Allosteric Activation of Bacterial Swi2/Snf2 (Switch/Sucrose Non-fermentable) Protein RapA by RNA Polymerase

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Members of the Swi2/Snf2 (switch/sucrose non-fermentable) family depend on their ATPase activity to mobilize nucleic acid-protein complexes for gene expression. In bacteria, RapA is an RNA polymerase (RNAP)-associated Swi2/Snf2 protein that mediates RNAP recycling during transcription. It is known that the ATPase activity of RapA is stimulated by its interaction with RNAP. It is not known, however, how the RapA-RNAP interaction activates the enzyme. Previously, we determined the crystal structure of RapA. The structure revealed the dynamic nature of its N-terminal domain (Ntd), which prompted us to elucidate the solution structure and activity of both the full-length protein and its Ntd-truncated mutant (RapAΔN). Here, we report the ATPase activity of RapA and RapAΔN in the absence or presence of RNAP and the solution structures of RapA and RapAΔN either ligand-free or in complex with RNAP. Determined by small-angle x-ray scattering, the solution structures reveal a new conformation of RapA, define the binding mode and binding site of RapA on RNAP, and show that the binding sites of RapA and σ70 on the surface of RNAP largely overlap. We conclude that the ATPase activity of RapA is inhibited by its Ntd but stimulated by RNAP in an allosteric fashion and that the conformational changes of RapA and its interaction with RNAP are essential for RNAP recycling. These and previous findings outline the functional cycle of RapA, which increases our understanding of the mechanism and regulation of Swi2/Snf2 proteins in general and of RapA in particular. The new structural information also leads to a hypothetical model of RapA in complex with RNAP immobilized during transcription.

Background: During transcription, RapA recycles RNA polymerase (RNAP) using its ATPase activity.

Results: The binding mode and binding site of RapA on RNAP are characterized.

Conclusion: The ATPase activity of RapA is inhibited by its N-terminal domain but stimulated by RNAP in an allosteric fashion.

Significance: Conformational changes of RapA and its interaction with RNAP are essential for RNAP recycling.

The Swi2/Snf2 (switch/sucrose non-fermentable) proteins mediate mobilization of nucleic acid-protein complexes during replication, transcription, DNA repair, recombination, and RNAP recycling (1, 2). As the only Swi2/Snf2 protein in bacteria, RapA is an RNAP-associated protein with ATPase activity (3, 4). After one or few rounds of transcription in vitro, RNAP becomes sequestered or immobilized in an undefined postranscription/posttermination complex (PTC), and RapA releases RNAP from the PTC for transcription reinitiation (5–7). As a Swi2/Snf2 protein, RapA uses its ATPase activity to remodel the PTC (8). However, the mechanism of RapA action and, particularly, how its ATPase activity is regulated is largely unknown.

In bacteria, initiation of transcription requires a family of σ factors; in Escherichia coli the major sigma factor is σ70 that binds to the core enzyme (Core) to form the holo enzyme (Holo) of RNAP (9). As abundant as σ70 in the cell, RapA is consistently co-purified with RNAP (3, 7). Using an in vitro competition assay, we have previously shown that RapA binds to the Core but is readily displaceable by σ70 (7). Therefore, it is plausible that RapA and σ70 either share a common binding site or have partially overlapping binding sites on RNAP.

The crystal structure of RapA (Protein Data Bank (PDB) ID 3DMQ) reveals that the protein contains seven domains: an N-terminal domain (Ntd), two RecA-like domains 1A and 2A, two Swi2/Snf2-specific domains 1B and 2B, a Spacer domain, and a C-terminal domain. Domains 1A, 2A, 1B, and 2B form the Swi2/Snf2-specific ATPase module of the protein. There are two molecules in the asymmetric unit. Of the two independent molecules, the relative positioning of the Ntd with respect to

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The abbreviations used are: Swi2/Snf2, switch/sucrose non-fermentable; Core, the core enzyme of RNA polymerase; Ec, E. coli; Holo, the holo enzyme of RNA polymerase; Ntd, N-terminal domain; PDDF, pair distance distribution function; PTC, postranscription/posttermination complex; RapAΔN, Ntd-truncated RapA mutant; RNAP, RNA polymerase; SAXS, small-angle x-ray scattering; T1TEC, T. thermophilus ternary elongation complex; TIC, transcription initiation complex; Tt, T. thermophilus; radius of gyration; TtTIC, T. thermophilus transcription initiation complex; DSS, disuccinimidyl suberate; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

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the rest of RapA is different, indicating the dynamic nature of the Ntd association with the rest of the protein (7).

In this study we engineered an Ntd-truncated RapA (RapAΔN), tested its Core binding and ATPase activity, and compared the results to those of the full-length protein. Using small-angle x-ray scattering (SAXS), we have also determined the solution structures of RapA and RapAΔN both standing alone and in complex with the Core. These results provide new insights into the regulation of the ATPase activity of RapA, shedding light on the mechanism of RapA-facilitated RNAP recycling during transcription.

**Experimental Procedures**

**Cloning, Protein Expression, and Purification**—The cDNA clone encoding *E. coli* RapAΔN (rapaΔN), containing His6, and covering amino acid residues 108–962 of RapA, was made by a PCR reaction using the forward primer with BamHI site (rapA-108 5′-TCAGGATCCATGTTCAGCAAGGCCGAGGCCGCTCTG) and reverse primer with HindIII site (rapA-31R 5′-GCAAAGCTTACTGATGCGTTACAAAC). The fragment was then cloned into the expression vector pQE-80L (Qiagen, Valencia, CA). The sequence of the rapAΔN clone was validated by DNA sequencing.

The EcRapAΔN protein was expressed and purified using the same protocol as described for the full-length protein (7). *Thermus thermophilus* (Tt) Core was obtained from the *T. thermophilus* HB8 strain (ATCC 27634; Manassas, VA). The cells were grown at 65 °C as described (10). Cells were lysed by sonication and spun down at 12,000 rpm for 45 min. This was followed by polyethyleneimine P and ammonium sulfate precipitation, and the TtCore was purified as described (11).

**Gel-shift Assay**—4 μg of TtCore was incubated with EcRapA and EcRapAΔN in molar ratios 1:0.5, 1:1, 1:2, and 1:3 in a 20-μl reaction mixture. The reaction buffer consisted of 20 mM Tris, pH 8.0, 200 mM NaCl, 5 mM MgCl2, and 5% glycerol. The reaction mixtures were incubated and shaken for 1.5 h at room temperature; 6% DNA retardation gel in 1× Tris borate EDTA buffer (Life Technologies) was run for 75 min at 2 watts followed by Coomassie staining.

**ATPase Assay**—The ATPase assay was performed to determine the amount of [α-32P]ADP released from [α-32P]ATP as described (4). The reaction mixture was prepared in 10× transcription buffer consisting of 40 mM Tris-HCl, pH 7.8, 40 mM KCl, 5 mM MgCl2, and 3 mM β-mercaptoethanol. The samples were prepared by mixing 1 μl of 10× transcription buffer, 1 μl of 500 mM NaCl, 1 μl of proteins (0.5 mg/ml), and 1 μl of 1 mM ATP containing 0.2–0.5 μCi of [α-32P]ATP, and the final volume was made to 10 μl with water. The samples were incubated at 37 °C for 60 min. 1.5 μl of each sample was applied on a poly(ethyleneimine)-cellulose plate (Sigma), and thin-layer chromatography was used to separate the ADP from ATP. The plate was dried for 15 min, and the sample spots were first washed with water and then with methanol. The TLC plate was dried for 30 min and developed in 1 M formic acid, 1 M LiCl solution in water. After developing, the plate was dried for 20 min and exposed for 10–60 min to a phosphorus screen. The screen was scanned using Typhoon 8600 PhosphorImager (GE Healthcare). The experiment was performed in triplicate under the same reaction conditions.

**RNAP Recycling Assay**—*In vitro* recycling of *T. thermophilus* RNAP (TtRNAP) was tested with EcRapA on a circular DNA template carrying *E. coli* Tac promoter, pTac (5). The assay was performed as described (5) except that the final reaction mixture contained 40 mM Tris-HCl, pH 7.9, 0.05 mM EDTA, 5 mM MgCl2, 3 mM DTT, 200 mM NaCl, 0.2 μg/ml acetylated bovine serum albumin, 1× SUPRase*In* RNase Inhibitor (Life Technologies), and nucleotide triphosphates mix (1/0.2/0.2/0.2 mM ATP/CTP/GTP/UTP) with 0.1–0.2 mM [32P]UTP (PerkinElmer Life Sciences). For a single-round transcription reaction, the DNA competitor heparin (50 μg/ml) and nucleotide triphosphates were added at the time of initiation. The reaction samples contained TtHolo alone, TtHolo plus RapA, and TtHolo plus RapA in the presence of heparin. For each sample, two mixtures (1.3 μg TtHolo, 10 μg plasmid DNA (pJD631) with or without 10.4 μg EcRapA in 90 μl of reaction mix (A)) and (nucleotide triphosphates in the presence or absence of heparin in 30 μl of reaction mix (B)), were pre-incubated at 37 °C for 5 min to form the open promoter complex (8). The *in vitro* transcription was initiated by mixing of A and B followed by collecting 10-μl aliquots after 5, 15, 30, 60, 120, 180, 240, and 480 min. The reaction was stopped by adding 12 μl of stop solution (10 μlurea, 250 mM EDTA, pH 8.0, and 0.05% xylene cyanol). Five microliters of each sample was analyzed on 8% (19:1) acrylamide sequencing gels (in 7 M urea, 1× TBE). Five microliters of each sample was analyzed on 8% (19:1) acrylamide sequencing gels (in 7 M urea, 1× Tris borate EDTA (TBE)) with 1× TBE running buffer. The gels were scanned using Typhoon 8600 PhosphorImager. The assay was performed in triplicate, and the results were plotted in the form of a bar graph (see below).

**Structural Analyses by Small-angle X-ray Scattering**—The EcRapA and EcRapAΔN samples for SAXS data collection were prepared by dialyzing 300 μg of each purified protein in 20 mM Tris, pH 7.9, 200 mM NaCl, 5 mM MgCl2, and 2 mM β-mercaptoethanol. The samples were concentrated to 100 μl at the final concentration of 3 mg/ml. The EcRapA-TtCore complex was prepared by incubating 240 μg of TtCore with 60 μg of EcRapA for 1 h at 4 °C in 1 ml of buffer containing 20 mM Tris, pH 7.9, 200 mM NaCl, 5 mM MgCl2, and 2 mM β-mercaptoethanol. After incubation, the complex was transferred to a 4-ml Amicon centrifugal filter with a membrane cut-off of 100 kDa (Millipore, Taunton, MA), washed with the buffer, and concentrated to a total volume of 200 μl. The complex was washed 5 times to exactly match the sample buffer with the reference buffer and concentrated down to 100 μl. The final concentration of the EcRapA-TtCore complex used for SAXS data collection was 3 mg/ml. The EcRapAΔN-TtCore complex was prepared using the same protocol as described above.

SAXS measurements were carried out at room temperature at beamline 12ID-B of the Advanced Photon Source, Argonne National Laboratory. The wavelength (λ) of x-ray radiation was 0.8856 Å. The scattered x-ray photons were recorded with a PILATUS 1M detector (Dectris) for SAXS and a PILATUS 100k detector (Dectris) for WAXS. The setups were adjusted to achieve scattering q values of 0.006 < q < 2.8 Å−1, where q = (4π/λ)sinθ, and 2θ is the scattering angle. Thirty two-dimensional images were recorded for each buffer and sample solu-
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...tion using a flow cell, with the exposure time of 1–2 s per image to minimize radiation damage and obtain good signal-to-noise ratio. The absence of systematic signal changes in sequentially collected x-ray scattering images confirmed that there was no radiation damage. The two-dimensional images were reduced to one-dimensional scattering profiles using the Matlab software package on site. The scattering profile of a sample solute was calculated by subtracting the buffer contribution from the sample-buffer profile using the program PRIMUS (12) following standard procedures (13). Concentration series measurements (4- and 2-fold dilution and stock solution) for the same sample were carried out to remove the scattering contribution due to interparticle interactions and to extrapolate the data to infinite dilution. The forward scattering intensity (I(0)) and the radius of gyration (R_g) were calculated from the data of infinite dilution at low q values in the range of qR_g < 1.3, using the Guinier approximation: ln I(q) = ln (I(0)) - R_g^2q^2/3. These parameters were also estimated from the scattering profile with a broader q range of 0.006–0.30 Å⁻¹ using the indirect Fourier transform method implemented in the program GNOM (14) along with the pair distance distribution function (PDDF, or P(r)) and the maximum dimension of the protein, D_max. The parameter D_max (the upper end of distance r) was chosen so that the resulting PDDF has a short, near-zero-value tail to avoid underestimation of the molecular dimension and consequent distortion in low resolution structural reconstruction. The Porod volume of solutes (V_Porod) and the volume-of-correlation (V_cor) were calculated using the program PRIMUS (12) and Scatter (15), respectively. The molecular weights of solutes were calculated on a relative scale using the Porod parameter, V_c = V_Porod/σ corr, where σ is the solvent density. The theoretical scattering intensity of the atomic structure model was calculated and fitted to the experimental scattering intensity using CRYSTAL (18), and the model-data agreement parameter, χ²_free was calculated using Scatter (15).

Low resolution ab initio shape envelopes were derived using DAMMIN (19), which generates models represented by an ensemble of densely packed beads, using scattering profiles within the q range of 0.006–0.30 Å⁻¹. A total of 32 independent runs were performed. The resulting models were averaged by DAMAVER (20), superimposed by SUPCOMB (21) based on the normalized spatial discrepancy criteria, and filtered using DAMFILT to generate the final model.

The all-atom rigid body modeling of RapA in solution was performed using the Xplor–NIH package (22, 23). The starting structure was prepared from the crystal structure of RapA (7). The Ntd (residues from 1–102) and the other six domains (residues from 121–962) of the molecule were defined as two rigid bodies, and the relative orientation of these two rigid bodies was optimized, resulting in models that best fit into the experimental scattering profile.

The flexibility of EcRapA was analyzed using the Ensemble Optimization Method (EOM) (24), which allows for coexistence of different conformations of the protein in solution. A pool of 10,000 random structures of EcRapA was generated with the same definition of rigid body domains as above. Scattering curves generated from these structures were filtered using a genetic algorithm against the SAXS scattering curves to select an ensemble of conformers consistent with the experimental data. Rigid body modeling for the EcRapA-TtCore and EcRapAΔN-TtCore complexes was first conducted using the program CORAL (25), which is applicable to SAXS-based rigid body modeling of multisubunit complexes, one or several of whose components lack some fragments (e.g. terminal portions or interdomain linkers are missing). The CORAL program combines rigid body and ab initio modeling; the missing terminal portions or interdomain linkers are treated as random polypeptide chains. The starting coordinates of RapAΔN and RapA were taken from the SAXS solution structures (this work), and the starting coordinates of TtCore were taken from the crystal structure of T. thermophilus transcription initiation complex (TtTIC) (26). A simulated annealing protocol was employed in CORAL to find the optimal positions and orientations of RapAΔN (or RapA) and TtCore and the approximate conformations of the missing portions of polypeptide chain(s) in TtCore, against the experimental scattering profiles.

To account for the polydispersity resulting from the association/dissociation equilibrium of the complexes, rigid body modeling for the EcRapA-TtCore and EcRapAΔN-TtCore complexes was performed using the recently developed program SASREFMX (27). The algorithm implemented in SASREFMX performs rigid body modeling of multisubunit complexes against the scattering profiles from polydisperse samples that contain dissociation products. The optimized parameters are the positions, orientations, and volume fractions of the intact complexes and dissociation products. The starting coordinates and scattering profiles used in SASREFMX were the same as those used in CORAL modeling.

Cross-linking Analysis—The cross-linking analysis was done using disuccinimidyl suberate (DSS) as the cross-linking agent (Sigma). 26 µg of TtCore was incubated with 8.4 µg of EcRapA and 6.25 µg of EcRapAΔN, respectively, in 45 µl of transcription buffer containing 40 mM KCl. The proteins were incubated for 1 h at room temperature to generate complexes. Five samples, 9 µl each of EcRapA, EcRapAΔN, TtCore, EcRapA-TtCore, and EcRapAΔN-TtCore were mixed with 1 µl of different concentrations of freshly prepared solution of DSS in dimethylformamide. These were incubated at 30 °C for 30 min. The final concentrations of DSS used were 0.025, 0.05, 0.1, and 0.25 mM. We optimized the DSS concentration by titrating increasing amounts of DSS to the protein samples followed by SDS-PAGE analysis. The highest concentration of DSS used was enough to lead to the disappearance of β'- subunit of TtCore and its complete cross-linking to higher molecular weight bands. The lowest DSS concentration was enough to partially cross-link the proteins and visualize all the subunits of TtCore and EcRapA by SDS-PAGE at the same time. The cross-linking was quenched by the addition of 1 mM ammonium bicarbonate to a final concentration of 100 mM and incubation for 30 min. The samples were fixed with 2 × SDS loading buffer, boiled for 10 min, and loaded to a 4–12% Bis-Tris SDS gel. The gels were run in MOPS SDS running buffer for 1 h and 20 min at 20 watts and stained with Coomassie Blue.

In-gel Digestion and Mass Spectrometry—Coomassie Blue-stained gel samples were destained and digested with trypsin...
(Promega, Madison, WI) overnight at 37 °C. Extracted peptides were lyophilized and reconstituted in 0.1% trifluoroacetic acid before being analyzed by nanoflow reversed-phase LC-MS/MS. The nanoflow reversed-phase LC-MS/MS was performed using a Thermo Scientific EASY-nLC 1000 nanoflow LC system coupled online to a Velos Pro dual-pressure linear ion trap mass spectrometer (ThermoElectron, San Jose, CA). The LC-MS/MS data were searched using Proteome Discoverer 1.4. Dynamic modification of methionine oxidation was included in the database search. Only tryptic peptides with up to two missed cleavage sites meeting a specific SEQUEST scoring criteria (delta correlation ($\Delta C_n$) $\geq 0.1$ and charge state-dependent cross-correlation (Xcorr) $\geq 1.9$ for [M+H]$^{+}$, $\geq 2.2$ for [M+2H]$^{2+}$, and $\geq 3.5$ for [M+3H]$^{3+}$) were considered as legitimate identifications. The normalized spectral abundance factor quantitative analysis function of Scaffold (Version 4.4.5, Proteome Software Inc., Portland, OR) was used to compare the abundance of proteins identified in the gel samples. For each of the EcRapA-TtCore and EcRapAΔN-TtCore complexes, two samples were chosen. To remove any bias in the quantitative detection of peptides resulting from the difference in sequence lengths of $\beta'$ and $\beta$, control samples for the two complexes were also analyzed. Bands of both EcRapA-TtCore and EcRapAΔN-TtCore complexes were cut from non-denaturing gels to get the absolute number of $\beta'$ and $\beta$ in a 1:1 complex that was not cross-linked. The results are normalized to the mass spectrometry (MS) data of the control samples.

**Structural Illustration**—All of the structural illustrations were generated using the PyMOL Molecular Graphics System, Version 1.3 Schrödinger, LLC.

**Results**

**EcRapA Forms a 1:1 Complex with TtCore**—As shown in lane 1 of Fig. 1A, TtCore by itself migrates as a diffused band in a non-denaturing gel, indicating a mixture of various oligomeric species (28). However, the EcRapA-TtCore complex migrates as a sharp band, indicating its homogenous state; furthermore, the titration of EcRapA in increasing amounts suggests reasonably tight binding of EcRapA to the TtCore (Fig. 1A, lanes 2–5). Using the same gel-shift assay, we observed that EcRapAΔN also forms a 1:1 complex with TtCore (Fig. 1B).

The Ntd of EcRapA contains two subdomains, each folded as a highly bent antiparallel $\beta$-sheet (7). The functional roles of Ntd homologs in other proteins suggest that the Ntd of RapA interacts with both nucleic acids and protein. In RapA, the Ntd contacts domains 1A, Spacer, and C-terminal domain, among which the 1A domain is part of the ATPase module. As aforementioned, RNAP recycling requires the ATPase activity of RapA, which is stimulated upon its binding to RNAP (5, 6). Contacting the ATPase module in a dynamic fashion, the Ntd may play a role regulating the ATPase activity of the protein. We then compared the ATPase activities of RapA and RapAΔN in the absence or presence of the Core RNAP.

**Ntd Inhibits the ATPase Activity of RapA**—The ATPase activity of RapA in the presence of Core is approximately four times that in the absence of Core (4). Strikingly, RapAΔN exhibits an ATPase activity approximately five times that of RapA in the absence of Core, and this activity is not significantly stimulated by the Core (Fig. 1, C and D). These observations suggest that the ATPase activity of RapA is regulated by the Ntd, the structural basis for which may be the relative positioning of Ntd with respect to the rest of the molecule as our structural data indicated. In the crystal structure, the Ntd is in close contact with the 1A domain (7), which may limit the internal motion of the ATPase module during ATP hydrolysis and thereby inhibit its activity. The dynamic nature of Ntd and the stimulated ATPase activity of RapAΔN (Fig. 1D) suggest that the observed stimu-
FIGURE 3. Structural analysis of EcRapA and EcRapAΔN by SAXS. A, top, Overlay of experimental scattering profiles with back-calculated scattering profiles for EcRapA and EcRapAΔN. The inset shows the Guinier analysis (30) for the experimental scattering curves of EcRapA and EcRapAΔN with q\textsuperscript{max} × Rg > 1.3. Bottom, residual differences in the scattering curves between the back-calculated and experimental ones. B, overlay of the model (blue) with experimental (green) PDDFs for EcRapA and overlay of the model (cyan) with experimental (magenta) PDDFs for EcRapAΔN. The PDDFs were not normalized to their molecular masses. The PDDFs for the models were calculated using Xplor-NIH (22, 23); 100 models were calculated, out of which an ensemble of 20 with the lowest χ\textsuperscript{2} values was averaged to obtain the representative curve. C, shown in two views, the EcRapAΔN structure (in cyan, residues 108–962 from the crystal structure of RapA; PDB ID 3DMQ) was docked into the SAXS envelope (in magenta). D, the crystal structure of EcRapA (in cyan with Ntd highlighted in red; PDB ID 3DMQ) was docked into the SAXS envelope (in green). E, the best-fit rigid-body model of EcRapA (in cyan with Ntd highlighted in red; PDB ID 3DMQ) using Xplor-NIH was docked into the SAXS envelope (in green).
ulation of ATPase activity by the Core is mediated by Ntd positioning. We speculated that the Ntd could move even further away from the ATPase core and utilized the SAXS method for an answer.

**EcRapA Facilitates the Recycling of TtRNAP in Vitro**—Next, we tested if EcRapA was active in the recycling of heterologous TtRNAP in vitro on the plasmid DNA template carrying E. coli Tac promoter (pTac), which was previously used for EcRNAP recycling assay (5). Because EcRapA was not functional at \( \geq 42^\circ C \) temperature (data not shown), we performed this experiment at \( 37^\circ C \) where TtRNAP (the optimal temperature 55—65°C) transcribed DNA at a >1000-fold slower rate compared with EcRNAP (29). We confirmed that TtRNAP efficiently initiated transcription at pTac under these conditions. Adding heparin prevented re-initiation at pTac, which revealed that a single round transcription required \( \sim 4 \text{ h} \) to complete in the presence or absence of RapA. Thus, the efficiency of initiation and elongation by TtRNAP were not significantly affected by EcRapA under the single-round conditions. In the multiround assay lacking heparin, the RNA synthesis continued beyond \( 4 \text{ h} \) in the presence, but not in the absence of RapA (Fig. 2). We concluded that RapA was active in recycling TtRNAP in a trend similar to its effect on the cognate EcRNAP on the same template (7).

**Solution Structures of EcRapA and EcRapAΔN by SAXS**—To elucidate the structure of EcRapA by the SAXS method, EcRapA and EcRapAΔN were exposed at multiple concentrations, and the experimental data were normalized and processed as described (13). The experimental scattering profiles, with the scattering intensity \( I(q) \) plotted versus momentum transfer \( q \) for each sample, along with PDDF are shown in Fig. 3, A and B. The Guinier region of the scattering profiles (Fig. 3A, inset) is linear, indicating that the two samples are monodisperse and homogeneous in solution (30). The overall structural parameters are summarized in Table 1, among which the molecular weights estimated from the SAXS data are consistent with those predicted, indicating that both proteins are monomeric in solution. The experimental scattering profiles for the two samples were fitted to scattering profiles back-calculated from the crystal structure for full-length EcRapA and the crystal structure with the Ntd removed for EcRapAΔN. The back-calculated scattering profile for EcRapAΔN fits well to the experimental data (\( \chi^2 = 1.64, \chi^2_{\text{free}} = 4.3, \)) indicating that EcRapAΔN under the SAXS conditions has a conformation very similar to that in the crystal structure. In contrast, the back-calculated scattering profile for EcRapA fits poorly to the experimental data (\( \chi^2 = 5.14, \chi^2_{\text{free}} = 36.8, \)). Furthermore, the predicted \( R_g \) value based on the crystal structure of EcRapA, is 35.5 Å, which is much smaller than that derived from the SAXS data (40.8 Å). Therefore, EcRapA must adopt a conformation significantly different from that observed in the crystal lattice.

To gain more specific information on the solution structures of EcRapA and EcRapAΔN, \textit{ab initio} shape envelopes were built for each sample using the program DAMMIN (19). The crystal structure of EcRapA with the Ntd removed can be well fitted into the SAXS envelope of EcRapAΔN (Fig. 3C), whereas fitting the crystal structure in its full length to the SAXS envelope of EcRapA indicates that the Ntd of EcRapA is positioned differently (Fig. 3D). Indeed, SAXS-based rigid body refinement of EcRapA using Xplor-NIH (22, 23), during which the relative positions of the Ntd and the other six domains of the EcRapA molecule were refined and optimized as rigid bodies against the SAXS data, resulted in an average conformation of RapA (Fig. 3E), which is much more extended than that of the crystal structure and fits well to the scattering data (\( \chi = 0.52, \chi^2_{\text{free}} = 0.91, \)).

**SAXS experiments have been used as a powerful technique to assess macromolecular flexibility. The visual features in the dimensionless Kratky plots of EcRapA and EcRapAΔN, which provide a direct means to qualitatively assess the degree of flexibility of the scattering solute on a common scale, show a more bell-shaped curve for EcRapAΔN but a significant enrichment in the curve at higher scattering angles and a decrease of peak height at \( qR_g = 1.73 \text{ for EcRapA} \) (Fig. 4A). This indicates that EcRapAΔN is more compact in solution than EcRapA, which has increased flexibility. This is further supported by the Porod-Debye plots (31) of EcRapA and EcRapAΔN, which show a plateau for EcRapAΔN (Fig. 4B). The loss of plateau for EcRapA suggests its increased flexibility. To further assess the flexibility of EcRapA in solution, we employed the Ensemble Optimization Method (EOM) (24). In this approach, the Ntd and all the other six domains of EcRapA were defined as two rigid bodies, which were used as inputs to generate a pool of 10,000 conformers. A genetic algorithm selection process was performed to generate ensembles containing multiple conformers that

### Table 1: Data collection and structural parameters derived from SAXS experiments

| Facility and Parameters | Settings and values |
|-------------------------|---------------------|
| Diffraction               |                    |
| X-ray Source              | \( \lambda = 1.54 \text{ Å} \) |
| Detector                  |                    |
| Software                  |                    |
| Data Collection           |                    |
| Experimental scattering   |                    |
| Scattering intensity      |                    |
| Porod Volume              |                    |
| Structural Parameters     |                    |
| EcRapA                    |                    |
| EcRapAΔN                  |                    |
| TtHolE                    |                    |
| EcRapAΔN-TCore            |                    |
| EcRapAΔN-TCore            |                    |

| Structural Parameters     |                  |
|---------------------------|------------------|
| EcRapA                    |                  |
| EcRapAΔN                  |                  |
| TtHolE                    |                  |
| EcRapAΔN-TCore            |                  |
| EcRapAΔN-TCore            |                  |

| Combination               |                  |
|---------------------------|------------------|
| EcRapA                    |                  |
| EcRapAΔN                  |                  |
| TtHolE                    |                  |
| EcRapAΔN-TCore            |                  |
| EcRapAΔN-TCore            |                  |

### Notes

- \( ^{a} \) The MW\text{pred} was calculated from the primary sequences of components.
- \( ^{b} \) The MW\text{saxs} was calculated using the mg/Rg power law developed by Rambo et al. (15).
- \( ^{c} \) The MW\text{saxs} was calculated from Porod volume using AUTOPOD (27).
- \( ^{d} \) The MW\text{saxs} was calculated using the web portal "SAXS MoW" (16).
best fit the experimental scattering curve. A plot of the fitting \( \chi \) between the experimental data and that calculated from the selected ensemble \( \text{versus} \) ensemble size \( (N_e) \) shows that an optimized ensemble with two conformers \( \text{versus} \) one single conformer significantly improves the fitting but that further enlarging the ensemble size does not, indicating that a minimal ensemble size of 2 is sufficient to describe EcRapA in solution (Fig. 4C). The frequency distribution of conformers for optimized ensemble of \( N_e = 2 \) as a function of \( R_g \) shows that the scattering curve is best described as the coexistence of two conformers with \( R_g \) value of 38.4 and 41.4, respectively (Fig. 4D). Interestingly, this is in broad agreement with the \( R_g \) values for EcRapA\( \Delta N \) and the EcRapA in its extended conformation derived above (see Table 1).

We wondered whether EcRapA adopts the open or closed conformation to bind to RNAP. To characterize the EcRapA-TtCore complex in solution, we first tested the applicability of the SAXS method to the RNAP system by determining the SAXS solution structure of TtHolo. The crystal structure of TtTIC available at the time of our SAXS experiment contained an incomplete \( \beta' \) subunit (32). Therefore, this experiment also provided an opportunity to test whether the SAXS method is able to reveal the missing structure.

**Applicability of SAXS to the RNAP System: Proof of Principle**—The experimental scattering profile and the PDDF for TtHolo are shown in Fig. 5A and B. The Guinier region of the scattering profile (Fig. 5A, inset) is linear, indicating that TtHolo is monodisperse and homogeneous (30). As shown in Table 1, the molecular weight estimated from the SAXS data is consistent with that predicted, indicating that the TtHolo is monomeric. The \( \text{ab initio} \) shape reconstruction for TtHolo by DAMMIN reveals a unique bowling pin shape (Fig. 5C). Fitting the incomplete TtHolo structure with the incomplete \( \beta' \) subunit (32) into the bowling-pin-shaped SAXS envelope revealed electron density for the missing portion of the \( \beta' \) subunit (Fig. 5C). Accordingly, we built the TtHolo with a complete \( \beta' \) sub-
unit, and our structure was soon validated by the recent crystal structure of TtTIC that contains a complete β′ subunit (26). Indeed, the head of the envelope corresponds to the missing portion of the β′ subunit (Fig. 5D). As shown in Fig. 5A, the experimental scattering profile of TtHolo can be well fitted into the back-calculated curve from the crystal structure of TtHolo (χ = 0.68, χ^2_free = 0.51). This experiment demonstrates the applicability of SAXS to the RNAP system.

Next, we used the SAXS method to determine the solution structures of EcRapA-TtCore and EcRapAβ′-TtCore.

**Solution Structures of EcRapA-TtCore and EcRapAβ′-TtCore by SAXS**—The experimental scattering profiles and the PDDFs for the two complexes are shown in Fig. 6, A and B. The molecular weights for the two complexes estimated from the SAXS data are consistent with predicted ones for 1:1 complexes (Table 1). The three-dimensional shapes generated with DAMMIN (19) for the two complexes are shown in Fig. 6, C and D. Whereas the envelope of EcRapA-TtCore shares a similar overall shape with that of TtHolo, the envelope of EcRapAβ′-TtCore is slightly different from those of the other two complexes (Fig. 6, C, D, and E). Superposition of the envelope of EcRapA-TtCore with that of EcRapAβ′-TtCore reveals the electron density for the Ntd in the EcRapA-TtCore complex (Fig. 6E), which is in agreement with the open conformation of RapA where the Ntd is disengaged from the rest of RapA. We further performed SAXS-based rigid body refinement for the two complexes using CORAL (25), However, the resulting structures could not fit well to the experimental data, as indicated by the χ values between experimental and back-calculated scattering curves of 1.8 and 2.2 for EcRapA-TtCore and EcRapAβ′-TtCore, respectively (Fig. 6A).

Both complexes were measured at a stoichiometry of 1:1:1, and so there was a small amount of free EcRapA or EcRapAβ′ in the sample. Therefore, the effects of dissociation products (EcRapA or EcRapAβ′) on the scattering profiles and rigid body refinement should be taken into account. Recently, the SASREFMX method (27) was developed to reconstruct three-
dimensional structures for transient multisubunit complexes from SAXS. This method not only provides low resolution three-dimensional structures but also estimates the volume fractions of both the complex and dissociation products. We thus used SASREFMX to probe the association behavior and conformations of the complexes. The resulting SAXS structures fit to the experimental data significantly better, with χ² values of 0.74 and 0.88 for EcRapA-TtCore and EcRapAΔN-TtCore, respectively. The final structures for the two complexes are shown in Fig. 6, C–E.
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The Binding Sites of RapA and $\sigma^{70}$ on RNAP Largely Overlap—As depicted in Fig. 7A, superposition of the SAXS structure of EcRapA-TtCore with the crystal structure of TtTIC (26) demonstrates that the binding sites of RapA and $\sigma^{70}$ on the Core largely overlap. The surface charge potential distribution for the contacting surfaces of RapA and Core in the solution structure of EcRapA-TtCore exhibits high complementarity. As shown in Fig. 7B, the RNAP-contacting RapA surface appears to be a positively charged island in the middle of a negatively charged sea, whereas the RapA-contacting RNAP surface looks like a negatively charged island in the middle of a positively charged ocean. Recently, the *E. coli* Holo structure became available (33). The superposition of EcRapA with the Eco$\sigma^{70}$ subunit embedded in the *E. coli* Holo structure also indicates the complementarity of surface charge potential distribution for the EcRapA-EcCore complex (not shown). The solution structure of EcRapA-TtCore suggests 77 amino acid side chains in EcRapA may interact with RNAP electrostatically. Among the 77 residues, 21 are conserved in TtRapA.

Fig. 7C shows that RapA mainly contacts the $\beta'$ and $\beta$ subunits of TtCore. The structure also suggests that 54 and 21 side chains from the $\beta'$ and $\beta$ subunit, respectively, may form electrostatic interactions with RapA. All of the 75 residues are conserved in EcCore. To validate the general contacting areas of RapA on RNAP, we performed a cross-linking assay (Fig. 7D). EcRapA, EcRapADN, and TtCore were used as controls. In the case of EcRapA-TtCore and EcRapADN-TtCore complexes, we observed that increasing the amount of DSS leads to gradual cross-linking of $\beta'$ more than $\beta$. Upon the addition of RapA, the $\beta'$ subunit cross-links to RapA to a higher degree than $\beta$, suggesting that RapA interacts more with $\beta'$ than $\beta$. We analyzed the products of cross-linking assay by in-gel digestion and MS to characterize the binding interface. Two bands, 1 and 2 (Fig. 7D), were cut from lane a of EcRapA-TtCore, digested with trypsin, and total unique peptide counts were determined by MS. A band with a 1:1 complex of EcRapA-TtCore on a 6% DNA retardation gel (lane 3, Fig. 1A) was analyzed as a control (see “Experimental Procedures”). MS analysis shows the presence of $\beta'$, $\beta$, and RapA in both the control and test samples. A comparison of the spectral count of peptides in the control and test bands shows ~1.28 times more $\beta'$ than $\beta$ peptides for EcRapA-TtCore. Comparable results (~1.35 times) were obtained for EcRapADN-TtCore using the test bands (1' and 2' in Fig. 7D) with the control band (lane 3 in Fig. 1B). This demonstrates that RapA has a more extensive binding interface with $\beta'$ than $\beta$, as suggested by our SAXS structures of the complexes (Fig. 6). In addition, we show here that the deletion of Ntd does not change the general binding area of RapA on RNAP (Fig. 7D).

Discussion

Accurate Structural Information from SAXS—Despite the resolution limit of SAXS, we have applied rigorous metrics and analyzed data in comparison to crystal structure information, which provides accurate structural analysis in solution (34, 35). Comparison of the *ab initio* shape envelopes of RapADN and RapA revealed electron density for the Ntd (Fig. 3, C and D), which was confirmed by rigid body modeling against the experimental data (Fig. 6, C–E). The PDDFs of SAXS solution structures of EcRapA, EcRapADN (Fig. 3B), TtHolo (Fig. 5B), EcRapA-TtCore, and EcRapADN-TtCore (Fig. 6B) are also in good agreement with those calculated from experimental data as judged on the basis of previous studies (36, 37). Furthermore, we show here that SAXS can be used to complete large, multisubunit structures with missing components. When we determined the SAXS solution structure of TtHolo, the available crystal structure of TtTIC contained an incomplete $\beta'$ subunit (32). Fitting the incomplete TtHolo taken from the TtTIC structure into the *ab initio* bowing-pin-shaped SAXS envelope revealed electron density for the missing portion of the $\beta'$ subunit (Fig. 5C). We completed the $\beta'$ subunit accordingly, and our SAXS solution structure of TtHolo was soon validated by the recent crystal structure of TtTIC (26), which contains a complete $\beta'$ subunit (Fig. 5D).

The Ntd of RapA Regulates the ATPase Activity of RapA via Conformational Transformation—In the crystal structure of RapA, the Ntd packs against the 1A domain of the ATPase module (Fig. 8A). We show here that the Ntd can indeed move farther away from the ATPase module, leading to the open conformation of EcRapA (Fig. 8B), which is dramatically different from the closed conformation in the crystal structure. We also show that removing the Ntd stimulates ATPase activity, which is not further stimulated in the presence of RNAP (Fig. 1, C and D). These data suggest that eliminating the physical contact between the Ntd and the ATPase module stimulates the ATPase activity of RapA. Hence, stabilizing the open conformation of RapA could also lead to the stimulated ATPase activity similar to the Ntd truncation.

The Ntd is tethered to the 1A domain in the ATPase module by Linker1, which is ~40 residues in length (7). The flexibility of...
Linker1, especially that of its N-terminal end, enables RapA to transform between the closed and open forms (Fig. 8, A and B). These two forms of RapA suggest that the protein exists in solution as an equilibrium between two major conformations. It appears that the closed form is suitable for forming the crystal lattice, and the consumption of the closed form shifts the equilibrium to sustain crystal growth. In the closed conformation, however, the Ntd appears to inhibit the ATPase activity. Only when the physical impact of Ntd on the ATPase module is completely removed, either upon the truncation of Ntd or upon the binding of RapA to the Core RNAP, the stimulated ATPase activity of RapA is observed (Fig. 1, C and D). Our SAXS structure of RapA-Core shows that RapA binds to the Core in the open conformation (Fig. 8C), and the overall shape of the RapA-Core complex resembles that of the $\alpha^{70}$-Core complex (Fig. 8D). Hence, stabilizing the open form of RapA is equivalent to

![Image](attached_image_url)
the removal of the Ntd, each resulting in the stimulated ATPase activity of the enzyme, which also explains why the ATPase activity of RapAΔN is not stimulated upon its binding to the Core (Fig. 1D). Together, these structural and functional data demonstrate that the Ntd of RapA inhibits the ATPase activity of RapA via its physical contact with the ATPase module and that RNAP stimulates the activity in an allosteric fashion.

The Functional Cycle of RapA—Our new structural and biochemical data together with previous findings provide critical insights into the functional cycle of RapA. As abundant as the housekeeping, promoter-specific transcription factor, RapA engages RNAP in its open or as short-lived intermediates between them. In fact, the closed form of RapA represents two slightly different conformations (7). RapA binds to the available Core RNAP in its open form to form the RapA-Core complex (Fig. 8, B to C), but RapA readily replaces RapA in RapA-Core, leading to the formation of the RapA-Core complex, i.e. the Core complex (Fig. 8, C to B) as we have previously shown in vitro using a competition assay (7). Using an in vitro transcription assay, we have also demonstrated that RapA binds to and remodels the PTC, mediating the recycling of RNAP (7). To connect these two processes, structural information for the RapA-Core complex is critical. Our SAXS solution structures show that the binding sites of RapA and RapAΔN largely overlap (Fig. 7A), providing the structural basis for the fact that RapA binds to the Core only and not to the Holo enzyme (7). Our SAXS structures also show that RapA binds to the core in its open form, which stimulates the ATPase activity for remodeling the PTC because the process depends on ATP hydrolysis (8).

Then, RapA displaces RapA and binds to the Core, resulting in the formation of the RapA-Core complex (70), i.e. the Holo complex, ready for transcription reinitiation (Fig. 8). Additional structural and biochemical data are needed to fully describe the functional cycle of RapA and complete the transcription cycle, among which high resolution structures of the PTC and RapA-PTC complexes are essential for the identification of nucleic acid components and characterization of protein-nucleic acid interactions.

A Hypothetical Model for the RapA-PTC Complex—Previously, we modeled a RapA-dsRNA complex showing how RapA may bind to and translocate on dsDNA (7). The up-to-date data, including the structural information we have gained in this study, allows us to extend the RapA-dsDNA model to include the PTC. The crystal structures of TtTIC (26) and TtTEC (39) exhibit consistent non-coding and coding DNA strands. However, the non-coding strand is longer in the TtTIC structure, and the coding strand is longer in the TtTEC structure. We modeled a hypothetical RapA-PTC complex by first including the non-coding DNA strand from the TtTIC and the coding DNA strand from the TtTEC to the SAXS structure of RapA-Core, resulting in a model of RapA-Core-DNA (Fig. 9). Intriguingly, superposition of the hypothetical RapA-dsDNA (7) and RapA-Core-DNA models indicates that the non-coding DNA strand joins a strand of the dsDNA meaningfully in terms of both strand polarity and structural arrangement, suggesting that RapA binds to the upstream dsDNA. All of the available structural data suggest that RapA translocates along the upstream dsDNA (Ref. 40 and this work). The gap between the upstream dsDNA and the coding DNA strand is ~6–8 nucleotides in length. Although no structural information is available for this gap, the DNA components of the RapA-PTC assembly may include the upstream dsDNA, the coding and non-coding strands in the transcription bubble, and the downstream dsDNA. This DNA architecture mimics the bent-and-open DNA model recently proposed for the T7 RNAP (41), and the polarity of the upstream dsDNA is consistent with the unified
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Hypothetical model of the RapA-PTC complex. The model is composed by using the SAXS structure of EcRapA-TC (this work, Fig. 8C), the dsDNA from the model of RapA-dsDNA (7), the non-coding DNA strand from the crystal structure of T. thermophilus transcription initiation complex (TtTIC, PDB ID 2PPB), Proteins are illustrated and colored as in Fig. 8. The nucleic acids are represented by tube-and-sticks, showing their polarity and colored in red for the coding or green for non-coding DNA strand. The dashed line represents a portion of the DNA coding strand for which structural information is not available.

mechanism for the Swi2/Snf2 enzymes and DEXX box helicases (42).

Our RapA-PTC model suggests that RapA facilitates a forward hypertranslocation of RNAP (Fig. 9). Weakening the transcription complex via forward hypertranslocation is a general mechanism for complex dissociation (43, 44). Another RNAP-associated transcription factor that promotes forward hypertranslocation of RNAP is the Mfd, an ATPase-dependent DNA translocase (45, 46). It binds to the upstream DNA and promotes the forward hypertranslocation of stalled RNAP, leading to the removal of RNAP from the DNA template (47).

RapA is the only Swi2/Snf2 protein in bacteria (3, 4). To establish the molecular mechanism of RapA action, high resolution structures of the PTC and the RapA-PTC complex are essential.

Author Contributions—D. J. J., X. J., M. K., and Y.-X. W. conceived the project and supervised the experiments. S. K. and G. X. S. prepared the RapA proteins and built the hypothetical PTC and RapA-PTC model complexes. X. F., S. K., and Y.-X. W. determined the solution structures of RapA proteins and RapA-RNAP complexes using the SAXS method. L. L. and S. K. purified the RNAP proteins, prepared the RapA-RNAP complexes, and performed the cross-linking experiment and gel-shift, ATPase activity, and in vitro transcription assays. Y. N. Z. cloned Ntd-truncated mutant of RapA and tested protein expression and solubility. X. J., S. K., X. F., L. L., D. J. J., and M. K. wrote the manuscript with inputs from all authors. All authors analyzed the results and approved the final version of the manuscript.

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