameric activator.

4. ATP hydrolysis cycles in hexameric bEBPs

Four possible types of ATPase cycles have been described for hexameric ATPases (Fig. 3) assuming continuous hydrolysis is required for their functionality. In the stochastic model proposed for ClpX [77], all protomers function independently from one another. In the synchronised model proposed for SV40-LTag [78] and Vps4P [79,80], all protomers are active and occupied by the same nucleotides simultaneously. In the rotational mechanism as proposed for F1-ATPase, three dimer pairs act as units and exist in different nucleotide states. In the sequential model proposed for P4 packaging ATPase [81], T7 gp4 [82], HslU (PDB entry:1DO0) [83] and E1 helicase [84], all protomers are active but only four protomers are occupied by nucleotides and opposite protomers are occupied by the same nucleotide at a given time. Recent structure of Rho with RNA bound show that five nucleotides were bound and a modified sequential mechanism was proposed [85].

The question regarding the type of ATPase cycle related to bEBPs is still open. Indeed, it is still very difficult to address this question directly in order to determine the amount of ATP that is required to efficiently remodel the RP_c and there is a question as whether a processive ATPase activity is required for bEBPs' activities. Most of the ATPase assays are performed in solution, sometimes in the absence of the targeted RP_c substrate. It is not yet possible to exclude a difference in the type of ATPase cycle in the presence or in the absence of RP_c or during the remodelling process. For PspF for which the ATPase activity has been studied extensively, Joly et al. [86] have demonstrated that the PspF hexamer contains ATP and ADP simultaneously. In addition, the presence of an excess of ATP (from 4 mM compared to the estimated 1–2 mM present *in vivo*) inhibited the PspF ATPase activity and that the presence of ADP stimulated the PspF ATPase activity. No significant difference in the steady state ATPase activity of PspF has been observed in the presence of its natural target RPc. Based on the above observations we propose that the ATPase activity of bEBPs does not adopt a stochastic or fully synchronised ATPase cycle. Zhang and Wigley have proposed that some AAA ATPases act as molecular switches to allow the full assembly of macromolecular complexes, during which the ATPase activity is suppressed. Upon full assembly, substrate binding stimulates ATP hydrolysis, which subsequently recycles the components [87]. For this type of reaction, continuous hydrolysis is not required. It is possible that bEBPs act in such a fashion to switch RP_c to RP_o and it is currently unclear how many nucleotides are required for this switch.

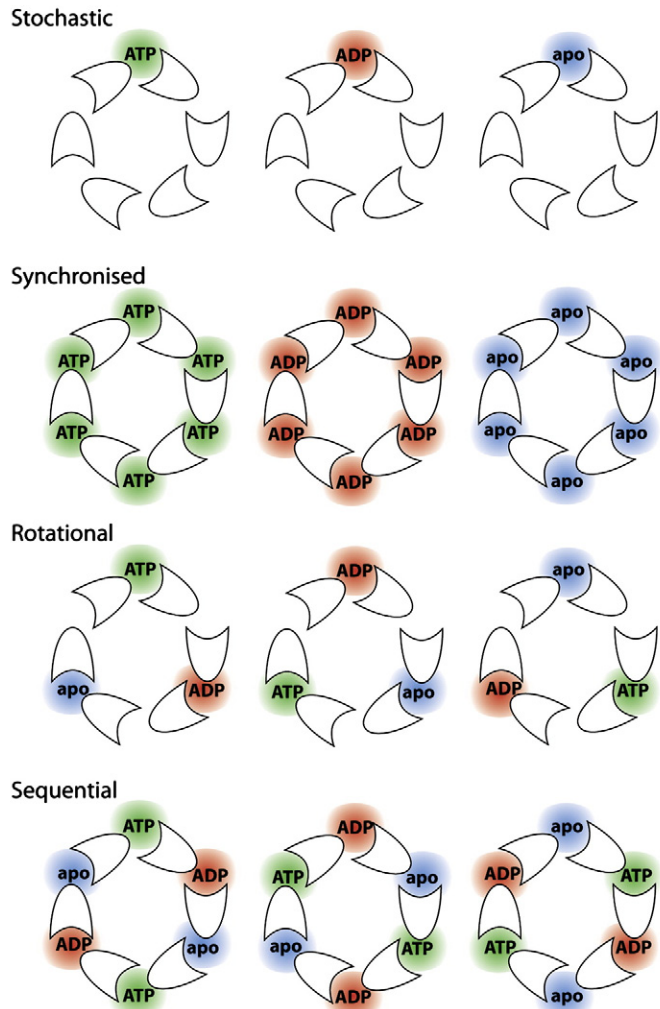

Fig. 3. The potential ATP hydrolysis cycles in hexameric ATPases. The protomer undergoes at least three stages of NTP occupancy: (i) ATP bound, (ii) ADP bound, and (iii) no NTP bound (Apo).

5. How bEBPs couple ATP hydrolysis to transcription activation

5.1. The ATP hydrolysis determinants

The nucleotide binding pocket of bEBPs contains Walker A, Walker B and R-fingers (Figs. 2B, 4A). The Walker A motif (GxxxxGKE) is directly involved in ATP binding. The conserved Lys (K) is thought to coordinate the β- and γ-phosphates [33,87]. Thus mutation of this residue (K42A in PspF) completely abolished ATP binding and consequently hydrolysis was not observed [88]. The Walker B motif (hhhhDE) is involved in ATP hydrolysis. The conserved aspartic acid participates in Mg^{2+} coordination, and the conserved glutamate polarises a water molecule to allow a nucleophilic attack of the γ-phosphate[87]. For structural arrangement in the catalytic site, see

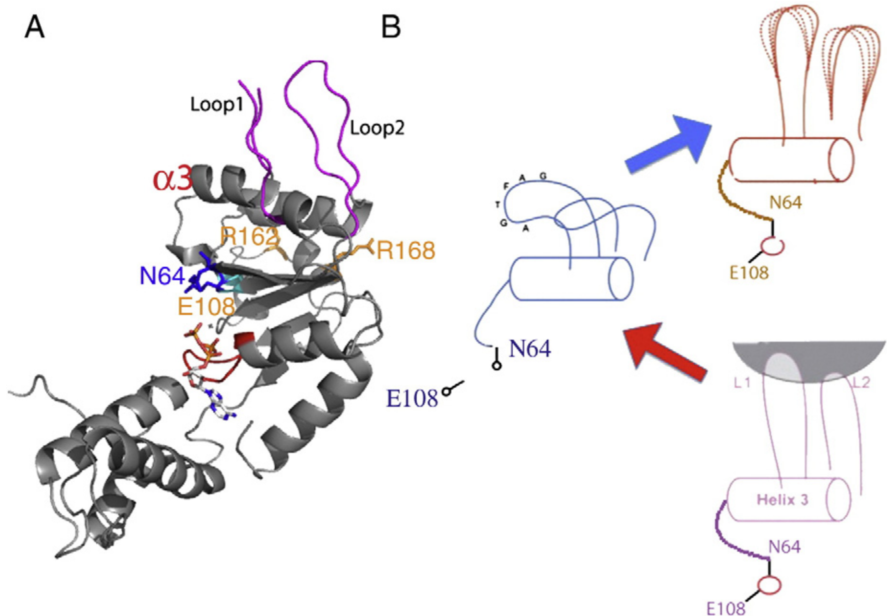

Fig. 4. Structural and functional motifs in bEBPs. A) Structure of PspF AAA domain with important motifs highlighted, Red: Walker A, Cyan: Walker B, Blue: the "Glutamate switch" E108 and N64, Orange: putative R-fingers R162 and R168. Loop1 and Loop2 are colored in magenta, ATP is shown in stick model. B) Proposed conformational changes that link the nucleotide binding pocket to the Loop1/Loop2 substrate interaction sites via the "Glutamate switch". Blue arrow indicates that nucleotides state can be relayed via the "Glutamate switch" to control substrate binding. Red arrow indicates that substrate interaction can control ATPase activity via the "Glutamate switch".

[87]. Walker B variants (of PspF) usually form constitutive hexamers with increased affinity for ATP binding and drastic reduction in ATP hydrolysis [71]. The Arg-fingers protrude into the catalytic site from an adjacent protomer, participating in ATP hydrolysis by stabilising the build-up of negative charge during the formation of the ATP transition state [33,66,89,90]. In bEBPs, two arginine residues appear as putative R-finger candidates. For example in the case of PspF, Schumacher et al. have proposed, from sequence analysis, that the two conserved arginines R162 and R168 could be putative Arg-fingers in PspF but so far neither has been directly established as the R-finger residue [64,65,88]. Using crystal structures of PspF monomer in complex with nucleotides [72,73], a hexamer model was constructed based on other AAA hexamers. In this hexamer model, R162 is closer to the γ-phosphate and has therefore been proposed to be the R-finger in PspF. However, Chen et al.[91] recently determined the crystal structure of the heptameric NtrC1 AAA domain E239A variant (E108A in PspF) in the presence of ATP (PDB entry:3M0E). They proposed that out of the two arginine candidates (R296 and R299), R299 was the R-finger residue of NtrC1 (R168 in PspF) as it is closer to the γ-phosphate. By comparing this structure with the wild type NtrC1 AAA domain ADP-bound structure, they identified conformational changes in R299 when interacting with the *trans* Sensor II residue (R357) to the γ-phosphate, hence they proposed the R299 (R168 in PspF) is responsible for larger scale conformational changes in the molecule. This apparent difference between NtrC1 and PspF is surprising and we note that there are three issues surrounding the observations made with NtrC1: (i) in the structure presented, the NtrC1 is adopting a heptamer conformation and (ii) the structural data are obtained from the Walker B variant of NtrC1 (E239A) which is incompetent for ATPase activity. Furthermore, this glutamate in wild type protein provides the negative charge for the active site, which requires a precise balance of negative and positive charges including those contributed by the R-finger. Mutating this glutamate would therefore likely cause reorganisation of residues, including the R-finger, in the active site. (iii) The authors compared the ADP structure of wild type NtrC1 with ATP structure of the mutant protein, hence it is unclear whether the differences observed are due to the mutations or different nucleotide states.

In the absence of a complete set of wild-type and mutant variant structures with ATP and ADP bound and in which the true functionally relevant oligomeric state is established beyond doubt, some caveats exit in assigning the R-finger and its potential associated functionalities in the ATP hydrolysis cycle.

5.2. Substrate interaction–σ^{54} interaction

bEBPs are specialised AAA ATPases. As discussed previously, the Loop1 contains the σ^{54}-interacting motif whereas the Loop2 seems to have more a "helper" role in coordinating the conformation of Loop1 (Fig. 4A). Rappas et al. [72,73] have proposed that in the ATP state, the loop is in an extended conformation allowing the efficient exposure of the GAFTGA (σ^{54}-interacting motif). While upon γ-phosphate release as in the ADP bound state, the loop adopts a more buried conformation, inaccessible for σ^{54} interaction (Fig. 4B). Based on this model, it was hypothesised that an inhibition of the ATPase activity but not ATP binding of the AAA protein should stabilise the interaction between the AAA ATPase and its specific substrate.

Indeed, Joly et al. [71] have shown that the substitution of the E108 residue led to the formation of a stable complex between the PspF and σ^{54} in the presence of ATP but not with ADP or in the absence of nucleotide. However, the substitution of the second acidic amino acid of the Walker B motif, D107, which drastically decreases the rate of ATP hydrolysis, does not allow the formation of such a nucleotide-dependent stable complex between the activator and its target σ^{54}. From these observations, Joly et al. [71] proposed a revised model in which the inhibition of ATPase activity is not directly responsible for the formation of a stable complex between the activator and σ^{54} but is a side effect of the modification of the nucleotide binding pocket. Nevertheless, it is clear that the ATP-bound state of the bEBP is the state that enables the engagement with its substrate (RP_c).

5.3. Signal coupling

The energy generated from ATP hydrolysis needs to be relayed from the catalytic site via an intra-molecular pathway to remodel

the RP_c. Comparison of $PspF_{1–275}$ crystal structures in different nucleotide-bound states (Apo, ATP and ADP) provided insights into how this energy coupling step successfully transforms chemical energy into mechanical motions.

In the nucleotide-free state, Loop1 is flexible and [73] upon ATP binding, the γ-phosphate is sensed by E108 that in turn interacts with N64. As a result of the N64–E108 interaction, a series of conformational changes occur that lead to the exposure of Loop1, ready for $σ^{54}$ contact (Fig. 4B, orange) [73]. After hydrolysis, E108 rotates nearly 90° to interact with Sensor I residue T148. Loop1 in the ADP bound state is thought to be locked in a folded conformation by the F85 ('GAFTGA' motif)-S75 interaction, unable to contact $σ^{54}$ (Fig. 4B, blue) [72]. The N64–E108 pair has therefore been proposed to switch PspF from an active to an inactive state and *vice verse*[69,71–73,87]. The N64–E108 pair is conserved in other AAA proteins that include members of all 7 AAA clades. The N64 residue has been proposed to relay nucleotide states to substrate interaction and controls ATPase activity through substrate binding either positively or negatively, hence termed the "Glutamate switch" [87] that links nucleotide states with substrate binding in a bi-directory fashion (Fig. 4B). Disruption of the interactions between N64–E108 would decouple these two events, thus substrate binding (or lack of binding) becomes less dependent of nucleotide states and/or ATP hydrolysis becomes less sensitive to substrate interactions. The removal of side chain interaction of either N64 (N64A) or E108 (E108A) still allowed Loop1-$σ^{54}$ interaction in a NTP-dependent manner [69,71], suggesting that other residues are also playing a role in relaying nucleotide states to substrate binding.

5.4. Substrate remodelling

bEBPs are required for activating $σ^{54}$-dependent transcription by converting the RNAP–$σ^{54}$ closed complex which is transcriptional incompetent to a transcriptional-proficient open complex. Using cryo-electron microscopy (EM) studies, Bose et al. [27] provided a structural explanation for why RNAP–$σ^{54}$ closed complex is unable to proceed to transcription. The closed complex is organised in such a way that the channel where template strand DNA needs to be loaded into the RNAP active centre is blocked and the −12 promoter element, where the transcription bubble starts, is located too far upstream. The physical blockage is contributed by $σ^{54}$. Specifically region I of $σ^{54}$, which is shown to interact with bEBPs, has been located in the vicinity of this blockage. Upon binding to the activator protein, as shown by the cryo-EM reconstruction of RNAP–$σ^{54}$–PspF in the presence of an ATP transition state analogue, the physical blockage is weakened and the promoter DNA is shifted in the downstream direction (Fig. 5). This is consistent with biochemical data showing that removal of $σ^{54}$ region I allowed transcription initiation in the absence of the activator proteins provided the transcription bubble is preformed. It appears that the roles of the bEBP activator proteins are two fold: 1) to use nucleotide driven conformational changes to engage RNAP–$σ^{54}$ which consequently relieves the physical blockage to DNA entry into RNAP, and 2) to induce domain movements within the RNAP–$σ^{54}$ that translocate the promoter DNA correctly. Although activator binding to RPc causes some reorganisation of RPc, complete nucleotide hydrolysis is required to completely remove the physical blockage, probably through the re-positioning of the Loop1 and Loop2 upon γ-phosphate release, allowing the region I of $σ^{54}$ to fully relocate.

6. Regulation of bEBPs activities

bEBP's activity is tightly regulated, either positively or negatively, either *in cis* through the N-terminal regulatory domain or *in trans* through another protein. For example, NtrC1 and NorR are negatively regulated by their N-terminal regulatory domains and deletion of

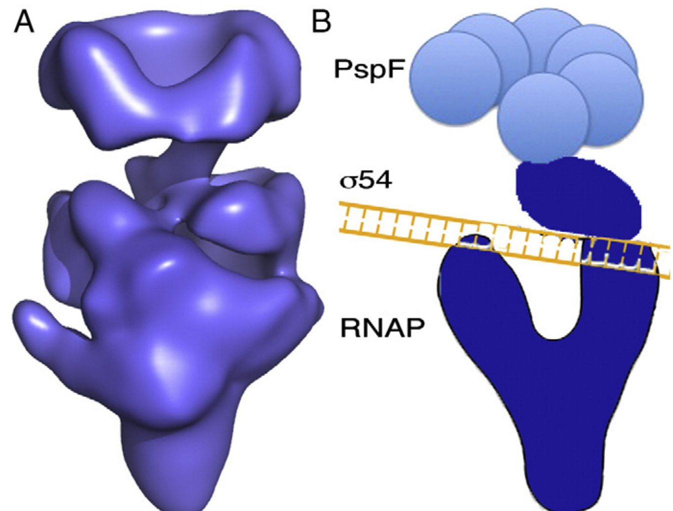

Fig. 5. Organisation of the bEBP with its substrate. From electron microscopy single particle reconstructions, bEBP is organised as a non-planar hexamer interacting asymmetrically with its substrate, the RNAP–$σ^{54}$ holoenzyme. A) cryo-EM reconstruction of $PspF_{1–275}$ in complex with RNAP–$σ^{54}$. B) Cartoon representation of various components, for illustrative purposes, DNA, which is not present in the cryo-EM reconstruction, is shown as orange tracks.

these domains results in a constitutively active variant that is independent of activation signals. PspF and HrpRS, both lack N-terminal regulatory domains, are also negatively regulated, by interacting with PspA and HrpV respectively. NtrC on the other hand, is positively regulated by its receiver domain upon phosphorylation, as deletion of the receiver domain results in a functionally dominant negative variant. Interestingly, studies on the bEBPs have revealed three different regulatory mechanisms: 1) through preventing or promoting oligomerisation, 2) interfering with ATPase activity *per se* or 3) preventing substrate interactions.

Groups I–III bEBPs all contain a N-terminal regulatory domain and many of the well-studied bEBPs, such as NtrC1, NtrC4, DctD and XylR, are negatively regulated by their N-terminal domains through the prevention of oligomerisation formations. Phosphorylation of the N-terminal domain relieves the repression, allowing the oligomerisation, thus the ATPase activity, of the AAA domain to occur. The N-terminal domain of NtrC, on the other hand, acts positively by promoting oligomerisation upon phosphorylation.

In contrast, in the case of the group IV and V bEBPs, which are constitutively active in solution, the regulation of the AAA+domain activity (usually negative) seems to be different. Biochemical experiments suggest that the negative regulation of Group IV bEBPs is not due to the inhibition of oligomer formation. Indeed, Joly et al. [92] have shown that PspA does not interfere with hexameric formation of PspF as PspA and PspF formed a "regulatory complex" constituted of six PspF monomers and six PspA monomers. Furthermore the PspA–PspF complex is still able to interact with the RNA polymerase closed complex, suggesting that PspA does not prevent substrate interaction (Joly et al., 2009). However, PspA severely affects ATPase activity of PspF. The Glu108-Asn64 pair have been proposed to link nucleotide bound states to substrate (or ligand) binding in AAA proteins. It was therefore proposed that PspA binding affects the "Glutamate switch", which consequently result in reduced ATPase activity [69]. Indeed, substitution of the Asn64, therefore disrupting the "Glutamate switch", has been shown to affect the ATPase activity of PspF but also to protect PspF from the negative regulation by PspA while PspF–PspA interaction was maintained, consistent with the idea that ligand binding controls ATPase activity through the "Glutamate switch" [33,87,90,93].

In the case of the regulation of HrpR/S activity by HrpV, the mechanism of regulation seems to be different again [94]. HrpR/S is the

only bEBP described so far which requires a hetero-hexamer for activity. This specific property of the complex strongly suggests the existence of an additional level of regulation compare to the homo-hexameric bEBP. In addition, Jovanovic et al. have shown that the negative regulator HrpV specifically targets the HrpS subunit but not the HrpR subunit, implying a subunit-specific negative control mechanism. It was proposed that the effect of HrpV could be (i) allosteric via topological effect on HrpR/S hexamer and/or (ii) steric, by blocking the interaction between HrpR/S hexamer and its substrate, the RNA polymerase closed complex [94].

NorR, which belongs to Group II bEBPs with a N-terminal domain that is regulated by nitric oxide, seems to adopt an altered mode of regulation. Like many other Groups I and II bEBPs, the activity of the AAA domain of NorR is negatively controlled through the repression of the N-terminal GAF domain, as the truncation of this domain results in a competent NorR for transcription activation in the absence of nitric oxide [95,96]. Interestingly, contrary to other bEBPs, the GAF domain in NorR does not affect the oligomerisation state of NorR, hence the regulatory domain does not repress its activity by interfering with the oligomerisation. Using mutagenesis approaches, Bush *et al.* [97] have identified mutations in the AAA domain (in helix 3, helix 4 or Loop1) that enable NorR to escape from the GAF repression. Surprisingly, two mutations have been identified in the highly conserved σ^{54}-specific interacting GAFTGA motif, both targeting the second Glycine of this motif (G266D and G266N). Bush et al. proposed that the GAF domain of NorR negatively regulates the AAA activity by preventing its interactions with RNAP–σ^{54} holoenzyme via blocking the surface interacting loops, Loop1 and Loop2 [44]. Furthermore, deletion of GAF domain showed elevated ATPase activity [98], suggesting that the GAF domain represses the ATPase activity of the NorR AAA domain *per se*, similar to that of PspA, possibly through the interference of the "Glutamate switch".

Although the oligomerisation of NorR is not regulated by its N-terminal GAF domain, NorR normally exists as dimers in solution and its oligomerisation is also highly regulated. Unlike many other bEBPs whose oligomerisation can occur in the absence of enhancer DNA, the oligomerisation and hence ATPase activity of NorR depend strongly on its binding to all three enhancer DNA sites. In fact, mutation of a single site reduced NorR's activity [97]. It was therefore hypothesised that the DNA binding domain of NorR prevents hexamerisation. Upon DNA binding, conformational changes occur in NorR that then promote hexamerisation of the AAA domain. Interestingly, a minimal sequence that contains all three enhancer sites is insufficient to maintain a stable NorR hexamer and a DNA sequence flanking the three sites is required for stable hexamer formation and enhanced ATPase activity [99]. NorR is therefore an unusual bEBP as it utilises all three known regulatory mechanisms: 1) oligomerisation negatively regulated by the DNA binding domain 2) its ATPase activity *per se* and 3) substrate interaction, both negatively regulated by the GAF domain (Fig. 6).

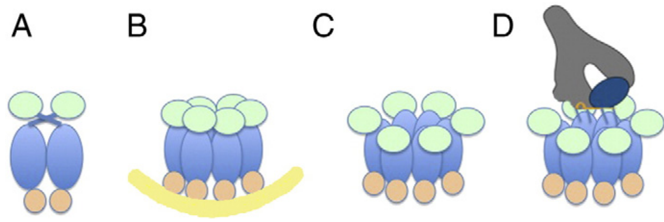

Fig. 6. The regulatory mechanism of bEBP NorR. Light green: N-terminal GAF domain, blue: AAA domain, brown: DNA binding domain, yellow: enhancer DNA, dark blue: sigma54, grey: RNAP. A) NorR exist as dimers in solution B) Upon enhancer DNA binding, NorR forms hexamers but incompetent for ATP hydrolysis and RNAP–σ^{54} interaction due to GAF domain inhibition, C) Upon activation, the repression of GAF domain is relieved which leads to D) RNAP–σ^{54} interactions.

7. Concluding remarks

In the last few years, a number of studies have advanced our understanding of how bEBPs utilise their ATPase activity to remodel their target, the RNAP–σ^{54} closed complex. It has been firmly established that a hexameric bEBP interacts with its substrate RPc through the two surface loops, Loop1 and Loop2, inserted into the AAA core domain. ATP binding is required for the exposure of the loops and stable engagement with RPc, while ATP hydrolysis is required to remove the repressive physical blockage imposed by σ^{54} on RNAP. Apart from the well established AAA motifs such as Walker A, Walker B and R-fingers, work on bEBPs also reveals the "Glutamate switch", which links substrate binding with the ATPase activity, as an important conserved feature among all AAA proteins. There is still much to determine including the exact conformation and coordination of subunits within the hexamer upon substrate binding, although it is widely believed that a non-concerted mechanism operates here and a non-planar hexamer arrangement is present upon interacting with its substrate (Fig. 5). Like many other AAA + proteins, the key to understand the detailed mechanisms behind bEBP's functionalities will require the high resolution details of the substrate bound complexes moving along the remodelling pathways from RP_c to RP_o.

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