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Receiver domains control the active state stoichiometry of *Aquifex aeolicus* σ^{54} activator NtrC4, as revealed by electrospray mass spectrometry

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Abstract

A common challenge with studies of proteins *in vitro* is determining which constructs and conditions are most physiologically relevant. σ^{54} activators are proteins that undergo regulated assembly to form an active ATPase ring that enables transcription by σ^{54} -polymerase. Previous studies of the AAA+ ATPase domains from σ^{54} activators have shown that some are heptamers, while others are hexamers. Because the active oligomers assemble from off-state dimers, it was thought that even-numbered oligomers should dominate, and that heptamer formation might occur when individual domains of the activators were studied rather than the intact proteins. Here we present results from electrospray mass spectrometry experiments that characterize the assembly states of intact NtrC4 (a σ^{54} activator from *Aquifex aeolicus*, an extreme thermophile) as well as its ATPase domain alone, and regulatory-ATPase and ATPase-DNA binding domain combinations. We show that the full-length and activated regulatory-ATPase proteins form hexamers, whereas the isolated ATPase domain, unactivated regulatory-ATPase and ATPase-DNA binding domain form heptamers. Activation of the N-terminal regulatory domain is the key factor stabilizing the hexamer form of the ATPase, relative to the heptamer.

Introduction

Bacterial RNA polymerases incorporate a subunit, termed the sigma factor, which is centrally involved in recognizing and binding to the correct promoters, and then opening DNA to initiate transcription.^{1; 2} There are two distinct classes of bacterial sigma factors, σ^{70} and σ^{54} , designated with the size of the founding members of the classes.³ σ^{70} and σ^{54} have very similar functional roles, but share no significant sequence homology. σ^{54} dependent transcription occurs only for a small percentage of genes, often controlling metabolic processes, such as the nitrogen and sulfate limitation responses^{4; 5; 6} and quorum sensing.⁷ Promoters under σ^{54} control also regulate genes involved in virulence and other host interactions.^{8; 9; 10} A key distinction between the two classes of sigma factors is that to reach the transcriptionally

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competent, open promoter state, σ^{54} requires the action of an activator protein, whereas σ^{70} is self-sufficient.¹¹ σ^{54} activators contact the σ^{54} RNA polymerase holoenzyme and couple ATP hydrolysis to a conformational change that enables transcriptional initiation.^{12; 13}

σ^{54} activators are typically composed of an N-terminal regulatory domain, a central AAA+ ATPase domain, and a C-terminal DNA binding domain. They bind DNA as ATPase-inactive dimers until they receive a cellular signal, whereupon they assemble into oligomeric rings that are active ATPases.¹⁴ The catalytic site for ATP hydrolysis is formed between subunits in the active oligomeric state. The N-terminal regulatory domain receives a cellular signal and serves as an allosteric switch to convert the protein from its inactive dimeric state to its active oligomeric state.¹⁵ Receiver domains are the most commonly observed type of regulatory domain and are activated by phosphorylation of a conserved aspartate residue by a histidine kinase.^{16; 17} All of the NtrC family proteins discussed here are of this type. Other σ^{54} activators use single or multiple regulatory domains¹⁵ such as GAF,^{18; 19} PAS,²⁰ FleQ,²¹ ACT,^{22; 23} or CBS²⁴ domains. The central AAA+ domain of σ^{54} activators forms an active oligomer, which contacts the σ^{54} RNA polymerase holoenzyme via a highly conserved GAFTGA sequence and uses the energy of ATP hydrolysis to change the conformation of the polymerase-promoter complex, after which initiation can occur.^{25; 26} Almost all σ^{54} activators also have a C-terminal DNA binding domain which targets the protein to specific promoter sequences 100–150 basepairs upstream of the targeted σ^{54} -Pol transcribed genes.¹⁵

Oligomeric σ^{54} activators have been found both as hexamers and heptamers.^{26; 27; 28; 29} The first structure determined of a AAA+ ATPase domain from a σ^{54} activator was that of *Aquifex aeolicus* NtrC1, which crystallized as a heptamer,²⁷ shown in Figure 1. However, subsequent structures of the isolated ATPase domain of *Escherichia coli* PspF²⁶ and the ATPase-DNA binding domain combination from *Salmonella enterica* var *Typhimurium* ZraR²⁹ showed that they form hexamers. Electron microscopy showed that the full length *S. enterica* NtrC is also a hexamer.²⁸ Most of the proteins studied were truncation mutants rather than intact proteins, and in other AAA+ systems truncation has been found to change the oligomerization state, leaving the assembly stoichiometry for most σ^{54} activators unclear.^{30; 31; 32}

S. enterica NtrC and *A. aeolicus* NtrC1 and NtrC4 are homologs, all composed of receiver, ATPase and DNA binding domains. However, previous structural studies have shown that their regulatory mechanisms are significantly different.^{28; 33; 34; 35} For NtrC, upon phosphorylation the receiver domain gains an interaction with a neighboring ATPase domain, stabilizing the oligomeric form.^{28; 35} For NtrC1 and NtrC4, phosphorylation or BeF_3^- - Mg^{2+} activation³⁶ of receiver domains causes a change in a dimer interface that releases them from a docked position that represses assembly of the ATPase domains.^{33; 34} For both NtrC and NtrC4, the DNA binding domains have been shown to be stable dimers with a helical fold and helix-turn-helix motifs^{33; 37} (Figure 1).

Electrospray ionization mass spectrometry (ESI-MS) has become an important tool for studying noncovalent complexes of biological molecules.^{38; 39; 40; 41; 42; 43; 44} Information about complex quaternary structure,³⁹ ligand binding,⁴⁰ assembly kinetics,⁴¹ and thermodynamics⁴² of biomolecular assemblies can be obtained using ESI-MS. The stoichiometries of non-covalent complexes can be determined with high sensitivity even for mixtures.⁴³ For example, Robinson and coworkers distinguished a 435 kDa subcomplex from a 438 kDa subcomplex of the eukaryotic translation factor eIF3, using less than 2 μg of protein.⁴⁴ Information about protein⁴⁵ and protein complex⁴⁶ conformations can be obtained from the charge state distributions observed in ESI mass spectra, where unfolded forms of a protein or protein subunits within a complex typically lead to higher charge states and more compact conformers take on lower charge states. An advantage of ESI-MS is that multiple conformers of a single analyte can be resolved from the resulting charge state distributions,⁴⁷ whereas

techniques such as circular dichroism and steady state fluorimetry reflect ensemble averages.
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Metal ion salts, such as sodium chloride or phosphate, that are frequently used to stabilize biomolecules in solution can adduct to complexes during ESI, leading to broad mass spectral peaks that are shifted to higher m/z values. This causes the measured molecular weight of a complex to be higher than that calculated for the bare complex when the masses of the constituent subunits are known.^{49; 50; 51; 52} Because of this adduction and because of the difficulty of obtaining signal from solutions containing these salts, volatile buffers, such as ammonium acetate or bicarbonate are often used. For complexes that require the presence of non-volatile salts, such as the ones studied here, “buffer loading”⁵³ with relatively high concentrations of ammonium acetate may be used to minimize the effects of salt adduction. For example, increasing the ammonium acetate concentration from 250 mM up to 1 M was shown to be effective for obtaining well-resolved mass spectra of the ~150 kDa tetramer of alcohol dehydrogenase in the presence of 10 mM Tris-HCl or HEPES.⁵⁴

To resolve how the different domains of the σ^{54} activators affect assembly, we used ESI-MS with “buffer loading” to determine the active state stoichiometry of full length *A. aeolicus* NtrC4, its central AAA+ ATPase domain alone, the receiver-ATPase domain combination, and the ATPase-DNA binding domain combination. We find that BeF_3^- activated (mimicking phosphorylated) full length NtrC4 and receiver-ATPase domains form hexamers, whereas constructs lacking the N-terminal receiver domain are mixed oligomers. Our results strongly indicate that the physiological active states of σ^{54} activators are hexamers.

Results

For convenience, we will refer to domains of NtrC family proteins using the following abbreviations: R domain for the N-terminal regulatory receiver domain, C domain for the central ATPase domain, and D domain for the C-terminal DNA-binding domain. In order to obtain information about the stoichiometry of these complexes, ESI mass spectra of solutions of NtrC4-RCD, NtrC4-RC, NtrC4-C and NtrC4-CD constructs were obtained and are shown in Figures 2–4. The stoichiometries of the higher-order complexes are the primary interest of these experiments, and the relevant experimental and calculated values for the dominant higher-order complexes of each construct are given in Table 1. The measured molecular weights are on average 0.65% higher than the expected molecular weights for these complexes. Correcting the measured masses for adduction using the method of Robinson and coworkers⁵⁵ results in an average deviation between the corrected experimental molecular weights and the expected molecular weights of -0.26%, suggesting a slight overcorrection. From these molecular weight measurements, the stoichiometries of the complexes can be unambiguously determined (Table 1).

ESI mass spectra of activated and unactivated NtrC4-RCD obtained from heated solutions (62 °C) are shown in Figure 2. The conditions used to acquire the two spectra were identical except for the presence of activating concentrations of MgCl_2 , BeF_3^- , and ADP in the solution used to acquire the spectrum shown in (b). In both spectra, two charge state distributions (labeled (1) and (2), Figure 2) corresponding to the mass of a dimer are observed. The two distinct distributions indicate that there are at least two conformations, or families of conformers, of the dimer, corresponding to a more open (1) and a more compact (2) form. A tetramer is also observed with low abundance in both spectra. In contrast, a hexamer is observed only in the activated solution. No signal for monomer or other higher-order oligomers were observed in either spectrum. At lower temperature (29 °C), the spectrum of the unactivated protein did not exhibit tetramer while the activated spectrum did exhibit tetramer and hexamer, but with much lower signal-to-noise (data not shown). A subtle increase in the average charge state of Dimer

1 was observed for unactivated (30.0+ to 31.3+) and activated (32.6+ to 34.8+) protein upon heating from 29 to 62 °C, whereas the average charge state of Dimer 2 remained essentially unchanged for unactivated (23.1+ to 23.2+) and activated (23.1+ to 23.4+) protein. This indicates that Dimer 1 undergoes a wider range of thermally driven conformational fluctuations than Dimer 2, allowing it to accommodate the increased charge.

Mass spectra of NtrC4-RC from unactivated and activated solutions are shown in Figure 3a and 3b, respectively. In the absence of MgCl_2 , BeF_3^- , and ADP, and at 29 °C, heptamer is the predominant higher-order oligomer in the mass spectrum of the RC construct (Figure 3a); signal for monomer, dimer, trimer, tetramer, and octamer is also observed. The unresolved peaks on the high mass side of the monomer charge state distribution are consistent with a single ADP molecule bound to the protein. Because ADP was not added to the purified protein solution before electrospray, it was likely carried through during protein purification. The mass of the low intensity distribution of peaks that overlaps with the heptamer series corresponds to an octamer. To determine if the heptamer would dissociate and reassemble to a hexamer at elevated temperature, mass spectra were continuously acquired while the solution was slowly heated from 29 to 74 °C. The only change observed in any of the spectra was a loss of signal intensity for the oligomers at 74 °C (data not shown). Similar to the results for activated NtrC4-RCD, the activated NtrC4-RC protein assembles to a hexamer upon heating the solution to 62 °C (Figure 3b) and exhibits no oligomers larger than dimer without increasing the solution temperature to ~60 °C. A shift to higher average charge for the monomer and dimer is observed as a result of activation and does not change significantly upon heating from 29 to 62 °C (data not shown). In contrast to NtrC4-RCD, monomer is the dominant component in the spectra of both the unactivated and activated solutions. A more quantitative comparison of relative concentrations of species present is complicated by differential ionization and detection efficiencies of the different oligomers. No significant change in the relative abundances of monomer and dimer was observed upon activation or heating.

Like NtrC4-RC, NtrC4-C predominantly forms a heptamer from a 29 °C solution that does not contain MgCl_2 , BeF_3^- , and ADP, and monomer, dimer, trimer, and hexamer are also observed (Figure 4a). Addition of MgCl_2 , BeF_3^- , and ADP had no effect on the observed protein oligomers or the heptamer to hexamer ratio (data not shown). ADP was not added to the solution so the ADP adducts to the monomer and dimer are likely formed during protein expression. There are two distinct charge state distributions of the heptamer, with average charge states of 39.1+ and 34.6+, and these distributions occur at lower m/z (~5250 to 6800) than the distribution of the hexamer (~7000 to 10,000) for which the average charge state is 23.3+. This is unusual because the m/z of noncovalent complexes typically increases with increasing oligomerization state. To verify that the low charge state hexamer originates from solution and is not formed by gas-phase dissociation of the heptamer into a hexamer and more highly charged monomer in the mass spectrometer, tandem mass spectrometry, via collisionally activated dissociation (CAD) with argon, was performed on the heptamer 34+ ion, roughly corresponding to the weighted average charge (34.7+) of the individual monomer (11.4+) and hexamer (23.3+). With the same instrument conditions used to acquire the mass spectrum in Figure 4a, no appreciable dissociation of the 34+ heptamer ion was observed (Figure 5a). Increasing the collision energy results in the expected dissociation into a hexamer and monomer with average charge states of 13.8+ and 20.3+, respectively, as a result of asymmetric charge partitioning^{56; 57} (Figure 5c). The CAD experiment was also performed with all of the ions in the lower charge state distribution of the heptamer, and the results were effectively identical with average charge states of 13.5+ and 21.8+, for the hexamer and monomer, respectively (data not shown). The much lower average charge state of the hexamer produced by CAD of the heptamer is clear evidence that the hexamer present in the mass spectrum in Figure 4a originates from the solution and is not formed by gas-phase dissociation of the heptamer. The bimodal distribution of charge states for the heptamer corresponds to two different conformers,

or families of conformers, that have more open forms and/or greater solvent exposed surface area than the hexamer (Figure 4a). The relatively broad charge state distribution for the hexamer suggests that it adopts a wider range of conformers, possibly due to non-specific aggregation owing to the relatively high concentration of the protein solution (500 μ M) prior to dilution for ESI-MS. Slow heating of this solution resulted only in a loss of signal intensity for the higher-order oligomers (data not shown).

The ESI mass spectrum of NtrC4-CD (Figure 4b) from a 29 °C solution that did not contain MgCl_2 , BeF_3^- , and ADP, shows predominantly dimer and two distinct charge state distributions of the monomer with average charges states of 13.0+ and 7.2+, consistent with more open and more compact forms, respectively. In addition, several higher-order oligomers are observed, including a tetramer, heptamer, octamer, and a tetradecamer. In contrast to NtrC4-RC and NtrC4-C, no adduction of ADP is observed. There is only one charge state distribution for each of the higher-order oligomers and the average charge of these distributions increases with increasing oligomerization state. As occurred for unactivated NtrC4-RC and -C, slow heating of this solution resulted only in loss of signal intensity for the higher-order oligomers, and like NtrC4-C, addition of MgCl_2 , BeF_3^- , and ADP did not change the observed stoichiometries (data not shown).

Discussion

Obtaining a crystal structure of the *A. aeolicus* NtrC1 central AAA+ domain was an important step towards understanding σ^{54} activator proteins.²⁷ However, NtrC1-C crystallized as a heptamer, and this odd number of subunits is not congruent with the receiver domains which are dimeric in both inactive and active states^{27; 34} (Figure 1). We have shown that the DNA binding domain of NtrC1 binds DNA as a dimer, but dimerizes only weakly as an independent domain [Maris and Wemmer, unpublished]. Studying the assembly of full length NtrC1 has not been feasible because it is difficult to activate quantitatively either by phosphorylation or by BeF_3^- addition. In our subsequent studies of NtrC1 homolog NtrC4 from the same organism, we found an inactive dimer for NtrC4-RC which was quite similar to that of NtrC1, although with a smaller and less ordered interface between the R and C domains.³³ Unlike NtrC1, the DNA binding domain of NtrC4 is a very stable dimer. We had anticipated that NtrC4-CD, which forms stable dimers through the DNA binding domains, might assemble into hexamers but EM studies indicated predominant formation of heptameric rings.³³ Because other studied σ^{54} activators form hexamers, we felt it was necessary to study full length NtrC4 to determine the stoichiometry of the active oligomeric form. To enable us to determine which other parts affect assembly, we studied the four ATPase domain containing constructs: NtrC4-C, NtrC4-RC, NtrC4-CD, and NtrC4-RCD.

Interactions of the N-terminal receiver domain, but not the C-terminal DNA binding domain stabilizes the hexamer, relative to the heptamer

The ESI-MS data show that oligomeric NtrC4-C is predominantly heptamer, consistent with the crystallographic studies of the homolog NtrC1, indicating that it is the most stable assembly for the AAA+ domain alone from these thermophilic proteins. A small amount of hexamer is also observed in the ESI mass spectrum, which is consistent with observation of a small number of hexamer rings seen previously in EM data for NtrC1-C.⁵⁸ The changes in structure required for packing into a hexamer ring rather than heptamer are relatively subtle, and there is a relatively small energy difference between these oligomeric states.

For activated, full length NtrC4-RCD, the dominant oligomer is the hexamer, with only a small amount of tetramer seen in addition to dimers. The low tetramer abundance is consistent with the fact that interaction of two dimers is through one inter-subunit interaction, while completion of the hexamer adds two equivalent interactions energetically favoring ring closure. This result

indicates that indeed the domains flanking the AAA+ influence the assembly stoichiometry, and that in cells, these thermophilic σ^{54} activators very likely assemble to the same hexamer state as the previously characterized mesophilic forms, NtrC²⁸, PspF²⁶ and ZraR.²⁹

Comparison of our ESI-MS results for NtrC4-RC and NtrC4-CD show clearly that the activated receiver domain is predominantly responsible for stabilizing the hexamer state, relative to the heptamer state. These results are consistent with previous observations about NtrC4. The regulatory receiver domains of NtrC4 behave somewhat differently than those of NtrC1. In the crystal structure of unactivated NtrC4-RC, the receiver domains form a dimer with a smaller interface than those of NtrC1, and lack the coiled coil extension.^{27; 33} The interface between the receiver domains and the dimer AAA+ domains is also much less well ordered than the equivalent region of NtrC1, consistent with the fact that NtrC4 is much easier to activate than NtrC1. The NtrC4 receiver domain, as an isolated domain, is a monomer, but forms a stable dimer upon BeF_3^- - Mg^{2+} activation.³³ Consistent with only weak interactions between unactivated receiver domains, the ESI-MS data for unactivated NtrC4-RC show a distribution of oligomers, but completed rings dominated by heptamer. However, when activated by BeF_3^- - Mg^{2+} addition, the only oligomer above a dimer observed is hexamer. This result shows that the active state receiver domain interactions are sufficient to stabilize the hexamer state, relative to the heptamer state.

The behavior of the NtrC4-CD construct shows that dimerization of the DNA binding domains is not sufficient to drive hexamer formation. The NtrC4-CD domain is a stable dimer, but the ESI-MS data show that it forms tetramer, heptamer, octamer and tetradecamer oligomers rather than hexamers that NtrC4-RC forms. The stability of the NtrC4-CD dimer is supported by the low abundance of the monomer relative to that in the spectrum of NtrC4-RC. The presence of the tetradecamer indicates that the ATPase ring has seven subunits, with one dimer bridging to a second heptamer ring, and suggests that the octamer may also be a heptameric ring with one excluded ATPase subunit on the outside. If the two ATPase rings of the tetradecamer stack on each other, and preferentially lay flat on EM grids (as seen for single rings), then they would not be apparent in the EM data. Single dangling ATPase domains might also be difficult to visualize.

Why do interactions of the N-terminal receiver domain, but not the C-terminal DNA binding domain, affect NtrC4's assembly? This may arise from differences in the two linker regions. In the NtrC4-RC crystal structure, the unstructured R-C linker extends from K122 to E135 and is 14 residues long.³³ Judging from structured regions in the NtrC4-RC and the NtrC4-D crystal structures, the unstructured C-D linker extends from C362 to E381 and is 20 residues long.³³ The shorter R-C linker, and the difference in attachment points to the ATPase domain, may lead to a stronger constraint from the receiver domain than the DNA binding domain leading to a preference for hexamer. In EM structures of *S. enterica* NtrC,²⁸ the DNA binding domains are positioned below the AAA+ ring, and activated monomeric receiver domains make specific contacts with residues on the sides of the AAA+ ring. Although the dimeric NtrC4's receiver domains cannot bind in an analogous manner (the surface involved in R to C interactions in NtrC are occluded by dimer formation in NtrC4-R), it is possible that they may make other specific contacts which help to stabilize the hexamer state for the AAA+ domain.

The alternate NtrC4-RCD dimer charge state distribution may represent an on-pathway assembly intermediate

We have identified two distinct charge state distributions for the NtrC4-RCD which correspond to a more open (1) and a more compact (2) form (Figure 2). Although we can not directly determine the nature of these alternate dimer conformations, we speculate that the more prevalent charge state distribution (2) corresponds to the previously structurally characterized front-to-front off-state dimer, shown in Figure 1, and the alternate charge state distribution (1)

corresponds to a front-to-back dimer with ATPase domain contacts similar to those seen in the active hexamer. We expect the front-to-back alternate dimer to have more exposed surface area, and thus the observed higher average charge, because this front-to back dimer conformation would be unable to form a stable R-C contact surface, exposing more of the R-C interface. This alternate dimer is not observed in NtrC4-RC, possibly because the alternate NtrC4-RCD dimer has a weaker dimer interface in the R-C region and dimerizes primarily through the DNA binding domain, which is not present in NtrC4-RC. At some point during hexamer assembly, the central domain must transition from off-state front-to-front contacts to on-state front-to-back contacts, and we may be directly observing both of these dimer states in our ESI-MS data.

The physiological active state of σ^{54} activators is likely a hexamer

Although the difficulty of activating NtrC1 makes direct characterization of its active oligomer impossible at this point, the overall similarity between it and NtrC4 suggests strongly that a full length activated construct of NtrC1 is also hexameric. The three other structurally characterized σ^{54} activators, specifically *S. enterica* NtrC, *E. coli* PspF and *S. enterica* ZraR, have all been shown to be hexamers in their active states.^{26; 28; 29} Because NtrC requires interactions of an R domain with a neighboring C domain to oligomerize,^{28; 35} it is not possible to study the state of the NtrC-C domain on its own. The PspF ATPase domain alone was found to form hexameric rings.²⁶ PspF is somewhat different from the others in that it lacks a regulatory domain, functioning as the equivalent of NtrC4-CD. The AAA+ domain is highly conserved, but there must be differences that lead to the preference for PspF-C to form hexamers, whereas NtrC1 and NtrC4 form heptamers. The complete ZraR does have a receiver domain, but the crystallized construct corresponded to ZraR-CD.²⁹ In this protein, the C-domain forms a hexameric ring, and the D domain forms a dimer that is relatively far from the ring. This behavior is quite analogous to PspF (though the DNA binding domains of PspF are not yet structurally characterized). Thus, to date, all σ^{54} activators are hexamers in their physiologically relevant forms, even when the ATPase domain alone prefers a heptameric state. This makes it seem very likely that all σ^{54} activators will have hexameric active states.

Heptamer assemblies of AAA+ proteins are prevalent, but in all cases the physiological state is likely a hexamer

Variable stoichiometry assemblies have been found for a variety of other AAA+-domain containing proteins. Heptameric rings were observed with McrB,⁵⁹ MCM,^{60; 61; 62} chelatase,⁶³ RuvB,⁶⁴ Lon,⁶⁵ ClpB,⁶⁶ HslU,⁶⁷ T7 primase,^{68; 69} p97's C-terminal domain,³⁰ and the Vps4P.^{31; 70} Hexamers were also observed for each of these proteins except McrB, which has been less completely studied.^{30; 59; 60; 61; 62; 64; 66; 68; 69; 70; 71; 72; 73} MCM and RuvB bind DNA in the central pore of the AAA+ ring. They are heptamers in the absence of DNA, but become hexamers when it is present.^{60; 61; 62; 64; 74} The initial heptamer state may facilitate loading of DNA. T7 primase is a heptamer in the presence of TDP but a hexamer in the presence of TTP.^{68; 69} Similarly, ClpB is a heptamer when no nucleotide is present, but shifts to a hexamer when ADP or ATP binds.⁶⁶ NtrC4³³, NtrC1²⁷, HslU⁶⁷, p97's C-terminal domain³⁰, and Vps4P^{31; 70} have heptameric AAA+ domains which are hexamers when expressed as part of the intact protein or, in the case of Vps4P, in the presence of its physiological cofactor Vta1P. Hexamer and heptamer rings have been observed in Lon and chelatase proteins from organisms in different kingdoms or phyla, respectively. The high frequency of observing variable oligomerization indicates a small energy difference between these assemblies. While there are cases in which both forms seem to be functionally significant, it appears more common that truncations of the proteins change interactions and constraints and lead to non-native assemblies. Some of these retain activity, and it remains to be determined whether they have altered catalytic or substrate binding properties. As such, it is important to understand the oligomerization determinants using analytical methods such as ESI-MS.

Materials and Methods

Protein expression and purification

NtrC4-RCD (residues 1–442), NtrC4-RC (residues 1–372), and NtrC4-CD (residues 131–442) were subcloned into a PSKB3 plasmid with a His₆ tag. NtrC4-C (residues 131–376) was subcloned into a pET21b3-2 plasmid (Novagen) with a His₆ tag which was not cleaved during protein purification. All proteins were expressed in *E. coli* BL21 (DE3) with a Rosetta.pLysS plasmid using Studier's auto induction protocol.⁷⁵ Cells were resuspended in 500mM ammonium acetate, pH 8.2, and 0.1mM PMSF with one Roche Complete EDTA-free antiproteolysis tablet. Cells were harvested by sonication and lysates were heated for 30 minutes at 70 °C. Lysate was spun at 30,000 r.p.m. Supernatant was run over a Ni-agarose column, eluted with 300 mM imidazole and then dialyzed overnight into 500mM ammonium acetate, pH 8.2.

Mass Spectrometry

Mass spectra of the protein complexes were acquired using a quadrupole time-of-flight (Q-ToF) mass spectrometer equipped with a Z-spray ion source (Q-ToF Premier™, Waters, Milford, MA). Ions were formed using nanoelectrospray emitters prepared by pulling borosilicate capillaries (1.0 mm o.d./0.78 mm i.d., Sutter Instruments, Novato CA) to a tip with an inner diameter of ~1 μm with a Flaming/Brown micropipette puller (Model P-87, Sutter). Solutions were prepared immediately prior to mass measurement with the following component concentrations: 100 μM protein; 1 mM ADP; 2 mM BeF₃⁻, 5 mM MgCl₂; and 1 M ammonium acetate, for NtrC4-RCD and -RC. The solutions of heptameric NtrC4-RC, -C, and -CD were prepared at ~25–60 μM protein in 200 mM–2M ammonium acetate, where the lowest concentration of ammonium acetate that gave stable ion current was used. A platinum wire (0.127 mm diameter, Sigma, St. Louis, MO) was inserted through the capillary into the solution and electrospray was initiated and maintained by applying 1–1.3 kV to the wire relative to instrument ground. The nanoelectrospray capillaries were resistively heated with NiCr wire wrapped around a cylindrical aluminum collar that is solid except for a small hole through which the capillary is held and the temperature was monitored continuously with a thermocouple and temperature meter (Omega, Stamford, CT).⁷⁶ Heated samples were held at elevated temperature for a minimum of five minutes before acquiring data. Raw data was smoothed three times using the Waters MassLynx™ software mean smoothing algorithm with a window of 50 *m/z* units for all spectra. The instrument was calibrated with CsI clusters formed by ESI of a 24 mg/mL solution of CsI in 70:30 Milli-Q:2-propanol prior to mass measurement. To obtain average molecular weights of the complexes, we used the method of Robinson and coworkers,⁵⁵ where the charge state of the most abundant peak in each distribution was initially assigned a value based on the *m/z* difference between it and the two adjacent peaks in the distribution. That value was incremented by ±3 to give seven possible charge state assignments from which the molecular weights and deviations in molecular weights were calculated. The molecular weight of each complex was assigned from the charge state distribution that resulted in the smallest standard deviation. Corrected molecular weights of the complexes were calculated using the method of Robinson and coworkers⁵⁵ in which the widths of the most intense peaks in a given charge state distribution were averaged and used to estimate the extent of adduction:

$$\text{adduction (\%)} = 160 * \text{fwhm} / (m/z) - 0.03$$

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Figure 1. NtrC4 and NtrC1 structures

σ^{54} activators exist as inactive dimers and active hexamers and heptamers. NtrC4 has dimeric DNA binding domains and dimeric activated receiver domains and NtrC1 crystallized in a heptameric active state, while PspF and ZraR crystallized as hexamers. PDB IDs **3DZD** (1), **3E7L** (2), **2JRL** (3), **1NY6** (4), and **2VII** (5).

Figure 2. Mass spectra of (a) unactivated and (b) activated NtrC4-RCD
In the presence of $MgCl_2$, BeF_3^- , and ADP, and heated to $\sim 62^\circ C$, the NtrC4-RCD protein forms hexamers. The tetramer observed in both spectra may be a non-specific aggregate of the dimer.

Figure 3. Mass spectra of (a) unactivated and (b) activated NtrC4-RC
 Multiple higher-order oligomers are seen for unactivated NtrC4-RC, while only hexamer is observed when the protein is activated with MgCl_2 , BeF_3^- , and ADP, and heated to $\sim 62^\circ\text{C}$.

Figure 4. Mass spectra of (a) NtrC4-C and (b) NtrC4-CD

Both the NtrC4-C and -CD constructs assemble to multiple oligomers at 29 °C without activation. Increasing the solution temperature results only in a decrease in signal-to-noise for the higher-order oligomers. Adding $MgCl_2$, BeF_3^- , and ADP to the solutions did not change the observed stoichiometries.

Figure 5.
Tandem mass spectra resulting from collisionally activated dissociation (argon target gas) of the 34+ ion isolated from the heptamer charge state distribution as a function of (a) low (30 V), (b) intermediate (50 V), and (c) higher (70 V) acceleration voltage.

Table 1
Table of measured and corrected molecular weights for the higher-order oligomers of the three NtrC4 constructs. The expected molecular weights were calculated with the measured monomer molecular weights (RC, C, & CD) or the measured dimer molecular weight (RCD).

	Measured MW (Da)	Avg. Peak Width (m/z)	Corrected MW (Da)	Expected MW (Da)	Deviation (%)
RCD-hexamer	328,897 ± 49	75	324,260 ± 48	324,060	0.06
RC-hexamer	275,217 ± 121	67	271,492 ± 119	272,166	-0.25
RC-heptamer	315,751 ± 81	48	312,879 ± 81	314,139	-0.41
C-heptamer	218,802 ± 53	52	215,993 ± 52	216,461	-0.22
CD-heptamer	273,030 ± 22	23	271,647 ± 22	272,685	-0.39
CD-octamer	312,107 ± 61	23	310,662 ± 22	311,960	-0.42
CD-tetradecamer	546,214 ± 108	21	544,435 ± 108	545,370	-0.17
			average		-0.26