Structural Basis of Transcription Inhibition by Antibiotic Streptolydigin

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Summary

Streptolydigin (Stl) is a potent inhibitor of bacterial RNA polymerases (RNAPs). The 2.4 Å resolution structure of the Thermus thermophilus RNAP-Stl complex showed that, in full agreement with the available genetic data, the inhibitor binding site is located 20 Å away from the RNAP active site and encompasses the bridge helix and the trigger loop, two elements that are considered to be crucial for RNAP catalytic center function. Structure-based biochemical experiments revealed additional determinants of Stl binding and demonstrated that Stl does not affect NTP substrate binding, DNA translocation, and phosphodiester bond formation. The RNAP-Stl complex structure, its comparison with the closely related substrate bound eukaryotic transcription elongation complexes, and biochemical analysis suggest an inhibitory mechanism in which Stl stabilizes catalytically inactive (preinsertion) substrate bound transcription intermediate, thereby blocking structural isomerization of RNAP to an active configuration. The results provide a basis for a design of new antibiotics utilizing the Stl-like mechanism.

Introduction

In all cellular organisms, transcription, the first and likely the most highly regulated step of gene expression, is carried out by DNA-dependent RNAPs. The bacterial RNAP core enzyme, a complex of five subunits (α2ββ′ω), must recruit an additional subunit, σ, to form a holoenzyme that recognizes promoter DNA sequences and carries out transcription initiation. After a stable 9–12 nucleotide (nt) primer is made, the transcription complex undergoes transition from initiation to a highly processive elongation phase (Krummel and Chamberlin, 1989). During elongation, ~14 base pairs (bp) of the transcribed DNA are unwound in the vicinity of the RNAP active site, forming a transcription bubble between the downstream and upstream portions of the DNA duplex. The unwound DNA template strand and the nascent RNA transcript form an eight to nine bp RNA-DNA hybrid, which is buried in the active site cavity.

RNAP must remain bound to the template DNA and the nascent RNA transcript until it reaches a termination signal. Thus, the nucleotide addition cycle (NAC), an elemental step in transcription, must be repeated thousands or even millions of times to complete the synthesis of a nascent RNA chain. The NAC may be divided into several steps (see Figure 1A in Temiakov et al. [2004]). At the beginning of the NAC, in the post-translocated state, the 3’ end of the nascent RNA transcript is hybridized with the template strand and occupies register (n − 1) immediately upstream of the active site. Base pairing of the substrate with the acceptor template base and subsequent nucleotide incorporation results in extension of the RNA 3’ terminus to reg-
Figure 1. The RNAP-Stl Complex Structure

(A) Structural formula of Stl. The distinct chemical groups with the Stl molecule are highlighted by colored boxes.

(B) The initial $|F_{\text{stl}} - F_{\text{nati}}|$ difference ED map (green, 3.5 Å resolution, contoured at 2.3 $\sigma$ level), where $F_{\text{stl}}$ and $F_{\text{nati}}$ are the structure factor amplitudes of the RNAP-Stl complex and the apo-holoenzyme, respectively, is superimposed on the ribbon diagram of the RNAP structure (yellow) and Stl (pink balls-and-sticks model) in the complex; the structure of the apo-holoenzyme is shown in white. The protein side chains which moved upon complex formation in the Stl binding site, as revealed by the substantial ED in the difference ED map, are modeled as balls and sticks.

Recent structural studies suggested two mechanisms of substrate loading into the active site. Structures of the single-subunit T7 RNAP elongation complex (EC) indicated that the incoming substrate is first recognized in the preinsertion site (PS) in an inactive “open” conformation of the enzyme and is subsequently delivered to the catalytic site (also called the insertion site [IS]) upon closing of the active site cleft (Temiakov et al., 2004; Yin and Steitz, 2004). In both T7 and yeast EC, binding of the NTP analog in the PS satisfies two major criteria for substrate selection: the substrate forms a Watson-Crick base pair with the DNA base, and its ribose is specifically recognized by the side chain of Asn479 (Kettenberger et al., 2004), in agreement with the recent evidence showing that its bacterial counterpart (β #Asn458 in E. coli) is crucial for the rNTP versus dNTP discrimination (Svetlov et al., 2004). Thus the primary role of PS in all RNAPs is likely to provide substrate selection prior to catalytic reaction, thereby maintaining fidelity of RNA synthesis.

ister (n) and conversion to pretranslocated state. The NAC is completed by translocation of the RNA-DNA hybrid one bp upstream, from n to n-1, to again form the posttranslocated complex. Reverse translocation is also possible and is known as backtracking (Komissarova and Kashlev, 1997).

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An alternative model, in which the substrate is loaded first to the E site, was also proposed (Westover et al., 2004). The E site overlaps with the RNAP active center and has been originally proposed to bind the noncomplementary NTPs that enhance the exonucleolytic activity of RNAP (Sosunov et al., 2003). In the E site, the substrate makes no base- and/or ribose-specific interactions and binds mostly through its phosphates. Subsequently, the substrate rotates to base pair with the DNA template base. Thus, according to the E site model, the substrate is directly loaded into the IS, eliminating the preinsertion step both structurally (as a distinct location) and functionally (no substrate selection is envisioned). The E site and PS site models would therefore appear to be mutually exclusive, but as we will show, this need not be true.

Though bacterial and eukaryotic RNAPs are homologous, they possess many distinct structural and functional features that make the bacterial enzyme an attractive target for drug design. However, among a wide range of known antibiotics, only a few target bacterial RNAP. The best studied examples include rifampicin, a front-line antituberculosis drug (Floss and Yu, 2005), a cyclic peptide microcin J25 (Adelman et al., 2004; Mukhopadhyay et al., 2004), CBR703 (Artisomovich et al., 2003a), and Stl (Figure 1A), which was purified from Streptomyces lydigus cultures ~50 years ago (Crum et al., 1955; Deboer et al., 1955). Several Stl-resistant substitutions in the RNAP β and β′ subunits have been isolated over the years (Heisler et al., 1993; Severinov et al., 1993, 1995; Yang and Price, 1995). Biochemical analyses implied that Stl affects substrate binding, catalysis, and DNA translocation (Cassani et al., 1971; McClure, 1980; Siddhikol et al., 1969). However, the mechanism by which Stl inhibits RNAP remains obscure.

Here, we report a 2.4 Å resolution structure of the T. thermophilus RNAP holoenzyme in complex with Stl, which, in combination with subsequent biochemical experiments, mutagenesis, and modeling, suggests a plausible mechanism for Stl action and may allow further improvement of the drug to increase its affinity and specificity. As most biochemical and genetic studies of Stl action were performed with the E. coli RNAP, we will use the E. coli sequence numbering throughout the manuscript.

Results and Discussion

Structure of RNAP-Stl Complex

The RNAP crystals used in these studies are characterized by the systematic differences in the unit cell parameters as compared to the previously reported crystals (Vassylyev et al., 2002). In addition to some interdomain movements and local differences in the side chain orientations, the β′ subunit bridge α helix (BH, β1 769–804) is uniform (straight), in contrast to previously observed locally distorted conformation (Figure S1 available in the Supplemental Data with this article online). Though this difference is likely crucial for Stl binding and function, it is observed in both the RNAP-Stl complex and the apo-holoenzyme structures of this crystal form. Therefore, straightening of the BH cannot be considered as a consequence of Stl binding. The experimental difference electron density (ED) map (Figure 1B) revealed a clear ED for Stl and local but marked alterations of the RNAP structure likely induced by Stl binding. First, two β subunit loops designated as STL1 (β538–552) and STL2 (β557–576) and the central portion of the BH move by ∼2 Å toward the Stl molecule to form a compact Stl binding site. Second, the trigger loop (TL, β932–1139) (Vassylyev et al., 2002) is displaced from its position in the apo-holoenzyme by the tetramic acid moiety of Stl (Figure 1), and its C terminus becomes disordered. The refinement of the RNAP-Stl complex converged to a final R factor of 23.0% (R free = 26.8%) at 2.4 Å (Table 1 and Figure S1). In the RNAP-Stl complex, Stl binds ∼20 Å away from the active site (Figures 1B and 2) along the BH, which is located at the junction between the downstream DNA and the RNA-DNA hybrid.

Stl Binding to RNAP

The Stl binding site is formed on the downstream DNA side of the BH and, in agreement with the previous studies (Heisler et al., 1993; Severinov et al., 1993, 1995; Yang and Price, 1995), comprises residues from both β and β′ subunits (Figure 2). This site can be divided into two subsites. The first, to which the streptolol moiety of Stl (Figures 1A and 2) is tightly bound through many hydrophobic interactions, is formed by STL1 and STL2 and the N-terminal portion of the BH (β769–782). The second subsite accommodates the bulky tetramic acid groups on the opposite side of the Stl molecule and consists of the central portion of the BH (β789–798) and the ordered segment of the TL; only a few contacts between RNAP and one (acetamide) out of three bulky branched tetramic acid groups are seen. The sugar makes no contacts with the protein and appears to be dispensable for binding—in fact, its presence may disfavor binding by competing for space with the disordered portion of the TL, which has no Stl binding determinants and would displace Stl to attain its original, more stable conformation (see below). The formation of a hydrogen bond between β Asn792 and the N2 atom of the acetamide group seems critical for the binding of the tetramic acid moiety of Stl (Figure 2). Indeed, RNAP is significantly less sensitive to the Stl analog tirandamycin, which lacks the acetamide group (Reusser, 1976), whereas the N792G substitution confers strong resistance to Stl (Figure 2A) (Yang and Price, 1995). Overall, the structure suggests that the Stl affinity for RNAP is largely determined by the streptolol group, whereas tetramic acid is lacking highly specific binding sites on the protein surface and thus contributes little, or might even weaken, the overall network of the RNAP-Stl interactions.

The Stl binding site is highly conserved among bacteria (Figure 2A), indicating that structural results obtained with T. thermophilus RNAP will be applicable to RNAPs from other bacterial species. To validate the structural model and to confirm the functional significance of Stl-RNAP interactions, structure-based E. coli RNAP mutants were constructed. In βL565–568G, four STL2 residues that form a portion of the hydrophobic streptolol binding pocket were replaced by glycine; in
<table>
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<th>Table 1. Data Collection and Refinement Statistics</th>
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<td><strong>Apo-Holoenzyme</strong></td>
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<td><strong>Resolution (Å)</strong></td>
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<td><strong>Reflections (total/unique)</strong></td>
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<td><strong>l/c (°)</strong></td>
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<td><strong>Rmerge (%)</strong></td>
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<td><strong>Completeness (%)</strong></td>
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<td><strong>Rfactor (%)</strong></td>
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<td><strong>Reflections used</strong></td>
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<td><strong>Rrmsd bond angles (°)</strong></td>
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<tr>
<td><strong>Rrmsd bond length (Å)</strong></td>
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<td><strong>Rrmsd improper angles (°)</strong></td>
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<td><strong>Number of Stl atoms</strong></td>
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<td><strong>Number of active site Mg&lt;sup&gt;2+&lt;/sup&gt; ions</strong></td>
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<tr>
<td><strong>Number of water molecules</strong></td>
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<tr>
<td><strong>Number of other Zn&lt;sup&gt;2+&lt;/sup&gt; ions</strong></td>
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<sup>a</sup> The crystals belong to P3<sub>2</sub> space group with a perfect (50%) merohedral twinning mimicking P6<sub>5</sub> space group. The data were therefore processed in P6<sub>3</sub> space group and were expanded to P3<sub>3</sub> space group for refinement.

<sup>b</sup> The data for the highest resolution shell are shown in brackets.

<sup>c</sup> The water molecules were added by the standard alternating cycles of the water pick (water molecules were picked at 3<sub>σ</sub> level in the |2Fobs − Fcalc| ED map and water delete (water molecules with correlation coefficients less than 0.45 and peak heights less than 0.12 σ in the |Eobs − Ecrys| ED map were deleted) procedures. A relatively large number of water molecules may be explained by the unique, unusually strong negative charge on enzyme surface likely attracting binding of the solvent molecules. Very mild, nearly physiological conditions in which crystals were grown might have additionally allowed for better specific hydration of the RNAP molecules.

βR548A, the residue that forms one of the two polar contacts with Stl is replaced with Ala (Figures 2B and 2C). In full agreement with structural predictions, the βR548A and βR548A enzymes were resistant to Stl (Figure 3A).

We have also constructed deletion β¹<sup>1238–1254</sup> of the TL in T. aquaticus RNAP (ΔTL, β¹<sup>1238–1254</sup> in E. coli; such a deletion could not be constructed in E. coli due to its high toxicity). The ΔTL enzyme displayed a substantially slower catalysis rate than the wild-type (wt) RNAP, implying an important role for the TL during transcription. Surprisingly, Stl stimulated rather than inhibited the ΔTL RNAP (Figure 3B). This suggests that although Stl still binds to ΔTL RNAP, its inhibition-inducing contacts with the enzyme are lost, and new interactions, which compensate in part for the TL absence, are made instead. Significantly, the response of ΔTL RNAP to Stl occurred at much lower (compared to the amido side chain of Asn (Figure 2). β¹<sup>N792D</sup> E. coli RNAP was constructed and was found to be ~75-fold more sensitive to Stl than the wt enzyme (Figures 3A and Figure S2). This suggests that the Asn residue could be in part responsible for the lower sensitivity of RNAPs from Gram-negative bacteria to Stl.

The data on the Stl effects on eukaryotic RNAPs are quite limited and contradictory (Logan et al., 1989; Reussner, 1976), leaving the question of whether Stl is specific for the bacterial RNAPs open. Structural and sequence comparisons show that whereas Stl binding segments in prokaryotic and eukaryotic RNAPs are well conserved, the STL1 and STL2 motifs are more divergent. Most notable are the ΔS453K and ΔF545A substitutions in the eukaryotic enzymes (Figure 2A) that likely preclude Stl binding because substitutions of these residues for, respectively, more and less bulky amino acids, confer strong Stl resistance in E. coli (Figure 2A).

Possible Pathways of Stl Action
As Stl does not induce dissociation of nucleic acids from RNAP (Siddhikol et al., 1969), it must inhibit a step(s) of the NAC. Stl may interrupt the NAC in several
Control of Transcription by Streptolydigin

Figure 2. The Stl Binding Site
(A) Alignment of regions of the $\beta$ and $\beta'$ subunits of RNAP that comprise the Stl binding site. Sequences used are E. coli (Ec), T. thermophilus (Tt), T. aquaticus (Ta), Bacillus subtilis (Bs), Bacillus anthracis (Ba), Mycobacterium tuberculosis (Mt), Chlamydia trachomatis (Ct), Neisseria meningitidis (Nm), Staphylococcus aureus (Sa), Brucella melitensis (Bm), Homo sapiens (Hs), and S. cerevisiae (Sc). Homology/identity level in the Stl binding region for RNAPs used in our experiments is indicated on the right side of the alignment. Residues that form van der Waals (blue) and polar interactions (green) with Stl in the bacterial enzymes and their important substitutions in eukaryotic RNAPs (red) are highlighted with colored boxes. Stl-resistant mutations in bacteria are indicated with arrows (Heisler et al., 1993; Severinov et al., 1995; Yang and Price, 1995).

(B) The Stl binding site. Structural elements of the $\beta$ (yellow) and $\beta'$ (white) subunits are shown as ribbon diagrams. The residues making hydrophobic (cyan) and polar (green) interactions with Stl (magenta) are shown as a balls-and-sticks model.

(C) Schematic drawing showing the RNAP-Stl van der Waals interactions (blue dashed lines) and hydrogen bonds (green arrows).

ways. First, it may directly compete with substrate binding, thereby decreasing substrate affinity for RNAP.

Second, Stl may inhibit the translocation reaction.

Third, Stl binding may induce a long-distance allosteric signal that would modulate the RNAP catalytic groups, for example by decreasing affinity of the active site Asp residues for the catalytic Mg$^{2+}$ ions, thereby directly inhibiting phosphodiestere bond formation. Fourth, the Stl binding may block structural isomerization of the RNAP active site from an inactive to an active conformation.

Stl Does Not Affect Substrate Binding but Decreases the Maximal Rate of Nucleotide Addition

Steady-state abortive initiation assays of the E. coli enzyme inhibition by Stl (Cassani et al., 1971; McClure, 1980) suggested a complex mechanism: Stl acted as a competitive inhibitor on some templates but as a non-competitive inhibitor on others, and the $K_i$ values were different depending on the NTP substrate used. This led McClure to suggest that Stl could both compete with the triphosphate binding and inhibit the product release. The complexity of the abortive initiation cycle, in which RNAP must bind the template DNA, form the open complex, bind two substrate NTPs (simultaneously or sequentially), form a phosphodiestere bond, and release the abortive products (with or without DNA translocation) many times over the course of the reaction could significantly complicate the elucidation of the inhibition mechanism. In contrast, pre-steady-state kinetic analysis of stable ECs allows us to monitor single-nucleotide addition reaction and thus to discern Stl effects on the substrate affinity and the rate of catalytic reaction; in the simplest case of a posttranslocated EC, even the translocation step shall be dispensable for substrate incorporation into the nascent RNA.

We assembled stable T. aquaticus EC on a synthetic scaffold (Supplemental Data) and monitored the extension of the radioactively labeled nascent RNA by one nucleotide using a quench-flow device. We found that although Stl reduced the rate constant of nucleotide addition ($k_{app}$), it did not significantly alter the substrate binding affinity ($K_i$) (Table 2). These results indicate that, in agreement with structural data, Stl neither
necessarily required for cleavage reactions.

posttranslocated) states, and (2) translocation is not

(1) these reactions must initiate from different (pre- or

location by Stl as the sole mode of Stl action, because

nov et al. [2003] argues against the inhibition of trans-

and exonucleolytic RNA cleavage; Figure 4A and Sosu-

RNAP (nucleotide addition, pyrophosphorolysis, endo-

over, the fact that Stl inhibits all catalytic reactions of

posed translocation mechanism exists thus far. More-

2002). In particular, the BH

in translocation (Cramer et al., 2001; Vassylyev et al.,

affects the BH and TL, which are thought to participate

consistent with observations that the inhibitor binding

The hypothesis that Stl blocks RNAP translocation is

Stl Does Not Inhibit DNA Translocation

The hypothesis that Stl blocks RNAP translocation is

occupies nor allosterically modifies the primary sub-

strate binding site on RNAP.

Stl Does Not Inhibit DNA Translocation

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Table 2. Influence of Stl on the Velocity of NTP and α-thio-NTP Addition by Wt RNAP and ΔTL and of Intrinsic Cleavage by the Wt Enzyme

<table>
<thead>
<tr>
<th>RNAP</th>
<th>NTP</th>
<th>k_{app} min^{-1}</th>
<th>K_{cat} μM</th>
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<tr>
<td></td>
<td>Stl, 0 γ/mL</td>
<td>Stl, 100 γ/mL</td>
<td>Stl, 0 γ/mL</td>
</tr>
<tr>
<td>ΔTL</td>
<td>CTP</td>
<td>0.25</td>
<td>0.59</td>
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<tr>
<td>Wt</td>
<td>CTP</td>
<td>3200</td>
<td>0.27</td>
</tr>
<tr>
<td>Wt</td>
<td>α-thio-CTP</td>
<td>2.4</td>
<td>0.012</td>
</tr>
<tr>
<td></td>
<td>Wt</td>
<td>3.6</td>
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</table>
Stl Does Not Target the Phosphodiester Bond Formation

Although Stl is positioned far away from the RNAP active site Asp residues, it could modulate catalysis allosterically. To investigate the effect of Stl on the phosphodiester bond formation, we used a slow-hydrolysable NTP analog, α-thio-NTP, which is thought to decrease the efficiency of nucleophilic attack by the nascent RNA's 3′ OH due to the lesser electronegativity and a larger size of sulfur compared to oxygen (Kaushik et al., 1996; Polesky et al., 1992). We found that at conditions when RNAP was saturated with either α-thio-NTP or the corresponding NTP, α-thio-NTP was incorporated more than three orders of magnitude slower than the NTP (Table 2). The large magnitude of the effect suggests that the chemistry of the catalysis is limiting the incorporation of NMP in the nascent RNA. In contrast, in the presence of Stl, the rate of incorporation of α-thio-NTP (as compared to that of the NTP) was decreased by only 20-fold. Barring the potential complications from steric effects of Stl binding, these results suggest that the chemical step is no longer rate limiting in the presence of the inhibitor (Kaushik et al., 1996; Polesky et al., 1992). These results are consistent with our structural data, which show that the RNAP active groups (catalytic Asps and Mg2+ ion) remain unaltered in the RNAP-Stl complex structure, as compared to that of free RNAP.

Active Site Isomerization Is a Likely Stl Target

The pronounced effect of Stl on the rate of the catalytic reaction along with the apparent lack of its effects on substrate binding, phosphodiester bond formation, and DNA translocation leave practically the only possibility for the inhibitor action: Stl may stabilize some inactive transcription intermediate, thereby precluding structural isomerization of RNAP to an active state. The ExolII footprinting analysis provides evidence for such a mode of the Stl action and additionally suggests that Stl stabilizes the complex in which the substrate selection did already occur. Indeed, addition of the cognate nonhydrolysable NTP shifted translocation equilibrium toward the posttranslocation state in the presence or in the absence of Stl, whereas the RNA extension was strongly inhibited by Stl (Figure 4D). Together with the lack of Stl effect on phosphodiester bond formation (Table 2), this strongly indicates that Stl freezes an inactive substrate intermediate equally stable to that of the catalytically active IS, in which the substrate would maintain the base pairing with the acceptor template DNA base, thereby stabilizing the posttranslocation conformation.

The experimental structural data revealed the substrates bound to three distinct sites (E site, PS, and IS) in the multisubunit RNAP ECs (Kettenberger et al., 2004; Westover et al., 2004). To understand which of the observed substrate intermediates would be likely affected by the Stl binding to presumably stabilize the inactive RNAP conformation, we superimposed these three eukaryotic structures as well as the bacterial RNAP-Stl complex and carried out detailed comparative structural analysis of the resulting bacterial RNAP model with bound substrates and Stl. The superposition of the eukaryotic complexes and the bacterial RNAP structure involved the Cα positions of the atoms from the BH, TL, STL2 (which are all likely fixed/displaced by the Stl binding in the bacterial system), and the active site Asp residues and showed the high structural homology of these structural fragments as revealed by the root-mean-square deviations of ~1.5 Å, suggesting that the conclusions drawn based on the analysis of the eukaryotic substrate complexes would be applicable to the bacterial enzyme and subsequently to the Stl mechanism.

The Substrate Preinsertion Complex

Based on the structural analysis, we argue that the substrate PS is the most likely Stl target (Figure 5A). Indeed, in this site, the substrate binds to the posttranslocated complex with high specificity as revealed by multiple polar and van der Waals interactions with the protein (Figure 5A) and by the base pair formed with the DNA template, whereas its phosphate groups are too far from the RNAP catalytic residues to achieve the reaction. Moreover, inspection of the preinsertion complex structure revealed that the three structural segments (the BH, TL, and STL2) that are structurally conserved between eukaryotic and bacterial RNAPs (Figure 5A) and that are likely affected by Stl in the bacterial enzyme are positioned near the bound substrate (Figures 5A and 5B). Among these, the BH and the TL are located within the interacting distance (3.3 Å and 4.2 Å, respectively), whereas STL2 is more distant (~5.5–6.5 Å) from NTP. However, a subtle (~2.5 Å) movement of STL2, which might not be detectable in the low-resolution structure of the preinsertion complex, would bring it into direct contact with the substrate.

The Substrate Entry Complex

In the light of the direct observation of the specific substrate binding in the PS, the existence of an alternative pathway (through the E site) for the substrate loading into the IS avoiding the PS step in the same enzyme (Westover et al., 2004) looks puzzling, particularly because the substrate in the E site does not have any rNTP-specific interactions. Given, however, that both the PS and the E site are validated by the experimental data, it would be reasonable to presume that the substrates bound to these sites represent two consecutive intermediates of the same process—loading of the substrate into the IS. In this scenario, the binding to the E site is likely the first step, as otherwise we have to...
Figure 4. Stl Does Not Inhibit Translocation

(A) Stl inhibits catalysis, pyrophosphorolysis, and endonucleolytic RNA cleavage by T. aquaticus RNAP. Scaffold ECs were incubated with 1 mM CTP, 5 mM sodium pyrophosphate (PPi), 20 pM T. aquaticus GreA, or in the absence of additives at 40°C for times indicated above each lane. Lanes 1 and 21 contain the starting EC. Stl was added at 100 μg/mL where indicated (lanes 21–40).

(B) Effect of pyrophosphate on EC14 and EC15. EC14 (lanes 1–9) and EC15 (lanes 10–12) were incubated with 50 μM Pi for 1–10 min at 60°C in the presence or absence of 100 μM of Stl. NTPs produced during the course of the pyrophosphorolysis reaction were readily incorporated into the transcript, leading to the extension of the 14-mer RNA (lanes 3–5).

(C) Inhibition of the T. thermophilus EC by Stl. EC14 and EC15 (1 μM) were incubated for 5 min with the indicated concentrations of Stl and extended with 10 μM ATP for 5 min at room temperature.

(D) ExoIII footprinting of the ECs. The nontemplate DNA strand and 13-mer RNA of EC13 were 5' end labeled. Stl was added to 100 μg/mL. 1 mM NTPs (where indicated) were added for the indicated times at 40°C. One unit of ExoIII (New England Biolabs) was added to each 15 μl reaction, followed by a 1 min incubation at 40°C before addition of the stop solution. The relative distribution of the front boundaries of the EC is shown by bar graphs above each gel panel.

assume that the base- and ribose-specific interactions established with the substrate in the PS would then be disrupted upon the substrate movement to the E site. The role of the initial substrate binding to the E site would be to shift equilibrium from the pre- to the posttranslocation state (Figure 4D). Although binding of any (cognate or noncognate) substrate to the E site may favor this shift, only cognate NTP would be able to stabilize the posttranslocated state through base pairing with the DNA template, consistent with the mechanism previously suggested for T7 RNAP (Huang and Sousa, 2000). However, this base pair cannot be formed in the
The pretranslocated state and therefore cannot be the driving force for translocation.

In the pretranslocated complexes, the substrate has little chance to bind directly to the PS and subsequently to stimulate translocation, for this will require a disruption of the base pair formed by the acceptor DNA template base in the (n + 1) register with the nontemplate strand. In contrast, the triphosphate moiety of the substrate bound to the E site may efficiently compete with the monophosphate of 3′ RNA nucleotide for the Mg²⁺-
mediated binding to the active site in register n (overlapping with the E site), thereby stimulating translocation. In posttranslocated complexes, the substrate might be directly loaded into the PS, bypassing the E site.

Structural superposition shows that the E site and the PS are overlapping and that the proposed pathway of the substrate loading from the E site to the IS by a single rotation (Westover et al., 2004) would first bring the NTP into the PS, where it would be likely fixed, given that the acceptor DNA base is in preinsertion orientation (Figure 5C). The proposed two-step substrate preinsertion mechanism (Figure 5D) resolves the principal discrepancy between the E site and the PS and harmonizes these originally mutually exclusive models.

The Substrate Insertion Complex
Theoretically, the IS substrate complex might be also affected by Stl; the Stl binding may alter the conformation of the RNAP structural fragments required for the proper positioning of the NTP in the IS, thereby inactivating the transcription complex. However, analysis of the IS substrate complex structure (Westover et al., 2004) shows that all structural segments (BH, TL, and STL2) likely affected by the Stl binding are located too far from the substrate to form direct interactions (in contrast to their proximity to the NTP bound in the PS), making this hypothesis unlikely. Surprisingly, comparison of the PS and IS complexes indicates that in the absence of the above interactions, as well as the ribose-discriminating contacts, the NTP in the active IS appears far more loosely bound to the protein than in the inactive PS—an observation that apparently contradicts the general structural principles of the substrate recognition and enzymatic reaction (see below). This inconsistency is likely attributed to a low resolution of the IS structure, in which subtle local rearrangements in RNAP might not be revealed by the ED due to a model bias and/or unavailability of the crystallographic refinement. It also further supports a recent hypothesis (Kettenberger et al., 2004) that a structural rearrangement ("closing") of the active cleft might accompany delivery of the substrate from the PS into the IS in multisubunit enzymes in a manner analogous to that found in T7 RNAP (Temiakov et al., 2004; Yin and Steitz, 2004) and template-independent RNAPs (Tomita et al., 2004; Xiong and Steitz, 2004). This structural transition would most likely involve the structural motifs already interacting with the substrate in the PS.

Plausible Mechanism of Inhibition of Transcription by Stl
Biological reactions catalyzed by various enzymes require a very precise arrangement of the reacting groups/substrates that is normally achieved through the formation of the compact closed conformation of the protein induced in many cases upon the substrate(s) binding to provide the best match between the substrate and the enzyme, thereby discriminating against noncognate substrates and increasing the rate of the catalytic reaction. As compared to other systems (in particular the closely functionally related single subunit RNAPs, template-independent RNAPs, and DNA polymerases), the active site of multisubunit RNAPs in all the available structures appears too open to provide an extensive network of specific interactions that are likely required for catalysis. By analogy with single-subunit T7 RNAP (Temiakov et al., 2004; Yin and Steitz, 2004), the binding of the substrate to the inactive RNAP conformation (PS) might be essential not only for the substrate selection and/or delivery to the IS but also to initiate a structural transition of RNAP to the active, presumably more compact configuration. In this scenario, the flipping (which is likely blocked by the Stl binding, Figure 2) of β Asn792 out of the BH, which is in close vicinity to the DNA acceptor template base in the eukaryotic preinsertion complex, may initiate substrate delivery from the PS to the IS by pushing the DNA base toward the active site and/or may stack on this base constituting a portion of the compact IS to provide better match of the substrate/DNA template base pair.

Altogether, the RNAP-Stl complex structure, biochemical data, and structural considerations suggest that in the presence of Stl, the incoming substrate may first bind to the RNAP E site that likely stimulates DNA translocation from the pre- to the posttranslocated state, after which NTP would be loaded into the inactive PS, where it forms a base pair with the DNA template that is crucial for both substrate selection and stability of the posttranslocated conformation. We suggest that Stl binding freezes this nonproductive intermediate presumably through restraining the mobility of the BH, TL, and STL2 motifs, which might assist the substrate delivery to the IS and/or RNAP isomerization to a more compact, active configuration (Figure 5D).

This discussion has been limited so far to the effect of Stl on nucleotide addition, whereas Stl strongly inhibits all catalytic reactions of RNAP. Assuming that all these reactions are mediated by the same active site (Sosunov et al., 2005) and/or can only occur in the closed, active state of the enzyme, the mechanism in which Stl freezes several structural elements in a nonproductive state explains why all these reactions are inhibited by the antibiotic.

Although the suggested mechanism of the Stl action is consistent with all available structural and biochemical data, the proposed conformational transition remains hypothetical. In this respect, the only decisive experimental data validating the Stl mechanism would be the crystal structures of the substrate bound ECs determined with and without Stl. We now have the crystals of the bacterial EC diffracting at the atomic resolution (D.G.V. and D.T., unpublished data) and consider the aforementioned complexes as our next target.

Concluding Remarks
Our results suggest several modifications leading to the design of new, not yet tested more efficient Stl derivatives. First, truncation of the sugar moiety might increase the affinity, avoiding competition with the trigger loop. Second, removal of the tetramic acid moiety may also increase affinity, as most of the RNAP contacts are located in the streptol tremoy moiety. Third, tetramic acid might be substituted for a chemical group that would not likely affect drug binding but might improve its cel-
lular permeability, for example for a rifamycin-like tail (correlations between the tail structure and drug permeability have been previously reported for rifamycins) (Brufarmi et al., 1982).

Another important issue raised by this work is that the substrate PS rather than the catalytic center is likely modulated by the inhibitor, and thus, the PS emerges as an attractive target for the design of novel antibiotics. Interestingly, α-amanitin, a highly specific eukaryotic toxin, occupies the binding site that is proximal to that of Stl and observed PS (Bushnell et al., 2002). Consequently, the mechanism of α-amanitin may also affect an active site isomerization analogous to that of Stl, in addition to its proposed effect on translocation (Bushnell et al., 2002). Notably, a large portion of the α-amanitin binding site is structurally divergent between bacterial and eukaryotic RNAPs—which may allow for the design of a bacterial-specific antibiotic with an α-amanitin-like mechanism.

Experimental Procedures

Structure Determination and Refinement
The T. thermophilus RNAP holoenzyme was purified and crystalized as described previously (Vassylyeva et al., 2002). To obtain the complex crystals, the crystals of the apo-holoenzyme were transferred for 8 hr into the drops containing harvest buffer and 2 mM of Stl. The data were collected at beam line BL5 at Photon Factory (Tsukuba, Japan) using an ADSC Quantum-315 CCD detector. Surprisingly, all the crystals, though belonging to the same space group, P32, exhibited somewhat distinct unit cell parameters, a = 240 Å, c = 253 Å, as compared to the previously studied T. thermophilus RNAP crystals (a = b = 236 Å, c = 250 Å) (Artisimovitch et al., 2004; Vassylyeva et al., 2002; Vassylyeva et al., 2002). The data were processed by using the HKL2000 data-processing package (Table 1) (Otwinowski and Minor, 1997). Refinement was carried out by using the CNS program (Table 1) (Brunger et al., 1998). To achieve adequate comparison of the RNAP-Stl complex with the apo-holoenzyme and the high quality of the initial "experimental" omit difference electron density (ED) map for Stl in the complexes, we collected diffraction data and refined the structure of the native, apo-holoenzyme in this crystal form at a 2.8 Å resolution (R factor = 23.1%, R free = 26.8%) in addition to the RNAP-Stl structure refined at 2.4 Å resolution. The Stl model was built into the initial experimental difference ED map (Figure 1B). The rigid body refinement (R factor/R free = 31.0%/31.8%) followed by several rounds of the B factor, positional, simulated annealing refinement, and water "pick" and water "delete" procedures, alternating by manual model building using the program O (Jones et al., 1991) yielded a final R factor of 23.0% and R free of 26.8% for the RNAP-Stl complex (Table 1). The final model was of high quality as revealed by the simulated annealing omit ED map calculated for Stl (Figure S1). Structural figures were prepared by using the programs Molscript (Kraulis, 1991), Bobscript (Essnouf, 1999), and Raster3D (Merritt and Bacon, 1997).

Wt and Mutant RNAPs
Wt T. thermophilus core RNAP, His-6 wt E. coli core, and holo RNAPs and wt T. aquaticus core RNAPs were purified as described previously (Artisimovitch et al., 2003b; Kuznedelov et al., 2003; Vassylyeva et al., 2002). E. coli RNAP mutants were constructed by site-directed mutagenesis in pΔ545 (PomCA)[His6]SSA and PM-C [His6] L117E, and plRLO62 (PomCA)[His6] 32P[ATP]. T. aquaticus mutant ΔSLT was obtained by substituting residues 1238-1254 to Gly in a coexpression system for recombinant RNAP (Kuznedelov et al., 2003). His-6 mutant RNAPs were purified by chromatography on Ni-NTA (Qiagen), Heparin HiTrap, and Q FF-Sepharose columns.

Transcription Conditions
ECS were assembled with wt and ΔSLT T. aquaticus RNAPs as described (Sidorenkov et al., 1998) with the only exception that transcription buffer was 40 mM Tris (pH 8.0), 40 mM NaCl. RNA was labeled at their 5’ end by using [32P]ATP (PerkinElmer) and T4 polynucleotide kinase (New England Biolabs). Stl was added before the reactions for 5 min at 40°C. Reactions were initiated by the addition of 10 mM MgCl2 (Sigma) and different concentrations of NTP, 20 μM T. aquaticus GreA (kindly provided by L. Minakhin), and 5 mM sodium pyrophosphate (Sigma). Reactions were incubated at 40°C for times indicated in the figures and stopped by the addition of formamide-containing buffer. Products were resolved in the denaturing acrylamide gels and visualized by Phosphoimager TM (Amersham).

Presteady state kinetics experiments were performed by using a KinTek Chemical Quench Flow Model RQF-3 mixer (KinTek Co., Austin, TX). The settings and calibration of the Quench Flow device were accomplished according to the standard procedure suggested by the manufacturer. One of the two 10 μl sample loops was loaded with solution containing various concentrations of CTP and 10 mM MgCl2, the other loop was loaded with EC assembled as above and eluted from the solid phase with 50 mM imidazole. The reactants were mixed in the reaction loop, and the reaction was allowed to proceed for the specified times and stopped by addition of 0.5 M EDTA. A control experiment where 0.5 M EDTA was provided through a sample loop and the CTP/MgCl2 solution through a quench line showed that reactions were fully quenched in less than 5 ms. The reaction products were then analyzed by using Phosphoimager. The calculated Kapp and Kds were in a good agreement with published data on presteady-state kinetics analysis of a stalled EC (Foster et al., 2001).

Supplemental Data
Supplemental Data include Supplemental Experimental Procedures and three figures and are available with this article online at http://www.molecule.org/cgi/content/full/19/5/655/DC1/.

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