The β Subunit Rif-cluster I Is Only Angstroms Away from the Active Center of Escherichia coli RNA Polymerase*

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Ribonucleotide analogs bound in the initiating site of Escherichia coli RNA polymerase-promoter complex were cross-linked to the β subunit. Using limited proteolysis and chemical degradation, the cross-link was mapped to a segment of β between amino acids Val536 and Arg586. This region (Rif-cluster I) is known to harbor many rifampicin-resistant (RifR) mutations. The results demonstrate that Rif-cluster I is part of the “5′-face” of the active center and provide structural basis for the long-known effects of RifR mutations on transcription initiation, elongation, and termination.

The cellular multisubunit DNA-dependent RNA polymerase is the central enzyme of gene expression and a target for genetic regulation. The two largest subunits of RNA polymerase (RNAP) display a remarkable degree of evolutionary conservation. The best studied RNAP is that of Escherichia coli. The catalytically competent core RNAP consists of the β (1,407 amino acid residues), the β (1,342 residues), and the two α (329 residues) subunits (3). The holoenzyme composed of the core and a σ subunit is capable of specifically initiating transcription from promoters (4). The initiation reaction begins with the binding of a priming substrate (usually a purine nucleotide) and the next nucleoside triphosphate to the holoenzyme stably anchored at the promoter DNA. RNAP then catalyzes the formation of the first phosphodiester bond, yielding a dinucleotide and pyrophosphate.

In order to identify amino acid residues participating in the active center of RNAP, we used the approach of mapping the sites of chemical cross-linking of substrate analogs. Using probes specific for Lys and His residues, we showed that evolutionarily conserved Lys520 and His523 near the C terminus of the β subunit are located at the “5′ face” of the priming ATP (5–7). Here, we use a broadly specific alkylating cross-linkable group placed on the 5′ side of the initiating NTP to implicate another site of the β subunit in the active center.

Three short regions in the middle of the β subunit defined by mutations of resistance to Rif, Rif-cluster I (amino acids 512–534), Rif-cluster II (amino acids 563–574), and Rif-cluster III (amino acid 687), have long been suspected of involvement in the RNAP active center. The assumption rested on the Rif ability to block extension of short initial transcripts (8). Yet, there is still no rigorous proof that mutationally defined Rif-clusters in the middle of β participate directly in Rif binding site or in the RNAP active center. Our present results directly position Rif-cluster I at the 5′ face of the initiating NTP site.

MATERIALS AND METHODS

Affinity Reagents

The iodo-containing derivatives of ATP, ADP, and AMP were obtained as follows. [N,N-bis-(2-iodoethyl)ammonobenzaldehyde—100 mg of 4-[N,N-bis-(2-chloroethyl)amino]benzaldehyde was placed in a solution containing 200 mg of NaI in 1 ml of dimethylformamide and incubated at 95°C for 4 h. The mixture was supplemented with 3 ml of water and extracted with an equal volume of ether. Organic phase was collected and evaporated to dryness under vacuum. The product was recrystallized from methanol.

Alkylation of ATP, AMP—10 mg of ATP, ADP, or AMP (tributylammonium salts) in 100 μl of water was mixed with 20 mg of [N,N-bis-(2-iodoethyl)laminobenzaldehyde in 200 μl of dimethylaminomethyl]phenol, and 10 μl of diisopropylethylamine was added. After 3–4 h at 60°C, the mixture was applied on a silica gel plate and developed in an acetonitrile/water (4:1) system. Reaction product was visualized under UV light and/or by color reaction with 2,4-dinitrophenylhydrazine, eluted with water, and lyophilized.

Affinity Labeling

10-μl reactions contained 40 mM Tris-HCl, pH 7.9, 100 mM KCl, 10 mM MgCl₂, ~1.0 μg of RNAP, and 0.5–1.0 mM derivatized initiating substrate. Reactions were supplemented with 10 mM BH₄ and incubated at 37°C for 10 min. Reactions were supplemented with 100 ng of 136-base pair T7 A1 promoter-containing DNA fragment (14) generated by polymerase chain reaction, [α-32P]UTP (3,000 Ci/mmol) was added to 0.3 μM, and incubation continued for 30 min at 23°C. In experiment shown on Fig. 4, affinity modification of RNAP was performed in the presence of promoter fragment, and reaction was scaled up 5 times. Control experiments demonstrated that the resulting 32P-labeling of RNAP depended on the addition of the template DNA (data not shown).
Cross-linked Product Analysis

Electrophoresis in the presence of SDS was carried out in precast polyacrylamide gels (Novex). Conditions of WT and VT989 RNAP trypsin treatment are described in detail elsewhere (15, 16). Purification of the cross-linked polypeptides from the gel and conditions of complete CNBr degradation were essentially as described (17).

For Ni\textsuperscript{2+}NTA agarose affinity purification, CNBr cleavage reactions were dried down under vacuum and resuspended in 200–500 μl of buffer containing 6 M urea and 100 mM Tris-HCl, pH 7.6. An aliquot was withdrawn for electrophoretic analysis, 25–50 μl of Ni\textsuperscript{2+} resin was washed in the urea buffer added, and reactions were gently mixed for 30 min at 4 °C. After a brief centrifugation, the sorbent was washed 3 times with 1.5 ml of the urea buffer and then 2 times with 1.5 ml of transcription buffer. The buffer was then removed and the bound protein was eluted in a small volume of SDS-containing Laemmli loading buffer in the presence of 25 mM EDTA. For BNPS-skatole treatment, the affinity-purified CNBr fragment was eluted from Ni\textsuperscript{2+}NTA agarose by addition of 3 volumes of glacial acetic acid. 1 volume of BNPS-skatole (10 mg/ml) in 70% acetic acid was added to the eluate, and reaction was incubated for 10 h at room temperature, extracted to dryness with ether, and the solid residue was dissolved in SDS-containing Laemmli loading buffer.

RNA Polymerases

RNAP was reconstituted as described (18). Detailed characterization of the reconstituted VT989 and VT\textsubscript{H6} enzymes is described in Refs. 16 and 19, respectively.

RESULTS

The Experimental System—The task of introducing and mapping of a cross-link on the RNAP molecule is complicated by the fact that the enzyme is composed of 5 polypeptides with the cumulative molecular mass of ~500 kDa. To introduce the cross-linking probe, we employ the highly selective affinity labeling protocol (5, 6) which leads to a radioactive dinucleotide reporter group. For the high resolution mapping of the cross-links, we employ genetically engineered catalytically proficient variants of RNAP with conveniently located sites that facilitate analysis, i.e. a highly labile proteolysis site about two-thirds down the length of the subunit (15, 16) and an insertion of six consecutive histidines between Rif-cluster 1 and Rif-cluster 2 (19). The inserted His-tag specifically absorbs to Ni\textsuperscript{2+} -chelating sorbents and allows one to selectively recover a subset of peptide fragments carrying the tag from a proteolytic digest.

The adenine nucleotide analogs used in this work as the primary cross-linkable reagents are shown on Fig. 1. The AMP(1065) reagent was used in the previous work (5–7). Its aldehyde group cross-links exclusively to the \textit{b} subunit Lys\textsubscript{1065} (7). The three other reagents are analogous to the previously described chloro derivatives (5). The reagents contain an alkylating group that is inactive because of a strong electron-acceptor effect of the aldehyde group. Reduction of the aldehyde group in the presence of NaBH\textsubscript{4} results in a 1000-fold increase of the alkylating activity. The iodo-containing derivatives used in this work are ~100 times more reactive than the corresponding chloro compounds, and the alkylating reaction proceeds with a half-life of 5 min at room temperature (data not shown). The alkylating group can react with any nucleophilic amino acid side chain (Cys, His, Lys, Tyr, Asp, Glu, Ser, Thr). However, alkylated Glu and Asp residues are unstable and do not survive SDS-polyacrylamide gel electrophoresis conditions.
that large subunits contained little or no radioactivity, testifying labeling reactions, the part of the gel corresponding to intact RNAPlargesubunits,which were not resolved at the 8% SDS-gel used in this experiment.

The faster migrating band comigrated with the radioactive band comigrated with RNAPlargesubunits, which approximately two-thirds down the length of the A1 promoter of bacteriophage T7 was affinity-labeled with the reagents shown in Fig. 1. The reactions were treated with trypsin as indicated and were then loaded on an 8% Tris-glycine polyacrylamide SDS-gel. The cross-linked RNAP subunits were visualized by autoradiography. In B, small amounts of radioactive βb fragment visible in the lanes corresponding to reactions that were not treated with trypsin are due to a very high sensitivity of T989 RNAP to proteolytic attack which occurs to some extent during the enzyme preparation. The proteolyzed enzyme retains its catalytic activity (16) and is therefore labeled in the assay.

Because only a small fraction of the WT RNAP is cleaved under single-hit conditions, we repeated the above experiment with small amounts of trypsin were added to completed modification reactions, two new radioactive bands with apparent molecular size of ~90 and ~40 kDa appeared (lanes 2 and 3). In the control experiment with AMP(1065) reagent, only the 40-kDa fragment was seen in the trypsin-treated reactions (not shown). Elsewhere we show that at the low concentrations used here trypsin predominantly attacks WT RNAP at positions 903 and 909 of β, to generate two fragments of ~90 (N-terminal) and ~40 (C-terminal) kDa (15). The results suggest that ATP* cross-linked both in the C-terminal third and in the N-terminal two-thirds of the β-polypeptide.

Because only a small fraction of the WT RNAP is cleaved under single-hit conditions, we repeated the above experiment with mutant enzyme carrying a highly labile proteolysis site approximately two-thirds down the length of the β-polypeptide. The mutant ΔT989 RNAP is indistinguishable from the WT enzyme in functional tests, but is much more sensitive to trypsin attack due to an insertion of ~130 amino acids at position 989 (15, 16). Fig. 2B shows the results of an experiment where ΔT989 RNAP was modified with different reagents and then subjected to mild treatment with trypsin. When ΔT989 RNAP modified with the lysine-specific reagent AMP(1065) (lane 1) was treated with trypsin, all of the radioactivity was found in the C-terminal fragment of mutant β (lane 2). The alkylation cross-linkable group positioned at the ε-, γ-, and λ-phosphates of initiating adenosine nucleotide, labeled both the smaller (C-terminal) and the larger (N-terminal) fragments of ΔT989 β (lanes 4, 6, and 8, respectively). In trypsin-treated affinity labeling reactions, the part of the gel corresponding to intact large subunits contained little or no radioactivity, testifying that β' was not appreciably labeled by any of the reagents.

Rifampicin decreased the overall efficiency of labeling with ATP* reagent (lane 9), but did not inhibit cross-linking of the N-terminal part of β (lane 10) (in fact, the ratio of the N- to C-terminal fragment labeling increased in the presence of rifampicin).

Mapping of the Cross-link in the N-terminal Part of the β Subunit—To map the cross-link site in the N-terminal part of the ΔT989 β subunit, the radioactive N-terminal fragments of β shown in Fig. 2B were excised from the gel and treated with CNBr. Reaction products were separated on a 16% Tris-Tricine polyacrylamide-SDS gel, and radioactive peptides were visualized by autoradiography.

Mapping of the Cross-link in the N-terminal Part of the β Subunit—To map the cross-link site in the N-terminal part of the ΔT989 β subunit, the radioactive N-terminal fragments of β shown in Fig. 2B were excised from the gel and subjected to complete CNBr degradation (Fig. 3). As a control, the C-terminal fragment of the ΔT989 β subunit labeled at Lys1065 with the lysine-specific reagent was also subjected to CNBr treatment (lane 5) yielding a single labeled polypeptide with an apparent molecular mass of 11 kDa (expectant molecular size ~10,200 kDa). Thus, under these conditions, CNBr degradation of β proceeded to completion.

Two major radioactive peptides were generated when N-terminal fragments of ΔT989 β-labeled with alkylation reagents were subjected to CNBr treatment. The larger peptide had an apparent molecular mass of ~18 kDa, the smaller, ~4 kDa. The ratio of the two peptides depended on the affinity

Fig. 2. Affinity labeling of RNA polymerase. Affinity labeling of WT (A) and ΔT989 (B) RNAP. RNAP in the open complexes at the A1 promoter of phage T7 was affinity-labeled with the reagents shown in Fig. 1. The reactions were treated with trypsin as indicated and were then loaded on an 8% Tris-glycine polyacrylamide SDS-gel. The cross-linked RNAP subunits were visualized by autoradiography.
labeling reagent. The smaller peptide predominated when AMP* was used (lane 1), while the larger was dominant when RNAP was labeled with ATP* (lane 4). In the presence of Rif, the amount of the smaller peptide cross-linked to ATP* decreased even further (lane 5).

Since CNBr cleavage was complete, the 18-kDa labeled peptide should come from between two Met residues separated by 100 amino acids. From the sequence of the N-terminal tryptic fragment of β, the likely candidate is the fragment between Met535 and Met553. This was further tested using a mutant ßH6 RNAP which carried a stretch of 8 amino acids, His(His)6-Trp, inserted between positions 540 and 541 (19). The ßH6 RNAP is fully functional in vivo and in vitro (19). Preliminary experiments showed that the modified ßH6 enzyme gave the same pattern of radioactive fragments upon trypsin cleavage as the control WT RNAP (not shown). If the site of the ATP* cross-link in the N-terminal part of ßH6 subunit were indeed between Met535 and Met553, we should be able to purify the histidine-tagged, cross-linked ßH6 CNBr fragment by affinity chromatography on Ni**-sorbent.

In the experiment of Fig. 4, the ßH6 RNAP was affinity-labeled using ATP*, and an excess of unlabeled ßH6 subunit carrier was added so that the material could be visualized both by staining (lane 1) and autoradiography (lane 6). The CNBr reaction products shown (lanes 2 and 7) were loaded on Ni**-NTA resin in the presence of 6 M urea and washed extensively. As expected, CNBr-generated peptide Val516-Met653 was retained on the sorbent as revealed by Coomassie staining (lane 3). Since the peptide was radioactively labeled (lane 8), we conclude that the site of the major ATP* cross-link in the N-terminal part of ß is contained within the fragment Val516-Met653. Control experiments established that no polypeptides were retained on Ni**-NTA resin when WT RNAP was used in the modification reaction (data not shown).

To further localize the site of the ATP* cross-link, we made use of the Trp residue inserted immediately past the six histidines in the ßH6 RNAP. The inserted Trp is unique to the Val516-Met653 peptide. The affinity-purified Val516-Met653 peptide was subjected to treatment with BNPS-skatole, a reagent which specifically cleaves polyptides after Trp residues. As is shown on Fig. 4, lane 4, BNPS-skatole generated two peptides with the apparent mobility of 5 and 11 kDa (expectant molecular sizes are 3,678 Da and 12,850 Da, respectively). The smaller peptide was radioactive, while the larger was not (Fig. 4, lane 9). The larger BNPS-skatole peptide was lost during the second round of affinity purification, while the smaller peptide as well as the initial Val516-Met653 peptide were retained on the sorbent (lanes 5 and 10). We conclude that the site of the ATP* cross-link is contained within the ß subunit fragment between Val516 and Arg540.

**DISCUSSION**

The impetus for this work came from an earlier study (20). The authors mapped a cross-linking site of a 5'-chloro derivative of ATP between residues 516 and 653 of the WT ß subunit. In the turmoil of disintegration of the Soviet Union, these data were irreversibly lost, and we decided to reinvestigate the issue using a more powerful family of iodo-derivatized reagents and recombinant mutant RNA polymerases.

Our present results demonstrate that the ß subunit segment between residues 516 and 540 contains the site(s) of cross-linking to ATP*. This site should be located within 5 Å of the γ-phosphate of the priming ATP, which is the effective range of the probe used. The distance between the Rif-cluster I and the C-terminal ß localities labeled by the same reagent should not exceed this distance more than twice. Hence, these two regions which are separated in the linear sequence of ß by more than 500 amino acids must be juxtaposed in the folded ternary complex.
structure of the enzyme. Elsewhere we show that part of the conserved domain 3 of \( \beta \), as well as positions 23 and 24 of the template DNA strand are in contact with \( \gamma \)-phosphate of initiating nucleotide (17, 13). In addition to these elements, other RNAP sites apparently participate in the 5'-face of the active center since another, yet unidentified site in the N-terminal part of \( \beta \) is efficiently cross-linked to ADP* and AMP* probes (see Fig. 3). Experiments aimed at mapping of these sites are in progress.

Even though Rif-cluster I appears to be in direct contact with the priming substrate, the cross-linked residue is not essential for the enzymatic reaction since the derivatized enzyme is still able to form the phosphodiester bond. The viability of many mutants isolated in this area also argues against its direct involvement in an essential function. On the other hand, the mutant RNA polymerases have been shown to have altered apparent \( K_m \) values for the reaction substrates during initiation and elongation (11), as well as changes in factor-dependent and independent termination (10, 21). These phenotypes are consistent with the notion that the priming nucleotide site overlaps with a larger site holding the RNA product in the active center during elongation. Structural disruption of this area by a mutation may affect the formation and/or proper positioning of the 3'-proximal portion of nascent RNA and, hence, result in defects affecting extension of RNA chains.

Our previous work positioned RNAP-bound Rif immediately upstream of the initiating nucleotide binding site (13). The present results demonstrate that Rif-cluster I is located in the same vicinity adding strength to the notion that Rif\( ^R \) mutations indeed define the Rif binding site.

REFERENCES

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