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Regulation and action of the bacterial enhancer-binding protein AAA+ domains

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Abstract

Bacterial EBPs (enhancer-binding proteins) play crucial roles in regulating cellular responses to environmental changes, in part by providing efficient control over σ^{54}-dependent gene transcription. The AAA+ (ATPase associated with various cellular activites) domain of the EBPs, when assembled into a ring, uses energy from ATP binding, hydrolysis and product release to remodel the σ^{54}–RNAP (RNA polymerase) holoenzyme so that it can transition from closed to open form at promoter DNA. The assembly, and hence activity, of these ATPases are regulated by many different signal transduction mechanisms. Recent advances in solution scattering techniques, when combined with high-resolution structures and biochemical data, have enabled us to obtain mechanistic insights into the regulation and action of a subset of these σ^{54} activators: those whose assembly into ring form is controlled by two-component signal transduction. We review (i) experimental considerations of applying the SAXS (small-angle X-ray scattering)/WAXS (wide-angle X-ray scattering) technique, (ii) distinct regulation mechanisms of the AAA+ domains of three EBPs by similar two-component signal transduction receiver domains, and (iii) major conformational changes and correlated σ^{54}-binding activity of an isolated EBP AAA+ domain in the ATP hydrolysis cycle.

Introduction

Bacteria must adapt to environmental changes quickly and accurately to survive. Among the many strategies used to regulate their adaptive behaviours, σ^{54}-dependent gene transcription often plays a crucial role (reviewed in [1]). The σ factors are essential components of bacterial RNA polymerase, recruiting it to specific promoter regions and melting the DNA to permit transcription initiation. The σ^{54} family of σ factors is unique. Its members do not show sequence similarity to the other σ factors and, unlike them, σ^{54} stabilizes RNAP (RNA polymerase) as a closed complex at σ^{54}-specific promoters. The closed complex cannot isomerize and open the DNA duplex to initiate transcription unless acted upon by EBPs (enhancer-binding proteins) (Figure 1). EBPs are highly modular proteins, which in most cases consist of three types of domain: an N-terminal regulatory domain, a central AAA+ (ATPase associated with various cellular activites) domain, and a C-terminal DNA-binding domain (reviewed in [2]). An EBP usually forms a dimer and binds to enhancer sequences located upstream of the σ^{54}-promoter region. The central AAA+ domain needs to assemble into ring-shaped oligomers (hexamer and heptamer examples have been observed [3]) before becoming functional in activating transcription. This assembly process is tightly regulated by various N-terminal regulatory domains, which most frequently are two-component signal transduction receiver domains (reviewed in [4]). Upon phosphorylation, the receiver domain undergoes conformation changes, and either de-represses or facilitates the assembly of the AAA+ ring ([3,5,6] and see below). Via DNA looping, which is sometimes facilitated by IHF (integration host factor), the assembled ATPase ring on the upstream enhancer region is able to approach the σ^{54}–RNAP closed complex on the promoter region [7,8]. Being bound to upstream DNA is not believed to be essential, because the ATPase appears to be able to interact with closed complexes of RNA polymerase directly from solution [6,9]. The assembled ATPase ring hydrolyses ATP, undergoes major conformational changes at distinct steps of ATP hydrolysis, with only ground and transition states able to bind to σ^{54} [10,11]. This interaction with σ^{54} is critical for coupling ATP hydrolysis with transcription activation, and requires the signature motif of EBPs, the GAFTGA loop. This loop is located in the central pore region on the top of the ATPase ring [3]. During or after release of P_i, the σ^{54}–RNAP–promoter complex is remodelled and released from the ATPase, and the closed complex isomerizes into an open one that is competent for transcription initiation. The order of events remains unknown. Many high-resolution structures for parts of this elegant transcription system are available to date (reviewed in [2]). Recent low-resolution structures by SAXS (small-angle X-ray scattering)/WAXS (wide-angle X-ray scattering) from our laboratory and by EM (electron microscopy) from colleagues Nogales (University of California, Berkeley, Berkeley, CA, U.S.A., and Lawrence

Key words: ATPase associated with various cellular activites (AAA+), enhancer-binding protein, σ^{54}, small-angle X-ray scattering/wide-angle X-ray scattering (SAXS/WAXS), transcriptional regulation.

Abbreviations used: AlF_x, aluminium fluoride; ATP[S], adenosine 5′-[γ-thio]triphosphate; BeF_x, beryllium fluoride; DLS, dynamic light scattering; EBP, enhancer-binding protein; EM, electron microscopy; I_0, forward scattering; R_G, radius of gyration; RNAP, RNA polymerase; SAXS, small-angle X-ray scattering; WAXS, wide-angle X-ray scattering.

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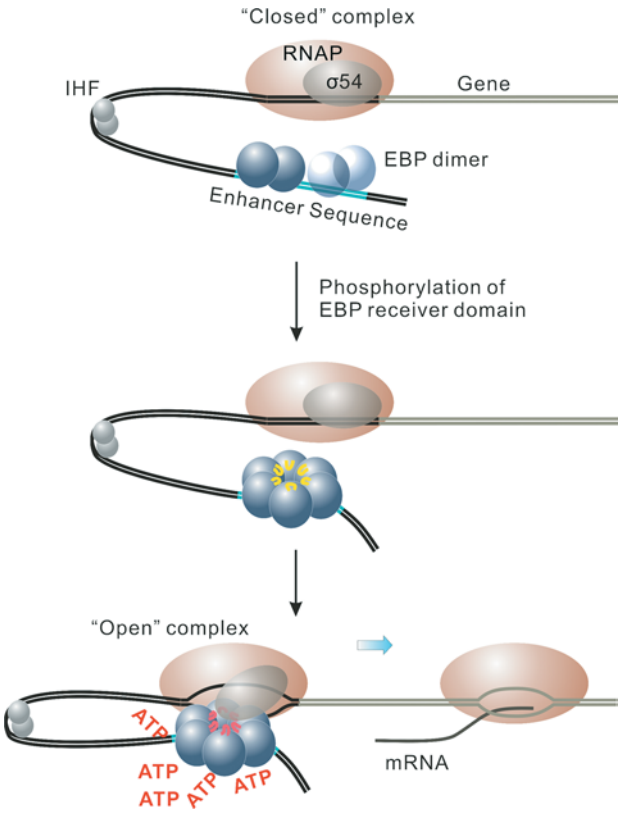
Figure 1 | σ^{54}-dependent gene transcription

The GAFTGA motifs are shown as yellow (ground state) or red (transition state) loops on top of the ATPase ring.



Berkeley National Laboratory, Berkeley, CA, U.S.A.) and De Carlo (University of Colorado, Boulder, CO, U.S.A.) provide further insights into the regulation and action of the EBP ATPases [5,6,10]. Here we describe our part of those studies.

SAXS methodology

SAXS has been significantly improved and has recently spurred a resurgence in low-resolution structural determination of biomolecules, thanks to the advent of high-intensity synchrotron X-ray beams and advanced data collection, processing and modelling tools (reviewed in [12]). Solution scattering has the advantage of providing structural information about proteins in close-to-physiological states. It has been used successfully to model tertiary organization of multi-domain proteins or protein complexes, conformational changes of proteins in different states, and folding–unfolding dynamics (reviewed in [13]). Although the experiment seems straightforward, obtaining high-quality data is essential for trustworthy modelling and requires informed and careful handling of protein samples and experimental setup.

First, aggregate-free protein solutions are required. Tiny amounts of aggregate could overwhelm protein scattering signals from low angles which are critical for correct estimation of a protein's overall size, information crucial for *ab initio* modelling [14]. Ways of removing aggregates vary depending on specific proteins, but generally include using fresh protein samples, careful sample handling, gel filtration, centrifugation and (spin) filtering. DLS (dynamic light scattering) and Guinier plots from SAXS data are sensitive tools to monitor for the presence of aggregates.

Secondly, modelling of SAXS data requires monodisperse solutions of non-interacting particles, a prerequisite for many modelling algorithms which consider the overall scattering equal to that from a single molecule in all possible orientations [14]. Serial dilution of protein samples may be used to find monodisperse conditions by showing that I_0 (forward scattering) is proportional to, and R_G (radius of gyration) is independent of, protein concentration [5,10]. DLS data for such samples should also yield single-species models. A convenient way to do this is to couple gel-filtration chromatography directly with exposure of samples to X-rays (Figure 2). Scattering is measured directly after samples elute from the column. Aggregates are clearly removed in this process. For monodisperse materials, one expects the I_0 across a protein elution peak to precisely track the absorbance profile, and the R_G corresponding to protein size should remain constant across each single elution peak. In our experience, close examination of the R_G shows a slow decrease across the peaks of the eluted proteins. This may indicate the presence of expanded and contracted forms of the molecules that are partially separated during size-exclusion chromatography.

Thirdly, the use of X-rays poses the problem of radiation damage to protein samples, especially at high-energy synchrotron beams. A dosage-dependent increase in R_G is a sensitive marker for radiation damage. Strategies to minimize it include shortening necessary exposure time, flowing samples while exposing them to X-rays and including 5–10 mM reducing agents or a small percentage of glycerol in the buffer [10].

Finally, careful matching of protein samples and control buffers is also important, especially for data from WAXS. This data provides higher-resolution structural information needed for some *ab initio* modelling algorithms [14]. Typically one subtracts scattering of buffer alone from the experimental samples containing protein. The signal-to-noise level is low for high-angle WAXS data, and, before subtraction, one needs to scale scattering from buffer controls to account for partial volume occupancy by protein in the experimental sample. Protein concentration thus needs to be known as precisely as possible, and extensive dialysis, gel filtration or multiple buffer exchange by spin-concentrators usually produces a satisfactory buffer match.

Regulated assembly of EBP AAA+ by two-component signal transduction

Despite fairly high sequence and structural similarities of EBPs whose assembly is controlled by two-component signal transduction receiver domains, the interactions between the receiver and the ATPase domains have evolved divergently into at least two distinct mechanisms. Previous studies had revealed a negative regulation that is typified by the DctD protein of *Sinorhizobium meliloti* and the NtrC1 protein

Figure 2 | **SAXS/WAXS data collection**

Top: experimental setup for SAXS and WAXS experiments. Bottom left: eluate from size-exclusion chromatography is delivered directly to the exposure capillary. Complex formation of protein A and B (B in excess over A) are traced by absorbance at 280 nm (solid line). Scaled forward scattering I_0 (open circles) and R_G (black circles) are superimposed on the elution profile. Bottom right: continuously collected scattering profiles from the gel filtration across buffer and different elution peaks.

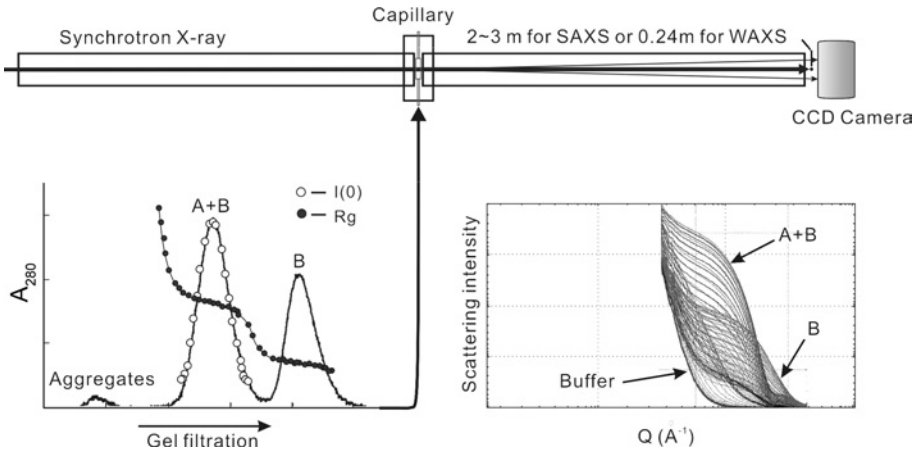
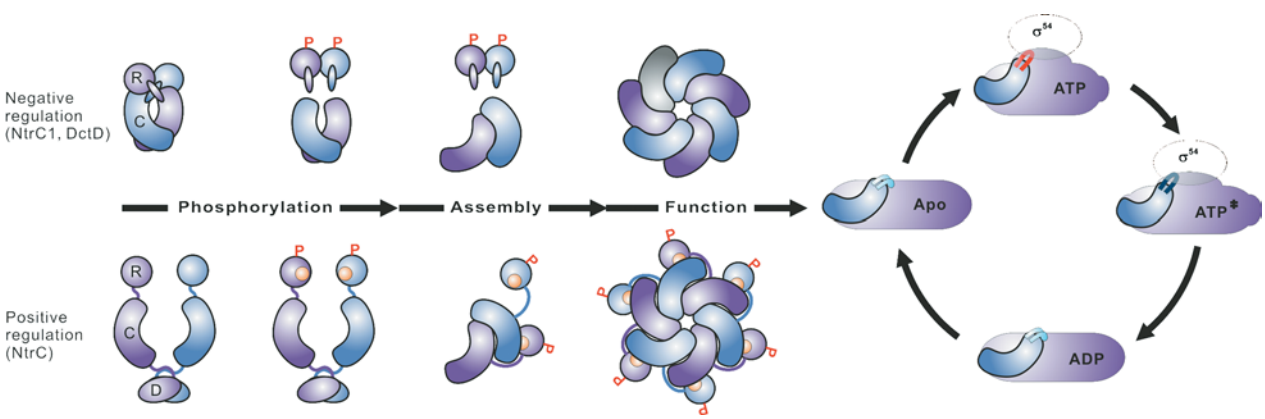


Figure 3 | **Regulated assembly and motor function of EBPs**

Negative regulation of ATPase assembly typified by NtrC1 and DctD (top left) and positive regulation by NtrC (bottom left). The side views of the assembled ATPase ring with an exemplary single subunit show conformations of the GAFTGA loop during the ATP hydrolysis cycle with accompanying binding to σ^{54} (right).



of *Aquifex aeolicus* (Figure 3) [3,6,15,16]. In this case, the unphosphorylated receiver domain forms a homodimer that interacts extensively with the ATPase domain, holding its intrinsically competent ATPase domain in a face-to-face juxtaposition. Phosphorylation stabilizes a reorganization of the receiver domains into a second homodimer that no longer stabilizes the face-to-face orientation of ATPase domains, leaving them capable of entering a more stable back-to-face reorientation that favours ring assembly. Consistent with this model, deletion of the receiver domain generates a constitutively assembled and active ATPase ring, and mutations weakening the repressive interactions lead to proteins that are more readily activated. However, this model fails to explain regulation of the well-studied NtrC protein. Its receiver domain is monomeric, and removing it produces a non-assembling non-functional ATPase [17,18]. Our recent SAXS/WAXS and EM data support a different positive-regulation mechanism for the NtrC protein [5]. The short life-time (~5 min) of phosphorylation was overcome by adding BeF_x (beryllium fluoride) to stably mimic phosphorylation [19], and a genetically engineered variant called NtrC S160F 3Ala from *Salmonella* Typhimurium was used to obtain high concentrations of fully activated protein [20]. The S160F substitution favours the fully assembled ring, and the three alanine (3Ala) substitutions in the DNA-binding domain (R456A, N457A and R461A) increase solubility and eliminate aggregation otherwise seen upon activation. We coupled size-exclusion chromatography to the X-ray beam as illustrated in Figure 2 to capture the scattering from the pure activated ring-form of NtrC, which effectively removed aggregates and

smaller sub-assemblies. P6 symmetry was imposed during *ab initio* modelling, as supported by negatively stained EM images. The averaged low-resolution SAXS structure was used for docking previously solved NMR and crystal structure models of isolated domains [3,21,22]. After the ATPase ring was docked into the central density, it became clear that the six density blobs around the ring periphery are occupied by phosphorylated receiver domain monomers. Previous genetic and biochemical data strongly suggested close juxtaposition of the α4 helix of the receiver domain and the α1 helix of a neighbouring subunit's ATPase domain [21,23]. These restraints and the SAXS-based model resulted in a single orientation that agreed well with previous cross-linking and Fe-BABE (iron chelate of bromoacetamidobenzyl-EDTA) cleavage data [23]. Finally, three copies of the DNA-binding domain dimer were placed into the remaining density beneath the ATPase ring. This model provides a good explanation for previous functional observations of the assembly and action of the NtrC protein. Each phosphorylated receiver domain binds to the periphery of the next subunit's ATPase domain, thus contributing to the stability of the whole assembly. The receiver domain is required for assembly, rather than inhibition, of the ATPase ring.

These two distinct regulation mechanisms are quite convincing, but some questions still remain unanswered. For example, (i) isolated NtrC1 ATPase is a heptamer both in solution and in the available crystal form, but the isolated NtrC1 receiver domain is a dimer. How does the full-length NtrC1 protein solve the apparent stoichiometry conflict? What is the organization of the activated receiver and ATPase domains? Do the receiver domains contribute at all to ring stability or function? (ii) Why does the isolated NtrC ATPase not self-assemble into an active ring like the NtrC1 ATPase? (iii) How does DNA binding or interaction with σ^{54} affect the ATPase assembly, which in some cases must occur with only a handful of ATPase molecules being present per cell.

Large conformational changes in the NtrC1 ATPase cycle

In earlier studies of two additional EBPs, the phage-specific protein PspF and the nitrogen fixation regulator NifA, the Buck group revealed tight binding between ATPase and σ^{54} only in the ATP hydrolysis transition state mimicked by ADP–AlF_x (aluminium fluoride) [11]. An EM structure of the PspF–σ^{54} complex indicated that, in the ADP–AlF_x state, PspF binds to σ^{54} via extended GAFTGA loops [24]. Negatively stained EM structures of NtrC S160F 3Ala also suggested that the GAFTGA loop region becomes ordered in the ADP–AlF_x state [5]. In the structures of PspF ATPase crystals soaked with different nucleotides, the GAFTGA loops were unfortunately not visible [25]. We recently obtained SAXS/WAXS solution structures of the NtrC1 ATPase that for the first time unambiguously reveal movement of the GAFTGA loops region during the ATP cycle. In this study, the isolated NtrC1 ATPase assembled ring is trapped at distinct stages of the ATP hydrolysis cycle using saturating concentrations of different nucleotides or nucleotide analogues, with ATP[S] (adenosine 5′-[γ-thio]triphosphate), p[NH]ppA (adenosine 5′-[β,γ-imido]triphosphate) and ADP–BeF_x as ATP ground-state mimics, ADP–AlF_x as a transition-state mimic, and ADP as product state. Note that ATP[S] and p[NH]ppA had to be freshly re-purified before use. Close comparison of models for the different states showed major conformational changes bearing three distinct features: extension of the GAFTGA region, spiking of the ring periphery, and the iris movement of the central pore region. Unexpectedly, ADP–BeF_x caused a conformational change at the GAFTGA loops region similar to that seen for the ADP–AlF_x state. This inspired us to re-check the role of the ATP ground state of EBPs, which was previously thought not to bind σ^{54}. We found that the NtrC1 ATPase, full-length NtrC and PspF ATPase all formed complexes with σ^{54} in the ADP–BeF_x state, and the integrity of the GAFTGA loop was required for this binding, but not for ATPase hydrolysis activity or conformational changes of the GAFTGA region. However, ADP–BeF_x does not stabilize the ATPase ring assembly or its complexation with σ^{54} as well as ADP–AlF_x. We conclude that ADP–BeF_x and ADP–AlF_x represent distinct ground and transition states for EBP ATPases. We also observed that changing the Walker B catalytic residue Glu^{239} to alanine allows the NtrC1 ATPase to extend the GAFTGA loop region and tightly bind σ^{54} when bound to ATP, which it can no longer hydrolyse. Together, these observations support a model for the EBP ATPase cycle as illustrated in Figure 3. ATP binding prompts an extension of the GAFTGA loop and formation of a relatively loose complex with σ^{54}, which isomerizes into a tighter complex at the transition state for ATP hydrolysis. When P_i is released, the ATPase retracts the GAFTGA loop and releases a remodelled σ^{54} that is competent to melt the promoter region, or that melting happens before release of σ^{54} from the ATPase.

Interestingly, the three ATP ground-state mimics led to different conformational changes, and although ADP–BeF_x supported complex formation with σ^{54} for all three EBPs, p[NH]ppA did so only for the NtrC1 ATPase and ATP[S] did not do so for any of them. We suggest that the active sites are sensitive to the exact chemical environment of the nucleotide γ-phosphate position, enabling them to distinguish between these analogues [26]. Specific analogues may only capture one or more of the sub-populations of the low-energy 'ground' state, only some of which show conformational changes in the GAFTGA region that enable binding to σ^{54}.

Conclusions

The improved SAXS/WAXS technique, combined with high-resolution structures and biochemical data, has advanced our understanding of regulated assembly and action of the EBP ATPase. Two distinct mechanisms, one a de-repression and the other an active facilitation, are used by similar two-component receiver domains to regulate the assembly of similar AAA+ proteins. The assembled ATPase ring undergoes major conformational changes when hydrolysing ATP,

binding to σ^{54} via the extended GAFTGA loops, first loosely at the ground state, and then more tightly at the transition state. The tension at the transition state is used in an uncharacterized way to remodel the σ^{54}–RNAP–promoter complex so that it can advance from closed to open form and start transcription.

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