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Conformational coupling, bridge helix dynamics and active site dehydration in catalysis by RNA polymerase

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ABSTRACT

Molecular dynamics simulation of *Thermus thermophilus* (Tt) RNA polymerase (RNAP) in a catalytic conformation demonstrates that the active site dNMP–NTP base pair must be substantially dehydrated to support full active site closing and optimum conditions for phosphodiester bond synthesis. *In silico* mutant β R428A RNAP, which was designed based on substitutions at the homologous position (Rpb2 R512) of *Saccharomyces cerevisiae* (Sc) RNAP II, was used as a reference structure to compare to Tt RNAP in simulations. Long range conformational coupling linking a dynamic segment of the bridge α-helix, the extended fork loop, the active site, and the trigger loop–trigger helix is apparent and adversely affected in β R428A RNAP. Furthermore, bridge helix bending is detected in the catalytic structure, indicating that bridge helix dynamics may regulate phosphodiester bond synthesis as well as translocation. An active site "latch" assembly that includes a key trigger helix residue Tt β′ H1242 and highly conserved active site residues β E445 and R557 appears to help regulate active site hydration/dehydration. The potential relevance of these observations in understanding RNAP and DNAP induced fit and fidelity is discussed.

1. Introduction

Multi-subunit RNA polymerases (RNAPs) synthesize RNA from a DNA template, but many features of the catalytic mechanism and its control remain unknown. For instance, although RNAP functions in an aqueous environment, the importance of active site hydration and dehydration in catalysis has not been fully described. X-ray crystal structures of ternary elongation complexes (TECs) reveal closed and open conformations of the RNAP active site [1–4]. Closing and opening, which are expected to affect, and to be affected by, hydration, rely on the conformation of the trigger helices–trigger loop assembly (*Thermus thermophilus* (Tt) RNAP β′ 1220 to 1265), which can assume a helical (closed, catalytic) or more looped (open, low activity) conformation. To underscore the importance of active site closing for catalysis, the antibiotic streptolydigin binds to a Tt RNAP TEC with an open trigger loop [2], and the potent Sc RNAP II inhibitor α-amanitin binds to a TEC with an open "wedged" conformation of the trigger loop [1]. In the catalytic structure, the closed trigger helices pack closely with the bridge α-helix, which is a prominent and dynamic feature of RNAP that borders the active site [1,5,6]. It has been suggested that bridge helix dynamics, which is expected to be regulated by trigger loop opening and closing, may provide the thermal driving force for translocation of nucleic acids through RNAP [7–9].

Water can regulate the specificity and fidelity of enzymatic reactions [10–15], and many enzymes including RNAP have a buried active site that can close to exclude water [2,3,16]. For example, the active site of DNA polymerase (DNAP)-β is substantially dehydrated by active site closing [16], and, in this manner, dehydration could potentially be a determining factor in catalysis and fidelity. Unfortunately, however, biochemical techniques are somewhat limited in their capacity to analyze water-mediated effects. For instance, lower resolution crystal structures may not account for bound waters, and loosely bound water may not be observed crystallographically. To

Abbreviations: TEC, ternary elongation complex; RNAP, RNA polymerase; DNAP, DNA polymerase; wt, wild type; Tt, *Thermus thermophilus*; Sc, *Saccharomyces cerevisiae*; Ec, *Escherichia coli*; AMPcPP, α,β-methylene adenosine triphosphate

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expose RNAP internal sites to hydration, therefore, the TEC structure was immersed in explicit water and subjected to full atomistic molecular dynamics simulations, as has previously been done for Sc RNAP II, T7 RNAP, and DNAPs [17–21]. In this work, simulations strongly indicate that water exclusion from the closed RNAP active site localized to the dNMP–NTP base pair is a determining factor in catalysis. In closing the active site, key trigger helix residues perform a central function in dehydration. In enzymatic mechanisms "induced fit" refers to the cooperative interaction between enzyme and substrates to accurately align reactive groups for chemistry. The current work strongly indicates that dehydration within an enclosed active site provides an essential component of induced fit mechanisms and that, for RNAPs, excessive localized hydration slows elongation by destabilizing the active site base pair, indicating that hydration/dehydration may be important in control of transcriptional fidelity.

A highly conserved residue Tt β R428 (corresponding to *Saccharomyces cerevisiae* (Sc) Rpb2 R512) is located about 20 Å from Mg^{2+}-I and just C-terminal to the fork loop. The Sc Rpb2 R512C mutation was initially identified in a genetic screen for suppressors of *ssu72-2*, which encodes a catalytically impaired form of the RNAP II carboxy-terminal domain (CTD) serine 5 (S5) phosphatase [22–24]. The CTD is a repeating heptapeptide unit (26 repeats in Sc) of consensus 1-YSPTSPS-7. The cyclin-dependent kinase, Kin28, which is a component of the RNAP II transcription initiation factor IIH, phosphorylates S5 of the CTD, and the S5-phosphate is removed by the Ssu72 and Rtr1 phosphatases [25,26]. The Rpb2 R512C substitution may result in slowing RNA synthesis during early elongation. Presumably, the reduction in elongation rate allows sufficient time for the *ssu72-2*-encoded phosphatase to remove S5-phosphate, thereby allowing RNAP II to pass through transcription cycle checkpoints. Consistent with this interpretation, *rpb2-R512C* mutants exhibit defects in transcription in vivo [22] and in vitro [23,27]. The transcriptional defects of R512C and R512A substitutions are very similar [27].

The β R428A *in silico* substitution in Tt RNAP was designed based on the Sc RNAP II Rpb2 R512C/A replacements, and Tt R428A provides an essential comparison to wild type (wt) RNAP in molecular dynamics simulations. Although Sc RNAP II (PDB 2E2H), with a closed trigger loop and loaded NTP analogue, might be a reasonable choice for simulations, we selected Tt RNAP (PDB 205J) because its structure was determined at a higher resolution, showing a straighter bridge helix and more planar geometries of active site i and i + 1 base pairs (in the catalytic structure, i indicates the position of the 3′-end of the RNA, and i + 1 indicates the position of the dNMP–NTP base pair). In addition, on inspection of the closed Sc and Tt TECs, the Tt RNAP active site appeared to show more favorable atomic contacts than the corresponding Sc RNAP II structure [2,3]. The closed trigger helix conformation was selected because we reasoned that the catalytic TEC might be more sensitive to mutation than an open, relaxed TEC. Comparing simulations of wt and β R428A Tt RNAP indicates: 1) long range conformational coupling between the bridge helix, the extended fork region, the active site and the trigger helix; 2) the importance of localized active site dehydration in catalysis and fidelity; 3) the importance and potential complexity of bridge α-helix dynamics; and 4) mechanisms for streptolydigin and microcin J25 inhibition of Tt RNAP and α-amanitin inhibition of Sc RNAP II.

2. Materials and methods

2.1. Purification of Sc RNAP II

Yeast strains YZS84 (*RPB2*) and YDP19 (*rpb2-100*), which encodes Rpb2 R512C [22], were used to TAP-tag the C-terminus of the RNAP II Rpb9 subunit, as described [28]. Whole-cell extracts from the resulting strains were prepared as described [29] and used to purify wt RNAP II and the Rpb2 R512C RNAP II complex, according to [28]. Purified RNAP II was dialyzed against buffer consisting of 20 mM HEPES, pH 7.6, 20% (v/v) glycerol, 10 mM EGTA, 10 mM $MgSO_2$, 5 mM dithiothreitol, and 1 mM phenylmethylsulfonylflouride and stored in aliquots at −80 °C.

2.2. In vitro TEC assembly

In vitro assembly of Sc RNAP II TECs was done as described [30–33]. The sequence of G9 RNA is 5′-AUCGAGAGG-3′. The DNA non-template strand was 5′-biotinyl- GGTATAGGATACTTACGCCATCGAGAGGGACACGGTGAAAAGAGAACCCAAGCGACACC-3′ (G9-sequence is underlined) and the DNA template strand was 5′-GGTGTCGCTTGGGTTCTCTTTTCACCGTGTCCCTCTCGATGGCGTAAGTATCCTATACC-3′ (G9-complement sequence is underlined). TECs were immobilized on Promega streptavidin-coated Magnesphere beads.

2.3. Rapid chemical quench flow

In vitro transcription experiments with wt and Rpb2 R512C Sc RNAP II were done using rapid chemical quench flow with the Kintek RQF-3 instrument, essentially as described [27,34,35]. RNAs were labeled by addition of α^{32}P-GTP (800 Ci/mmol) for 10 min at 25 °C in transcription buffer containing 60 mM KCl and 8 mM $MgCl_2$. TECs were then set aside on ice until addition of ATP for the running start to A11. Using this protocol, only active TECs are tracked in the reaction because GTP must be incorporated to visualize RNA on gels. Running starts for ^{32}P-labeled G10 TECs were by addition of 5 μM ATP for 30 s on the bench top. During the 30 s incubation, samples were transferred to the left sample port of the KinTek RQF-3 (KinTek Corporation) rapid chemical quench flow instrument. Reagents for elongation were loaded at twice their working concentration in transcription buffer containing 60 mM KCl and 8 mM $MgCl_2$, in the right sample port. The valves to the sample ports were set to the "fire" position, and the computer-regulated mixing program was initiated. Reactions were stopped with EDTA (500 mM) or HCl (1 M), as indicated. RNA products were analyzed on gels.

2.4. Rationale and strategy for molecular dynamics simulations

These studies demonstrate the requirement for molecular dynamics simulations to adequately interpret mutational structure–function analyses and to gain insight into wt mechanisms. For instance, without dynamics, the analysis of Sc RNAP II Rpb2 R512C/A substitutions would not be complete. Simulations may be particularly informative because the starting Tt RNAP TEC structure (PDB 205J) is in a strained, catalytic conformation that responds very sensitively to the R428A substitution but is stable for wt RNAP. Although not yet done, simulation of R428A in a structure with a relaxed trigger loop conformation (PDB 205I or 2PPB) might be much less informative, because the R428A substitution may not as strongly disrupt an already relaxed TEC. For instance, opening the trigger loop is expected to remove constraints on bridge α-helix dynamics by reducing contacts between the bridge helix and the trigger helices [9]. Based on these results and ideas, we suggest that wt simulations may require a reference structure (in this case R428A) to be most useful and that the choice of starting wt structure (in this case a strained catalytic structure) may be crucial.

Molecular dynamics provides a model based on optimized force field assignments, and, therefore, results of simulations must be considered with some caution. Molecular dynamics provides a picosecond (ps) to nanosecond (ns) time-resolved atomistic model of protein structure and movement with explicit water hydration and counterions. Genetic, biochemical and structural approaches rely on ns scale atomistic events in a hydrated environment but are limited in their capacity to provide detail comparable to simulations. Using the