Dissecting the ATP hydrolysis pathway of bacterial enhancer-binding proteins

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Abstract

bEBPs (bacterial enhancer-binding proteins) are AAA+ (ATPase associated with various cellular activities) transcription activators that activate gene transcription through a specific bacterial σ factor, σ 54, σ 54–RNAP (RNA polymerase) binds to promoter DNA sites and forms a stable closed complex, unable to proceed to transcription. The closed complex must be remodelled using energy from ATP hydrolysis provided by bEBPs to melt DNA and initiate transcription. Recently, large amounts of structural and biochemical data have produced insights into how ATP hydrolysis within the active site of bEBPs is coupled to the re-modelling of the closed complex. In the present article, we review some of the key nucleotides, mutations and techniques used and how they have contributed towards our understanding of the function of bEBPs.

Introduction

bEBPs (bacterial enhancer-binding proteins) are AAA+ (ATPase associated with various cellular activities) family members involved in regulating bacterial gene expression. Regulated transcription in bacteria requires multisubunit RNAP (RNA polymerase) to interact with σ factors, a family of proteins that bind to RNAP forming holoenzymes and direct it to promoter DNA [1,2]. bEBPs are essential for transcription initiation using the major variant σ factor, σ 54, commonly involved in tightly regulated bacterial responses to stress [3–5]. This is functionally analogous to enhancer-dependent initiation of eukaryotic RNA polymerase II, which requires an input of energy from ATP hydrolysis provided by TFIIH (transcription factor IIH) [6,7]. ATP hydrolysis by bEBPs provides energy for remodelling the σ 54–RNAP closed complex, resulting in further DNA melting and loading of the template strand of DNA into the RNAP active site [8,9]. Energy is transferred to the closed complex through a physical interaction between σ 54 and the AAA+ domain of the bEBP [10,11].

A number of bEBPs are well characterized, including NtrC, ZraR, PspF, NorR, DctD, NifA and HrpR/S [12,13]. They typically comprise three domains. An N-terminal regulatory (R) domain acts either positively or negatively on ring formation and ATPase activity [14]. Positive regulation (e.g. NtrC and ZraR) is when the modification of the R domain actively promotes oligomerization, whereas, in negative regulation, the modification of R domain releases the inhibition of oligomerization (e.g. NtrC1 and DctD) [15–18]. In some cases, the R domain is dispensable with, and regulation is achieved through an interaction with a secondary protein (e.g. PspF with PspA and HrpR/S with HrpV) [19–21]. The C-terminal DNA-binding domain enables specific promoter recognition by allowing bEBPs to bind to enhancer-like sequences located approx. 100–150 bp upstream of the transcription start site [3,22]. bEBPs bound to upstream DNA contact the closed σ 54–RNAP complex by a DNA-looping mechanism, often aided by DNA-bending proteins [23]. Interactions with DNA may also promote oligomerization of the AAA+ domain (e.g. NorR) [24]. The AAA+ domain in isolation is often sufficient to activate σ 54-dependent transcription in vitro and in vivo [25,26]. Apart from the well-conserved AAA+–specific motifs Walker A (GXXXGKT/S), Walker B (ΨΨΨΨDE, where Ψ is hydrophobic) and the SRH (second region of homology) motif, bEBPs also contain the highly conserved signature GAFTGA motif, which mediates interactions with σ 54 [27–31]. Nucleotide binding occurs at the interface of two subunits, and residues from adjacent subunits contribute to hydrolysis, partly explaining the dependence of ATP hydrolysis activity on oligomerization [5,32,33]. The AAA+ domain also contains the L1 and L2 loops, which are specific to bEBPs and undergo a series of movements in response to stages of the ATP hydrolysis cycle [10,34]. L1 contains the GAFTGA motif at its tip and forms an insertion in the third helix of the AAA+ domain, present as an uninterrupted helix in other AAA+ family members [27]. L2 is present at the C-terminus of the fourth helix, and is thought to be responsible for co-ordinating the movements of the L1 loop [10]. bEBPs are members of a subclass of AAA proteins known as the pre-sensor I β-hairpin superclade, which share the insertion of a β-hairpin in the position of loop L2, and include the helicases RuvB, Ltag and MCM (mini-chromosome maintenance), as well as proteases HslU, ClpX and Lon [35].
The nucleotide state in the active site is communicated to the L1 and L2 loops, causing loop movements and affecting interactions between the bEBP and σ54–RNAP [36,37]. The transition from closed to open complex is likely to involve at least two transient intermediate states, possibly as a result of changing interactions with the GAFTGA motif during the ATP hydrolysis cycle [38–40]. A wide variety of techniques and approaches have been employed to study the intricate events that accompany the ATP hydrolysis cycle within bEBPs. In this review, we describe our current understanding of the bEBP-mediated transition from closed to open complex, and examine some of the important techniques, nucleotide analogues and mutations employed to dissect the stages of the ATP hydrolysis cycle that are responsible for this transition.

**Structural studies**

A number of high-resolution structures of bEBPs have revealed large amounts of information regarding the coupling of nucleotide hydrolysis to functional interactions with σ54 within bEBPs, and, together with information from other AAA+ family members, have enabled the design of rational mutations to study these processes further. To date, structures exist for NtrC1, PsPF and ZraR in different nucleotide states and varying mutant forms [10,16,27,34]. A number of the nucleotide-bound structures of PsPF were obtained by soaking crystals with nucleotides, detailing the localized changes occurring in the active site during hydrolysis, and suggesting a network of interactions linking the active site to loops L1 and L2 [10,36]. Lower-resolution methods such as SAXS (small-angle X-ray scattering)/WAXS (wide-angle X-ray scattering) and single-particle cryo- and negative-stain EM (electron microscopy) have been used in conjunction with nucleotide analogues to provide a glimpse of the macromolecular changes occurring during the hydrolysis cycle, and the cryo-EM structure of PsPF in complex with σ54 highlighted the importance of the L1/L2 loops for coupling nucleotide hydrolysis to remodelling of the closed complex [10,15,41].

**Nucleotide states**

**Ground state (ATP/ADP–BeF/p[NH]ppA/ATP[S])**

Binding of ATP in the active site is communicated through a network of interactions causing a relocation of Linker 1 and subsequent rotation of the third helix of the AAA+ domain [36]. This causes the L1/L2 loops to move upwards, positioning the GAFTGA motif in an exposed conformation able to interact with σ54 (Figure 1A) [10]. X-ray structures of PsPF bound to ATP were obtained by soaking crystals of wild-type PsPF with ATP in the absence of Mg2+ to prevent hydrolysis, or by using the hydrolysis-deficient mutant form of PsPF (PsPFβ27γ52) in the presence of Mg2+. The structure of wild-type PsPF soaked with the ground-state analogue p[NH]ppA was also solved [9]. The structures show the localized interactions made between ATP and the active site of PsPF, notably the Walker B aspartate residue (Asp127) positions a water molecule for nucleophilic attack of the γ-phosphate, while the glutamate residue (Glu128) senses the γ-phosphate of the bound nucleotide and forms a tight interaction with the highly conserved asparagine residue (Asn64) at the N-terminus of Linker 1 (Figure 1B) [36]. Although crystal lattice constraints limit the observation of larger-scale conformational changes, studies using NtrC1 with ADP–BeF as a ground-state analogue indicate that the initial contact with σ54 is indeed formed during the ground state [41]. ADP–BeF causes a pronounced raising of the L1/L2 loops in NtrC1 that can be observed using SAXS/WAXS analysis, a result consistent with the local changes observed in the ATP-bound crystal structure of PsPF [36,41]. Furthermore, σ54 can form complexes with NtrC1 and PsPF in the presence of ADP–BeF [41]. Interestingly, p[NH]ppA (adenosine 5′-[(β,γ-imino)tri phosphate] and ATP[S] (adenosine 5′-γ-thio)triphosphate) do not cause any loop movements observable by SAXS/WAXS, although p[NH]ppA can stabilize complexes between σ54 and NtrC1 or PsPF [41] (Table 1). The results suggest that ATP binding can cause the release of the L1/L2 loops and enables an initial unstable interaction with σ54.

**Transition state (ADP–AlFx)**

Use of the ATP hydrolysis transition-state analogue ADP–AlFx, (where x is either 3 or 4) has been critical for understanding the function of bEBPs (Table 1). The ability of ADP–AlFx to form stable complexes with σ54 led to observations implicating the importance of a direct interaction between GAFTGA and σ54-region I for the isomerization of the closed complex [8,32,42]. Most importantly, the stabilizing effect of ADP–AlFx on the complex between PsPF and σ54 enabled reconstruction of this complex using single-particle cryo-EM [10]. Together with the X-ray structure of PsPF, the reconstruction revealed that at the point of ATP hydrolysis, the L1/L2 loops elevate above the plane of the hexameric ring and make significant interactions with σ54, probably through the GAFTGA motif [10,11]. Reconstructions of NtrC and NtrC1 bound to ADP–AlFx, solved using SAXS/WAXS and negative-stain EM, show a pronounced ring of density above the plane of the oligomeric bEBP, corresponding to the raised L1/L2 loops in the transition state [15,41]. Stable complexes with σ54 have been observed in the presence of ADP–AlFx for NtrC1 and NifA, and stable complexes form between PsPF and σ54–RNAP [41,42]. Interestingly, the NtrC1–σ54 complex formed with ADP–AlFx is significantly more stable than that formed with ADP–BeF, suggesting that the initial complex formed with the ground state is stabilized upon transition-state formation [40,41]. ADP–AlFx–bound PsPF has a greatly reduced ATP-hydrolysis activity and is incapable of initiating transcription [42]. However, it is capable of inducing conformational changes of both σ54–RNAP and promoter DNA within the closed complex similar to those observed during open complex formation [39,40]. Consequently, the interaction stabilized by ADP–AlFx is likely to represent a genuine intermediate on the isomerization pathway between closed and open complexes.
**Figure 1** | Conformational changes during ATP hydrolysis

Structures of PspF\(^{1-275}\) bound to ATP (A, C) PDB code 2C96/2C9C and ADP (B, D) PDB code 2C99/2C9C. Important motifs are highlighted: Walker A (red), Walker B (blue), Sensor I (orange), R-finger (green), Sensor II (magenta), Linker 1 (pale green), Loops L1 (red) and L2 (blue), conformational signalling between Asn\(^{64}\) and L1 (yellow). Monomeric PspF\(^{1-275}\) in (A) ATP state and (B) ADP state, showing raised L1 conformation in ATP state and lowered conformation in ADP state. Active-site interactions with (C) ATP and (D) ADP. (E) Switching mechanism of Walker B Glu\(^{108}\). In ATP state, Glu\(^{108}\) (blue) interacts with Asn\(^{64}\). In ADP state Glu\(^{108}\) (teal) interacts with Thr\(^{148}\).

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**Table 1** | Nucleotide states

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Nucleotide state</th>
<th>Properties when bound to bEBP</th>
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</thead>
<tbody>
<tr>
<td>ATP</td>
<td>Ground state (ATP)</td>
<td>Promotes hexamerization; high concentrations inhibit catalytic activity</td>
</tr>
<tr>
<td>ADP-BeF</td>
<td>Ground state (ATP)</td>
<td>L1/L2 loops in raised position to contact (\sigma)(^{54}). Stabilizes complex with (\sigma)(^{54}) in NtrC1 and PspF</td>
</tr>
<tr>
<td>(\beta)[NH]ppA</td>
<td>Ground state (ATP)</td>
<td>Can stabilize complex of NtrC1 and (\sigma)(^{54})</td>
</tr>
<tr>
<td>ATP[S]</td>
<td>Slow hydroysis</td>
<td>No complex stabilization</td>
</tr>
<tr>
<td>ADP-AIF(_x)</td>
<td>Hydrolysis transition state</td>
<td>L1/L2 loops in raised position. Forms stable complex with (\sigma)(^{54}) with improved stability over ADP-BeF complex. Reduced hydrolysis activity. Can re-model closed complex, forming the first of two proposed intermediate complexes</td>
</tr>
<tr>
<td>ADP</td>
<td>Post-hydrolysis (ADP)</td>
<td>L1/L2 loops in lowered conformation. High concentrations inhibit hydrolysis, but maximum catalytic activity when bound at physiological concentrations with ATP in PspF</td>
</tr>
</tbody>
</table>

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Post-hydrolysis (ADP)
The ADP-bound state has been a more tractable prospect to study structurally, with crystal structures solved for PspF, NtrC1 and ZraR [10,16,34,36]. The structure of ADP-bound PspF indicates that release of the $\gamma$-phosphate causes a 90° rotation of the Glu108 side chain, which forms a new interaction with the Sensor I threonine residue (Thr148) (Figure 1E) [36]. This is communicated through Asn64 and Linker 1, causing a rotation of the third helix [36]. The structure of ADP-bound NtrC1 shows the L1/L2 loops locked into a position pointing towards the central pore of the bEBP, unavailable for $\sigma^{54}$ interactions (Figure 1B) [34]. This conformation is maintained by hydrophobic interactions between the phenylalanine residue of GAFTGA and hydrophobic residues in L2 and in the third helix [34]. The Walker B glutamate residue appears to act as an atomic switch causing movement of the L1/L2 loops from a raised position in the ATP and ADP–AlF<sub>x</sub> states to the lowered position observed in the ADP state [27,36]. Binding of ADP can stimulate formation of PspF hexamers and has a stimulatory effect on the ATP-hydrolysis activity of PspF [43]. This partly reflects the role of ADP in hexamer formation, but also involves intersubunit communication [43].

Functional determinants

Walker B motif
Mutations of the Walker B motif (Asp–Glu) have dramatic effects on the ATP hydrolysis activity of AAA proteins [27]. The aspartate residue is highly conserved and principally involved in hydrolysis, consequently mutations of the residue typically result in hydrolysis-deficient proteins [33,44]. In the absence of nucleotide, the aspartate residue appears to negatively regulate hexamer formation, as substitutions of Asp107 in PspF can result in formation of constitutive hexamers [40]. Mutations of the glutamate residue can uncouple nucleotide hydrolysis from functional output, preventing hydrolysis, but not the conformational movements resulting from nucleotide binding [45,46]. The mutation of PspF Glu108 to alanine or glutamine (E108A or E108Q) results in mutant forms capable of forming nucleotide-independent hexamers, albeit with a greatly reduced nucleotide hydrolysis activity [40]. More significantly, the Glu108 mutants are also capable of forming a relatively stable complex with $\sigma^{54}$ and $\sigma^{54}$–RNAP in the presence of ATP, a state only obtainable with ADP–BeF and ADP–AlF<sub>x</sub> in wild-type protein [40–42]. Observation of a stable complex with $\sigma^{54}$ when ATP is bound, but not hydrolysed, is consistent with the complexes seen with NtrC1, PspF and $\sigma^{54}$ when bound to ADP–BeF [40,41]. The side chain of Glu108 in PspF switches between interactions with Asn64 and Thr148 in response to ATP hydrolysis, controlling the movements of L1 and L2 [36,47]. The ability of E108A to bind stably to $\sigma^{54}$ suggests that the raised conformation of L1 and L2 is not wholly dependent on a tight interaction between Glu108 and Asn64 [40]. In contrast with the ADP–AlF<sub>x</sub> complex, the Glu108 variant complexes are transcriptionally active, despite having a low rate of ATP hydrolysis [40]. Similar results have been observed for mutation of the equivalent residue in NtrC1 (Glu239) to alanine [41]. Mutations of Glu108 in PspF also enabled identification, on the basis of altered DNA conformations, of at least two of the intermediate complexes formed en route to full open complex formation. The first is more similar to that observed with the trapped ADP–AlF<sub>x</sub> complex, whereas the second represents a conformation closer to that of the open complex [37,39,40]. The presence of a side chain at position 108 appears to be essential for formation of these isomerized complexes, reflecting the importance of a switching mechanism during ATP hydrolysis [40].

SRH (Sensor I, R-finger)
SRH contains the sensor I motif and the R-finger, a catalytic arginine residue that acts in trans between adjacent promoters [30,31]. Mutation of the R-finger (PspF Arg168) to alanine abolishes nucleotide hydrolysis, but enables PspF to form nucleotide-independent hexamers [33]. Mutation of either Sensor I Thr148 or Asn149 in PspF to alanine also results in a reduced ATP-hydrolysis activity [47]. However, the T148A mutation is additionally defective in making stable interactions with $\sigma^{54}$ and consequently for open complex formation, highlighting the potential importance of the interaction between Sensor I (Thr148) and the Walker B residue Glu108 for communicating nucleotide state to the L1/L2 loops [36,47]. Interestingly, this interaction (which occurs through a water molecule present in every nucleotide-bound structure) could provide one structural basis for ATP-hydrolysis-driven movements of the L2 loop. T148A mutants display similar functional phenotypes to those observed with L2 loop mutants R131A and V132A, which do not interact with $\sigma^{54}$ (Table 2). Movements of L2 in response to the post-hydrolysis ADP state initiated by a Glu108–Thr148 interaction and propagated through a second signalling pathway could amplify the effect of the ‘power stroke’ as L1 and L2 relocate to the lowered position [47].

Order of hydrolysis
The cryo-EM structure of the PspF–ADP–AlF<sub>x</sub>–$\sigma^{54}$ complex reveals an asymmetric distribution of contacts made to $\sigma^{54}$, raising the question of how hexameric bEBPs interact with monomeric asymmetric $\sigma^{54}$–RNAP [10]. This asymmetry immediately disfavours a concerted mechanism, where every subunit has the same nucleotide state, in favour of either a stochastic, sequential or rotational hydrolysis mechanism, characterized by heterogeneous nucleotide occupancy. Recent work with PspF has addressed how the order of hydrolysis and interactions with substrate affect hydrolysis. ADP has a stimulatory effect on the ATP-hydrolysis activity of PspF, which, together with the observation that saturating concentrations of ATP inhibit hydrolysis, suggests heterogeneous nucleotide occupancy and communication of nucleotide states between subunits [43]. Indeed, heterogeneous nucleotide occupancy in PspF correlates with maximal catalytic activity at physiological concentrations of
Table 2 | Functional determinants

<table>
<thead>
<tr>
<th>Residue (PspF)</th>
<th>Mutations</th>
<th>Mutant phenotype</th>
<th>Potential as tool</th>
</tr>
</thead>
<tbody>
<tr>
<td>Walker A</td>
<td>Lys152</td>
<td>K42A</td>
<td>Deficient for ATP binding, hydrolysis, hexamer formation and initiation</td>
</tr>
<tr>
<td>Walker B</td>
<td>Asp107</td>
<td>D107A, D107E</td>
<td>Decreased ATP hydrolysis. D107E shows increased ATP binding. Forms constitutive hexamers</td>
</tr>
<tr>
<td>传感器 I</td>
<td>Thr148</td>
<td>T148A</td>
<td>Slightly reduced hydrolysis, defective for transcription activation. Deficient for isomerization of the closed complex, but able to bind σ^54</td>
</tr>
<tr>
<td></td>
<td>Asn149</td>
<td>N149A, N149S</td>
<td>Slightly reduced hydrolysis, able to bind σ^54 and isomerize closed complex</td>
</tr>
<tr>
<td>Loop 2</td>
<td>Arg131</td>
<td>R131A</td>
<td>Slightly reduced hydrolysis, defective for transcription activation. Deficient for isomerization of the closed complex, but able to bind σ^54</td>
</tr>
<tr>
<td></td>
<td>Val132</td>
<td>V132A</td>
<td>Slightly reduced hydrolysis, defective for transcription activation. Deficient for isomerization of the closed complex, but able to bind σ^54</td>
</tr>
<tr>
<td>GAFTGA</td>
<td>Thr86</td>
<td>T86S</td>
<td>Deficient for initiation with wild type σ^54, but rescued by mutants of region I</td>
</tr>
<tr>
<td>Sensor II</td>
<td>Arg227</td>
<td>R227A</td>
<td>Binds ATP, but deficient for hydrolysis, nucleotide-independent hexamer formation and initiation</td>
</tr>
</tbody>
</table>

nucleotide [43]. This argues against concerted mechanisms and also stochastic mechanisms, as nucleotide state is communicated between adjacent subunits [43]. Rotational or sequential mechanisms are therefore most likely for bEBPs. A non-symmetric mechanism in bEBPs would permit asymmetrical contacts to form with σ^54 in the closed complex, while reducing cycles of unproductive hydrolysis.

Hexameric bEBPs probably use a non-concerted mechanism for ATP hydrolysis. Studies of the detailed conformational changes and co-ordination within the hexameric ring during an ATP hydrolysis cycle, and the interactions made with σ^54–RNAP holoenzyme that ultimately lead to DNA melting and open complex formation, remain the great challenges for the coming years.

Summary

In summary, structural and biochemical studies of bEBPs have revealed a multistep process during the ATP hydrolysis cycle, which correlates with intermediate complexes formed during open complex formation. Conformational signalling propagating from the Walker B glutamate residue communicates changes in nucleotide state to the L1/L2 loops.

References


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