X-ray Crystal Structure of Escherichia coli RNA Polymerase σ70 Holoenzyme

Katsuhiko S. Murakami

From the Department of Biochemistry and Molecular Biology, Center for RNA Molecular Biology, Pennsylvania State University, University Park, Pennsylvania 16802

Background: A crystal structure of Escherichia coli RNA polymerase (RNAP) has not been determined.

Results: The σ1.1 and α subunit C-terminal domain structures have been determined in the context of an intact RNAP.

Conclusion: σ1.1 localizes within the RNAP DNA-binding channel and must disengage from this site to form an open complex.

Significance: This work enables future structure determination of bacterial RNAP mutants.

Escherichia coli RNA polymerase (RNAP) is the most studied bacterial RNAP and has been used as the model structure for screening and evaluating potential RNAP-targeting antibiotics. However, the x-ray crystal structure of E. coli RNAP has been limited to individual domains. Here, I report the x-ray structure of the E. coli RNAP σ70 holoenzyme, which shows σ region 1.1 (σ1.1) and the α subunit C-terminal domain for the first time in the context of an intact RNAP. σ1.1 is positioned at the RNAP DNA-binding channel and completely blocks DNA entry to the RNAP active site. The structure reveals that σ1.1 contains a basic patch on its surface, which may play an important role in DNA interaction to facilitate open promoter complex formation. The α subunit C-terminal domain is positioned next to σ domain 4 with a fully stretched linker between the N- and C-terminal domains. E. coli RNAP crystals can be prepared from a convenient overexpression system, allowing further structural studies of bacterial RNAP mutants, including functionally deficient and antibiotic-resistant RNAPs.

RNA polymerase (RNAP) is the central enzyme of gene expression, and all life forms have RNAPs that function as multisubunit protein complexes (multisubunit cellular RNAP). The common core of the multisubunit RNAPs is composed of five subunits that are conserved in bacteria, archaea, and eukaryotes. Bacterial RNAP is the simplest form of this family (composed of the core enzyme α4ββ′ω subunits), whereas in eukaryotes and archaea, RNAP possesses additional polypeptides to form 11–15-subunit complexes.

In bacteria, one of several different σ factors binds to the core enzyme to form the holoenzyme, which is responsible for recognizing promoter DNA. σ70 in Escherichia coli and SigA in other bacteria belong to the group 1 (primary or housekeeping) σ factor family. These σ factors contain distinct regions of highly conserved amino acid sequence and are composed of four domains: σ1.1 (region 1.1), σ2 (regions 1.2–2.4), σ3 (regions 3.0–3.2), and σ4 (regions 4.1–4.2) (3). Group 1 σ factors can bind to promoter DNA as part of the holoenzyme; once it binds to the core enzyme, the σ2, σ3, and σ4 domains are ideally positioned to recognize the promoter DNA sequences of –10, extended –10, and –35, respectively (4, 5).

In addition to the σ2, σ3, and σ4 domains, the group 1 σ family contains an ~100-amino acid N-terminal extension, σ1.1, which is a negatively charged α helical domain (6). The σ1.1 domain has been shown to accelerate the formation of the open complex at some promoters and suggested to reside inside the RNAP main channel (7). This channel is positively charged to accommodate nucleic acids in the open complex and the transcription elongation complex. It has been proposed that during open complex formation, signals from DNA may induce opening and closing of the RNAP clamp, causing σ1.1 to eject from the RNAP main channel (4, 8). Given its flexible nature, σ1.1 has not been solved in all Thermus RNAP holoenzyme crystal structures that have been reported (5, 9–12). Only an NMR structure of σ1.1 from Thermotoga maritima has been reported, and it consists of three α helices with a compact hydrophobic core formed by highly conserved hydrophobic residues (6).

Since the first discovery of RNAP in the early 1960s (13), the RNAP from E. coli has been the primary model system of choice for understanding functions of cellular RNAPs for many reasons. For example, active E. coli RNAP can be conveniently reconstituted in vitro from its individual subunits using either wild-type or mutant proteins (14, 15), and its mechanism can be easily probed in vitro in the presence of purified template DNA, σ factors, and transcription factors. A simple and robust E. coli transcription system also makes it an excellent model for single-molecule studies of RNAPs (16).

X-ray crystal structures of bacterial RNAPs have been determined only from the Thermus genus. Because of the high sequence conservation among RNAPs from all species of bacteria, the most insight derived from the Thermus RNAP has been generalized to represent the transcription apparatus in all bacteria (4, 5, 9–12, 17–19). Nevertheless, without the structure of E. coli...
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RNA Pol available, it is difficult to fully interpret the enormous amount of data that have been collected on E. coli RNA Pol. The structure of E. coli RNA Pol will also generate new insight about structural domains and motifs, as well as interactions with some ligands (e.g. ppGpp) and antibiotics (e.g. lipiarminycin) that specifically affect E. coli but not the Thermus RNAPs (20, 21). These structural insights are important to identify their binding sites and to understand the mechanisms of action.

EXPERIMENTAL PROCEDURES

Preparation and Crystallization of the E. coli RNA Pol Holoenzyme—The polycistronic plasmid pGEMABC was created for overexpressing the rpoA (encoding the a subunit), rpoB (encoding the b subunit), and rpoC (encoding the b subunit) genes as follows. The plasmid pGEMABC expressing rpoA under the control of an IPTG-inducible T7 RNA Pol promoter (22) was digested at a BamHI site downstream of rpoA. A DNA fragment containing the rpoB-rpoC genes was isolated from the pPNE2017 plasmid3 by BamHI digestion and inserted at the BamHI site of pGEMABC. pGEMABC expresses a single mRNA containing the rpoA-rpoB-rpoC genes.

All core RNAP subunits were expressed in E. coli BL21(DE3) cells transformed with pGEMABC (encoding rpoA, rpoB, and rpoC) and pACYCduet-1Ec_rpoZ (encoding rpoZ). Core RNAP was purified as follows. ~16 g of cell paste was suspended in 50 ml of lysis buffer (50 mM Tris-HCl (pH 8 at 4 °C), 1 mM EDTA, 5 mM 2-mercaptoethanol, 1X protease inhibitor mixture, and 2 mM PMSF), and cells were lysed using an EmulsiFlex C3 homogenizer (Avestin Inc.) at 20,000 p.s.i. After a low-speed spin, RNAP in the soluble fraction was precipitated by 1 M NaCl and then precipitated by ammonium sulfate (final 60% saturation). The pellet was suspended in TGED buffer and dialyzed against TGED buffer and dialyzed against TGED buffer + 50 mM NaCl. Core RNAP was purified by Bio-Rex 70 (Bio-Rad), Resource Q (GE Healthcare), and Superdex 200 (GE Healthcare) column chromatography. E. coli σ70 was expressed in BL21(DE3) cells transformed with pGEMD (22). After cells were lysed by sonication, σ70 was purified by HiTrap Q HP (GE Healthcare) and Superdex 200 column chromatography.

The RNAP holoenzyme was prepared by adding a 3-fold excess of σ70 to core RNAP, followed by incubation at 30 °C for 30 min and purification by Superdex 200 column chromatography. Crystals were obtained by hanging drop vapor diffusion by mixing equal volumes of RNAP holoenzyme solution (~20 mg/ml) and crystallization solution (0.1 m HEPES-HCl (pH 7.0), 0.2 m calcium acetate, and ~15% PEG 400) and incubating at 22 °C over the same crystallization solution. For cryocrystallography, crystals were soaked in crystallization solution containing 25% PEG 400. Seleniummethionyl-substituted proteins, including core RNAP and σ70, were prepared by suppression of methionine biosynthesis (23). The crystals belong to the primitive orthorhombic space group (Table 1) containing two 440-

| PDB code | 4IGC |
|----------|------|
| Data collection | Space group P212121 |
| | Cell dimensions (Å) a 187.308, b 205.901, c 309.185 |
| | Resolution (Å) 30–3.60 |
| | Total reflections 592,860 |
| | Unique reflections 123,448 |
| | Rwork 0.242 |
| | Rfree 0.285 |
| | Bond length (Å) 0.003 |
| | Bond angles 0.77° |

3 The highest resolution shell (3.66 to 3.6 Å) is shown in parentheses.

The data sets were collected at MacCHESS beamline A1. PDB, Protein Data Bank; r.m.s.d., root mean square deviations.

RESULTS AND DISCUSSION

E. coli σ70 RNA Pol Holoenzyme Preparation and Crystallization—Endogenous E. coli RNA Pol can be purified from cells by a combination of RNAP-DNA co-precipitation using Polymym P and column chromatography (32). However, the yield and purity of endogenous E. coli RNA Pol are inadequate to

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3 N. Fujita and R. E. Glass, personal communication.
obtain high-quality crystals for x-ray crystallography (<1 mg of RNAP is generated from 1 liter of cell culture). Therefore, I developed a co-overexpression plasmid (pGEMABC) that expresses the rpoA (encoding the α subunit), rpoB (encoding the β subunit), and rpoC (encoding the β′ subunit) genes under a single T7 RNAP promoter. This overexpression system drastically improves the yield and purity of RNAP (10 mg of RNAP from 1 liter of cell culture). The σ70 holoenzyme can be prepared by adding recombinant σ70 to core RNAP. Both the core and holoenzyme formed crystals, but neither diffracted beyond 10 Å resolution. pGEMABC overexpresses the α, β, and β′ subunits but not the ω subunit; thus, purified RNAP contains a substoichiometric amount of the ω subunit. The importance of the ω subunit for RNAP assembly and formation was suggested by a biochemical experiment (33) and by the Thermus RNAP crystal structure, which shows that the ω subunit binds the C-terminal tail of the β′ subunit (see Fig. 3b and supplemental Movie S4) (17).

To prepare RNAP containing a stoichiometric amount of the ω subunit, all RNAP subunits were overexpressed by pGEMABC and pACYCDuet-1_Ec_rpoZ, which overexpresses the ω subunit. The E. coli RNAP holoenzyme was prepared in vitro by addition of σ70, which produced better quality crystals that allowed determination of the structure by x-ray crystallography.

Structure Determination of the E. coli σ70 RNAP Holoenzyme—The crystals contain two 440-kDa RNAP holoenzyme molecules, designated RNAPα and RNAPβ, per asymmetric unit. The structure was solved by molecular replacement with an E. coli RNAP core enzyme model (24). After density modification, the resulting electron density map had several deviations from the molecular replacement solution, including the following regions: 1) β insert 4 (βi4, residues 225–343, previously named β dispensable region 1/BDR1/SI1), 2) β insert 9 (βi9, residues 938–1042, previously named β dispensable region 2/BDR2/SI2), 3) β insert 11 (βi11, residues 1122–1180, present between β conserved regions H and I), 4) β′ insert 6 (β′i6, residues 942–1129, present in the middle of the highly conserved β′ trigger loop/helix), 5) β′ residues 515–597 (present between β′ conserved regions B and C), and 6) the C-terminal tails of the β′ and ω subunits (Fig. 1; see Fig. 3a). The overall structures of βi4 and βi9 are similar to the structures in the previously reported E. coli core enzyme model (24), but their orientations relative to the main body of the RNAP are different. The crystal structures of the E. coli σ70 domains (27, 28) were manually placed in the Fo − Fc map, resulting in good fits of σ2, σ3, and σ4. Anomalous signals from SeMet sites from both the core enzyme and σ70 were used as guides for model building and refinement.

Structure of the E. coli σ70 RNAP Holoenzyme—The overall structure of the E. coli RNAP holoenzyme is similar to the structure of Thermus RNAP, resembling a crab claw with two pincers that constitute the DNA-binding cleft and the active site (Fig. 1 and supplemental Movie S1) (17). The β′ subunit...
forms one pincer, called the “clamp,” and the β subunit forms the other pincer. The clamp changes its position by swinging between open and closed states (34). Comparison of the E. coli RNAP structure with the Thermus RNAP structures, including E. coli subunit N terminus. The structural homolog in the Thermus RNAP crystal structure from this study enables a direct comparison of the β, β', and δ subunits between E. coli and Thermus (Fig. 2).

The entire architecture of the E. coli β subunit (Ecoβ) can be superimposed on the T. aquaticus β subunit (Taβ), with deviations around Ecoβ4 (residues 225–343), Ecoβ9 (residues 938–1042), and Ecoβ11 (residues 1122–1180) (Fig. 2, a and b). Ecoβ11 comprises three α helices, with a long loop connecting the second and third α helices, and it is located near the β subunit N terminus. The Ecoβ11 structural homolog in the Thermus RNAP is β12 (Taβ12, residues 919–969), but it is located ~20 Å away from the relative position of Ecoβ11 and other RNAP crystal structures, including E. coli and Thermus RNAPs.

FIGURE 2. Structure comparisons of the β and β' subunits of the E. coli and T. aquaticus RNAPs. a, superposition of Ecoβ and Taqβ RNAPs. Ecoβ (cyan) and Taqβ (black) are shown as α-carbon backbones in addition to the molecular surfaces of other E. coli RNAP subunits (α, yellow; δ, green; β', pink; αβ, orange; and αδ, red). b, magnified view of the boxed region in a. c, superposition of Ecoβ and Taqβ RNAPs. Ecoβ' (pink and magenta), Taqβ' (black and white) are shown as α-carbon backbones in addition to the molecular surfaces of other E. coli RNAP subunits (α, yellow; δ, green; β', pink; αβ, orange; and αδ, red). d, magnified view of the boxed region in c. e, the bridge helix (yellow), TLH (light blue), and jaw (yellow green) are highlighted on the α-carbon backbone of the Ecoβ' (pink) structure. Ecoβ'16 (purple) was modeled using the E. coli core enzyme model (24).

See Fig. 4a and supplemental Movie S1).
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FIGURE 3. Structure comparisons of the ω subunits of the E. coli and Thermus RNAPs. Shown are close-up views of the ω (gray) and β′ (pink) subunit interactions of the E. coli (a) and Thermus (b) RNAPs. The positions of α helices (E. coli, α₁–α₅; and Thermus, α₁–α₅) of the ω subunits are indicated, and the C termini of the β′ subunits are also indicated.

does not associate with the N-terminal tail of Taqβ (Fig. 2b). The structures of Ecoβ4i and Ecoβ9i have been determined and described previously (24).

In the case of the β′ subunits of E. coli and Thermus, there is structural conservation distributed throughout the entire subunit (Fig. 2c). However, Ecoβ′ has several insertions that are not present in Taqβ′ and vice versa. These insertions include a 13-amino acid insertion between Thr-553 and Thr-567 of E. coli residues 1130–1148) (Fig. 2). The folding of the TLH into two regions (TLH1, residues 915–941; and TLH2, residues 934–941) (Fig. 2d). Taqβ′ also has a large insert (Taqβ′12) between conserved regions A and B (Figs. 1c and 2c) (24, 38).

The β′ subunit trigger loop/helix (TLH) plays a critical role in the nucleotide addition cycle (39, 40). The front edge of the TLH (residues 930–941 and 1130–1137) is highly flexible, but it becomes a rigid “trigger helix” structure when an incoming nucleotide is present at the active site. The middle of the E. coli TLH has a large insert (β′16, residues 942–1129) that separates the TLH into two regions (TLH1, residues 915–941; and TLH2, residues 1130–1148) (Fig. 2e). The edges of TLH1 and TLH2 of E. coli RNAP are in loop conformations (residues 930–933 in TLH1 and residues 1133–1138 in TLH2; residues 934–941 and 1130–1132 are disordered). Ecoβ′16 plays an important role in all stages of transcription, including open complex formation, transcription pausing, and termination, and its location was proposed to be near the β′ subunit jaw (41). However, β′i6 in the E. coli holoenzyme structure is completely disordered, without any trace of electron density map, indicating that β′i6 is highly mobile in this crystal structure and possibly in an apoform holoenzyme.

The β′ subunit bridge helix separates the deep groove of RNAP into a DNA-binding main channel and an NTP entry secondary channel (Figs. 1 and 2e) (17). The eukaryotic RNA polymerase II structure shows a straight-form bridge helix (39, 42), whereas the Thermus RNAP structures show a bent-form bridge helix (5, 17). Further crystallographic studies of the Thermus RNAP complex with the antibiotic streptolydigin (11), as well as a transcription elongation complex (18), have shown that an alternative straight-form bridge helix can exist in the Thermus RNAP. Based on these structures, it was proposed that alternate straight-form and bent-form bridge helix conformations are important for the nucleotide additional cycle, including NTP binding and DNA/RNA hybrid translocation (43, 44). The E. coli RNAP holoenzyme structure presented here possesses a straight bridge helix (Fig. 2e).

Structure and Function of the ω Subunit of E. coli RNAP—The ω subunit of E. coli RNAP is composed of five α helices (α₁–α₅) (Fig. 3a), and the first three α helices (α₁–α₃) can be overlaid with the first three α helices of the Thermus ω subunit (Fig. 3b). The folding of the ω subunit N-terminal tail in the E. coli RNAP is different in the Thermus RNAP structure. The E. coli ω subunit C-terminal tail, including α₄ and α₅, is fully extended; the E. coli ω subunit makes no interaction with the C-terminal tail of the β′ subunit, in contrast to the Thermus RNAP, which has an extensive interaction between the ω subunit and the C-terminal tail of the β′ subunit (supplemental Movie S4).

Functionally, the ω subunit is the least understood subunit, but there is a clear link between the ω subunit and ppGpp-dependent transcription (45, 46). The finding that the ω subunit structure is so different in the E. coli and Thermus RNAPs may be related to the observation that E. coli RNAP can respond to ppGpp only in the presence of the ω subunit (46, 47). Thus, the E. coli holoenzyme structure can be used as an ideal system for understanding the relationship between the ω subunit and ppGpp-dependent transcription regulation and may finally reconcile 4 decades of experimental data, especially in understanding the cause of the stringent response and growth control by ppGpp in E. coli cells (48–51).

Structure and Function of σ₇₀—Strong and traceable electron density maps of σ₇₀ were attainable from σ₁,2 to the C terminus. In one of two E. coli RNAP molecules (RNAPₜ) in the asymmetric unit, the Fₒ – Fᵣ electron density map calculated using CNS version 1.3 (30) showed rod-like densities for σ₁,1, which is adjacent to σ₁,2. A homology model of E. coli σ₁,1, which was constructed by SWISS-MODEL (52) based on the T. maritima σ₁,1 NMR structure (6), was placed on the σ₁,1 electron density map, and the positions of three α helices were manually adjusted. An additional α helix (H4) was then built based on a rod-like density next to the third α helix (H3). The σ₁,1 structure was refined in the holoenzyme. The final σ₁,1 structure contains four α helices (residues 6–64), and the electron density of residues from position 65 to σ₁,2 (residue 95) is completely disordered. The higher B-factor and weak electron density were also noted in σ₇₀.
density map of $\sigma_{1,1}$ in the *E. coli* holoenzyme structure indicate that $\sigma_{1,1}$ is highly mobile in the holoenzyme.

The structure shows that $\sigma_{1,1}$ is surrounded by $\sigma 2$, the $\beta$ lobe, the $\beta'$ clamp, and the $\beta'$ cleft (Fig. 4a and supplemental Movie S3). The $\sigma_{1,1}$ location in the *E. coli* RNAP crystal structure is consistent with the *E. coli* RNAP model derived from systematic FRET and distance-constrained docking (8).

The acidic residues of $\sigma_{1,1}$ mask the basic residues of the $\beta$ lobe and $\beta'$ clamp, and $\sigma_{1,1}$ fits snugly in the DNA-binding main channel of RNAP, thereby preventing access of either double- or single-stranded DNA to the RNAP active site. Therefore, $\sigma_{1,1}$ must disengage from this binding site, or the RNAP clamp must open further (34) to form an open complex.

The three basic residues (Lys-10, Arg-15, and Lys-17) found at the $\sigma_{1,1}$ N terminus are surface-exposed and face the outside of the RNAP main channel (Fig. 4b and supplemental Movie S3). These $\sigma_{1,1}$ basic residues, together with other positively charged regions, including $\sigma 2$, the $\beta$ lobe, the $\beta'$ clamp, and the $\beta'$ jaw, form a continuous path of negative electrostatic potential for promoter DNA and downstream DNA binding. This region may also serve an important role in bending DNA to form the early stage intermediates between the closed and open promoter complexes (7). Although the presence of basic residues at the $\sigma_{1,1}$ N terminus is common in the group 1 $\sigma$ family, the function of this basic region for transcription has not been tested. This basic region in $\sigma_{1,1}$ could make a contribution to open complex formation.

**The $\alpha$ Subunit C-terminal Domain within *E. coli* RNAP—**The C-terminal domain of the $\alpha$ subunit ($\alpha$-CTD, residues 250–329) is a DNA-binding element and a major target of transcription factors for regulation (22, 53). The two $\alpha$-CTDs of the RNAP holoenzyme, connected to their N-terminal domains ($\alpha$-NTD) by linkers (54), can interact independently with transcription factors that bind to DNA 40–100 bp upstream from the transcription start site (55, 56). The structure of an $\alpha$-CTD in the context of an intact RNAP has not been solved because it is dynamic. In the *E. coli* RNAP structure presented in this study, electron density was visible for only one of the four $\alpha$-CTDs in the asymmetric unit (RNAP$_A$ $\alpha_i$). The map enabled
a model of the α-CTD to be fitted (57). Furthermore, the map included density of the linker region that allows modeling of the linker (Fig. 5a). Arg-265 in the α-CTD is ~60 Å away from the α-NTD (residue 233), with the linker fully stretched and without any secondary structure (Fig. 5a and supplemental Movie S2), indicating that the near-maximum length that Arg-265 in the α-CTD can reach DNA from its N-terminal domain is ~60 Å.

Previous biochemical studies suggested that surface-exposed residues in σII, interact directly with the α-CTD (Fig. 5b) (58, 59). Although these residues are partially involved in making the α-CTD–σII complex in the E. coli RNAP crystal structure, the orientation of the α-CTD relative to σII is different compared with the cryo-EM model of the RNAP-catabolite activator protein-DNA complex (60) and the predicted models of the α-CTD–σII-DNA complex based on biochemical studies (Fig. 5b and supplemental Movie S2) (58, 59). The structure of the α-CTD in this holoenzyme structure may be one of several possible conformations of free holoenzyme and would have to rearrange itself for promoter DNA binding.

Concluding Remarks—The crystal structure of the E. coli RNAP holoenzyme presented here provides an ideal model for analyzing the functional data that have been generated for over 50 years and for designing future experiments that will uncover the transcription mechanisms. My E. coli RNAP structure reveals the molecular features of the α-CTD and σII, for the first time in the context of an intact bacterial RNAP. Furthermore, I have shown that the E. coli RNAP prepared from a co-overexpression vector can generate sufficient quantities of active RNAP for crystallization and high-quality diffraction. This methodology will facilitate the structure determination of the large collection of mutant RNAPs that have been generated for E. coli transcription and regulation studies. Finally, because the sequence and antibiotic sensitivity of E. coli RNAP are similar to those of pathogen-related RNAPs, including Mycobacterium tuberculosis and Staphylococcus aureus, E. coli RNAP can now be used to readily study RNAP-antibiotic interactions by x-ray crystallography.

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