Unraveling the Role of Silent Mutation in the ω-Subunit of Escherichia coli RNA Polymerase: Structure Transition Inhibits Transcription

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Supporting Information

ABSTRACT: The bacterial RNA polymerase is a multi-subunit enzyme complex composed of six subunits, αββ′σω. The function of this enzyme is to transcribe the DNA base sequence to the RNA intermediate, which is ultimately translated to protein. Though the contribution of each subunit in RNA synthesis has been clearly elucidated, the role of the smallest ω-subunit is still unclear despite several studies. Recently, a study on a dominant negative mutant of rpoZ has been reported in which the mutant was shown to render the RNA polymerase defective in transcription initiation (ω6, N60D) and gave an insight on the function of ω in RNA polymerase. Serendipitously, we also obtained a silent mutant, and the mutant was found to be lethal during the isolation of toxic mutants. The primary focus of this study is to understand the mechanistic details of this lethality. Isolated ω shows a predominantly unstructured circular dichroism profile and becomes α-helical in the enzyme complex. This structural transition is perhaps the reason for this lack of function. Subsequently, we generated several silent mutants of ω to investigate the role of codon bias and the effect of rare codons with respect to their position in rpoZ. Not all silent mutations affect the structure. RNA polymerase when reconstituted with structurally altered silent mutants of ω is transcriptionally inactive. The CodonPlus strain, which has surplus tRNA, was used to assess for the rescue of the phenotype in lethal silent mutants.

INTRODUCTION

Molecular machines pass on the information stored in the genetic material to a functional protein, and the sequence of events is known as the central dogma. One of the key players of the information transfer is the enzyme RNA polymerase, catalyzing the first step transcription from DNA to mRNA. The mRNA molecule is then ultimately translated to protein molecules with the help of ribosomes and tRNA. Transcription is one of the key processes of the central dogma and is intimately tied to the ability of cells to "make decisions" about the genes that should be expressed. The bacterial RNA polymerase (RNAP) holoenzyme is a multi-subunit complex (αββ′σω) that performs the essential function of gene transcription. Given the role of RNA polymerase in the central dogma, it was assumed that all the subunits of RNA polymerase would be essential for the survival of the bacteria. However, this is not the case with the smallest subunit ω encoded by the rpoZ gene. ω is a small protein with 91 amino acids and has a molecular mass of 10 kDa.

With the increasing availability of the genome sequences of bacteria, archaea, and eukaryote, it has become clear that the ω-subunit is conserved in all branches of life. In eukaryotes, there exists a structural and functional homologue of ω, RPB6, which is found to be associated with RNAP I, II, and III. Further, in archaea, there exists the ω homologue termed RpoK. However, over the years, ω was unaccepted as an essential subunit in bacteria mainly because of the following two major observations. In vivo deletion of rpoZ is tolerated in bacterial cells unlike the other subunits of RNA polymerase. In addition, reconstitution of Escherichia coli RNA polymerase was achieved with purified α, β, and β′ subunits, thereby...
pointing at the redundancy of \( \omega \). Eventually, \( \omega \) was validated as the integral part of RNA polymerase machinery in vivo when the direct association of \( \omega \) with the \( \beta' \) subunit of RNA polymerase was shown. The subsequent crystal structure of *Thermus aquaticus* RNA polymerase supported this idea. Experiments with RNA polymerase isolated from \( \omega \)-less strain showed that the multi-subunit enzyme complex copurifies with GroEL, a global chaperone protein, whose removal results in loss of activity of the RNA polymerase enzyme complex. This observation indicates that GroEL substitutes for the function of \( \omega \) in protection and recruitment of \( \beta' \) during RNA polymerase assembly in a \( \omega \)-less strain. In support of this argument, it was observed in the RNA polymerase crystal structure that the \( \omega \)-subunit is latched on to the C-terminal of the \( \beta' \) subunit in such a fashion that a chaperone role of \( \omega \) in the folding \( \beta' \) subunit can be envisaged. Thus, to examine a system where the functional role of \( \omega \) can be elucidated without the effect of accessory protein(s), a toxic mutant screen was employed. Mutants were isolated in a way that they would thwart the entry of GroEL in RNA polymerase. This would render the RNA polymerase bound with mutant \( \omega \) (\( \omega_6\), N60D) inactive during the downstream process. This toxic mutant screen led to the serendipitous discovery of a silent mutant \( \omega_9 \), which was lethal. Interestingly, the silent mutant of \( \omega \) was structured as compared to wild type \( \omega \) protein, which is mostly disordered.

The genetic code is degenerate, which means that there are multiple codes for the same amino acid. Silent mutations occur when the change of the DNA sequence within a protein-coding portion of a gene does not affect the sequence of amino acids that make up the protein. This change typically takes place at the third position of the codon also known as the wobble position. A change in one or two nucleotides, however, does not inevitably alter the triplet’s meaning; the mutated triplet may still code for the same amino acid. Initially thought to be irrelevant, studies have shown that the occurrence of synonymous codons is nonrandom. In an organism, recurrently used codons are translated rapidly and are termed as frequent codons, while the scarcely used codons are translated at a slower rate and are known as rare codons. Thus, this
nonuniform distribution of codons in a gene and abundance of the corresponding isoacceptor tRNA hints at the nonuniform rate of mRNA translation.20–26 Protein expression studies in Escherichia coli suggested that translation kinetics and synonymous codon usage can affect protein folding and functions.27–32 There are also reports that have shown that silent polymorphism can lead to the altered structure and function of the protein.33

Anfinsen’s pioneering work showed that a polypeptide chain adopts the native structure, spontaneously, or in other words, the amino acid sequence determines the structure.34 Protein folding obeys a sequential model with a distinct path and intermediates. However, kinetic and thermodynamic parameters of this path are unamenable to measurements in vivo. Attempts to achieve refolding of denatured protein in vitro were partially successful.35 In vivo nascent proteins acquire native structures co- and post-translationally, with their half-lives in the range of seconds to minutes, whereas the rate of protein folding varies from seconds to hours in vitro.36–39

After the seminal experiment of Anfinsen on reversible denaturation of ribonuclease, many experiments have suggested cotranslational folding of proteins, small or large in vivo.24,40–45 Protein biosynthesis without cotranslational folding is likely to be energetically unfavorable.46 The addition of amino acid on a growing polypeptide chain is dependent on the readability of the frequency of codon, rare or frequent.24,44–46 Though recent studies suggest that silent mutations can affect the final protein structure, it is unknown if its position within the sequence is relevant. Silent mutation at different positions of the genes will address a few critical questions: (a) will a protein synthesized from silent mutation at different positions necessarily have the same structure as that of wild type protein? (b) does the substitution of a frequent codon to rare codon lead to drastic structure alteration? (c) what is the exact nature of the mutant proteins synthesized from silent mutation in varying positions?

In the present study, we generated several silent mutants by replacing frequent codons to rare codons at different positions of the rpoZ gene. This allowed us to determine the delicate balance that exists between the positional effects of codon usage in ω. The important function that was monitored here was the reconstitution of active RNA polymerase with ω variants.

### RESULTS

**Characterization of the Silent Mutant ωp.** The principal aim of this work was to understand the mechanism behind the structural changes in the ω silent mutant that leads to transcriptionally inactive RNA polymerase. We have shown before that a point mutation results in inactive RNA polymerase with concomitant changes in the helicity of the ω.16 We have also shown in a recent work that this structural alteration manifests themselves in the strength of binding of β′ with the ω-subunit.48 We would like to emphasize that the silent mutation here for alanine (GCC → GCT) showed no change in the protein sequence yet we observe a lethal phenotype upon induction of protein synthesis (Figure 1A). The ωp mutant was obtained as described in Sarkar et al.16 It must be mentioned here that, at the beginning, we ensured that the overexpression system was not harmful due to the aggregation of proteins and they were purified through overexpression plasmid pET-ω with the T7 promoter. However, we observed no difference in phenotype upon expression of native type and mutant proteins with various promoters. We did not want to alter the chromosomal copy of the rpoZ since the polar effect of the mutation plays an important role in Escherichia coli.

There are four codons coding for alanine, and the codon usage indexes for all of them are shown in Table 1. It has been reported before that concentration of the cognate tRNA for rare codon (GCT) is 5 times less concentration than the other tRNA for alanine.47,49 To evaluate the significance of this silent mutant, CD spectroscopy was performed with purified proteins (Figure 1B). We used CD spectroscopy to measure the amount of secondary structure in both wild type ω and silent mutant ωp. We observed that ωp is more structured (45% helical) than wild type ω (16% helical) (Figure 1C). Mass spectroscopic analysis of the purified protein was performed to eliminate the possibility of misincorporation of an amino acid at the mutation site (Figure S1).

**In Vitro Refolding To Determine the Minimum Energy Structure of a Protein with the Same Amino Acid Sequence.** As mentioned previously, it is established that “the final 3D structure of a protein is defined from its primary amino acid sequence in a given environment.”46 Here, it was observed that, despite possessing the same amino acid sequence, silent mutant ωp acquires a different structure from the wild type ω. The proteins were His-tagged and purified from the soluble cell extract fraction by Ni (II) affinity chromatography. We were interested to find out whether the altered CD profile is in a stable conformation, and thus, urea-dependent denaturation and renaturation experiments were performed. The proteins were completely denatured at time point 0 (urea concn 7 M) (Figure S2). At the 10th hour, the protein refolded back with removal of urea (urea concn 10 mM), as shown in Figure 2. Another ω-variant ωs, a lethal mutant (N60D) that shows α-helicity, was used as a control. ωp upon going through the urea denaturation–renaturation cycle reverted to its α-helical conformation. However, ωp reverted to a disordered state like native ω. This observation hints that purified ωp, to start with, was not in its stable conformation.

Subsequent analysis by size-exclusion chromatography experiments showed that the wild type, as well as mutant proteins, existed as monomers as shown in Figure S3. This rules out the possibility that the change in the structure of the protein is due to the change in the oligomeric status of the protein.

**Analyzing Codon Usage in rpoZ Gene.** We wanted to find out the influence of the rarity of the codons as well as their positions in shaping the secondary structure of the protein. Codon optimality represents a function of the stochastic nature.
of ribosome decoding of mRNA and the variability of tRNA concentrations. A codon can be defined either as a frequent or a rare codon depending on how efficiently the appropriate cognate tRNA can be selected from the cytoplasmic pool of tRNAs. As follows, codons can be a powerful determinant of translational rates. As shown in Figure 3, rpoZ, which remain a class I gene in Escherichia coli, hardly has any nonoptimal or rare codons. The rare codon for alanine, GCU, is present 5 times in the frame and are located close to each other (Figure 4A). Upon generation of silent mutation at the 82nd position, GCC to GCU, one more time, the rare codon for alanine is introduced proximal to the previously mentioned positions. Our subsequent attempt was to alter the next position, that is, 83rd for valine (GTT → GTA). We altered the third base to introduce the rare codon in place of a frequent codon for valine. However, mutation at this position did not show lethality in the growth curve upon protein induction or CD profile of the protein (Figure S4).

**Positional Effect of Introduction of Rare Codon in rpoZ.** It is well recognized that the local rate of translation correlates with the codon usage, which in turn correlates with tRNA population in cells. There is no absolute definition of the rare or frequent codon, but a relative definition can be described. As follows, we decided to investigate the effect of rare codons in rpoZ in Escherichia coli MG1655. Two rare codons, AGA and AGG, which code for arginine, occur 123 times in the essential genes and 4288 times in the entire genome. On the other hand, isoleucine encoded by AUA is another rare codon that occurs 148 times in essential genes as opposed to 5620 times in the entire genome. Codon usage indexes for all codons encoding arginine and isoleucine are presented in Table 2. Reports showed that these codons are translated at a lower rate. To our benefit, none of these rare codons are present in rpoZ, as shown in Figure 3. We generated a series of silent mutants with these rare codons introduced at a different position along the rpoZ. Such changes
allowed us to investigate the positional effect of a rare codon. Positions of silent mutants on the DNA sequence of rpoZ are shown in Figure 4A. They are equally marked in the overall structure of $\omega$ (Figure 4B), along with the list of mutants with codon positions and amino acids. The CD spectra for two mutants are shown in Figure 4C. Mass spectroscopic analysis was performed with purified protein to confirm the protein sequence (not shown).

It can be noted from Figure 4C that the mutant $\omega_7$ like $\omega_9$ is more structured than native $\omega$. There are two sites where a frequent isoleucine codon is mutated to the rare codon, and there are only three codons in between the sites of mutations. However, $\omega_1$ has a mutation in the N-terminal of the protein and does not demonstrate any structural change (Figure 4C). It can be seen from Figure S5 that the mutants $\omega_2$, $\omega_10$, and $\omega_5$ like $\omega_9$ are more structured than native $\omega$. All these mutations occur at the C-terminal unstructured region of the proteins. All other mutants ($\omega_3$, $\omega_11$, $\omega_12$) show little structural element. $\omega_7$, $\omega_10$, and $\omega_9$ have this characteristic feature where the rare codons are present repetitively. They are also present close to each other near the C-terminal and fall in the disordered region predicted by PONDR54 (Figure 5A). Mutant-like $\omega_{11}$ (arginine silent mutant at the 90th and 91st positions) does not show any change in phenotype or CD spectra of the purified protein. It appears that they fall at the end of the

Table 2. Codon Usage Index for Arginine and Isoleucine

| amino acid | codon | fraction in all genes | fraction in essential genes |
|------------|------|----------------------|-----------------------------|
| arginine   | AGG  | 0.022                | 0.003                       |
| arginine   | AGA  | 0.039                | 0.006                       |
| arginine   | CGG  | 0.098                | 0.008                       |
| arginine   | CGA  | 0.065                | 0.001                       |
| arginine   | CGU  | 0.378                | 0.643                       |
| arginine   | CGC  | 0.398                | 0.330                       |
| isoleucine | AUA  | 0.073                | 0.006                       |
| isoleucine | AUU  | 0.507                | 0.335                       |
| isoleucine | AUC  | 0.420                | 0.659                       |

“AUA (isoleucine) and AGG and AGA (arginine) codons are rarely used in Escherichia coli. Their usage reduces in the constitutively expressed genes.”50,51

Figure 4. Effect of frequent to rare codon mutation with respect to position: (A) rpoZ DNA sequence with highlighted codons for arginine (orange in color) and isoleucine (blue in color). (B) Crystal structure of $\omega$ in RNAP complex, with a few mutant positions highlighted in red ($\omega$ structure retrieved from 4JKR) and list of mutants with codon positions and amino acids. (C) CD profile for the silent mutants. The rest of the CD profiles are in Figure S8.
protein synthesis and might not impact the overall sequential folding of the protein.

**Phenotype Rescue in CodonPlus Strain.** ω is unstructured in its soluble state, as shown in Figure 1B. Moreover, many algorithms have predicted unstructured segments in ω (12–18%) (Figure 5A). However, when we inspected the crystal structure of *Escherichia coli* RNA polymerase, we observed that ω is more structured in its bound state (63% helical) (Figure 5A). These observations prompted us to think that there is binding-induced folding of ω, and this is important for proper assembly and functioning of RNA polymerase.

There occurs a correlation between codon usage and tRNA population present in the cells, which occupies a crucial role in fine-tuning the local translation kinetics. The tRNA population cognate for the frequent codon is present in abundance in cells, and that for the rare codon is infrequent in the cellular milieu, which in turn affects the translational kinetics.46,47 This prompted us to think that the manipulation of the local concentrations of tRNA can influence the translation rate and finally the folding of the protein. Thus, if cells are provided with surplus tRNA copies for the cognate rare codon, folding of the protein can be altered.

The silent mutants, which have acquired structures as opposed to that of wild type ω, were expressed in an *Escherichia coli* CodonPlus strain, which carries an extra gene encoding tRNA cognate for codons AGG, AGA (arginine), and AUA (isoleucine). ω7 was expressed in the CodonPlus strain (isoleucine silent mutant at the 49th and 54th positions), and CD spectroscopy was performed on the purified protein. It can be seen from Figure 5B that purified ω7 from the CodonPlus strain shows an unstructured CD profile as against that of ω7 isolated from BL21. Expectedly, the lethality of the dominant-negative phenotype of *Escherichia coli* cells with ω7 (BL21) was rescued in the ω7 (CodonPlus) strain in the CFU assay (Figure S6).

**Transcriptional Activity of RNA Polymerase Reconstituted in Vitro with Wild Type ω and Its Silent Mutants.** ω is a disordered protein, and it has been shown that its flexibility is crucial for the purpose it serves. Studies have shown that the high intramolecular flexibility and plasticity of disordered proteins permit them to bind to their target with high specificity and modulate their function. Structured mutants of ω are rigid as compared to the flexible native protein. We envisaged that the inherent flexibility of the native ω is important for the proper assembly and mobility necessary for the multi-subunit complex RNAP. To verify this, we reconstituted RNA polymerase from individual subunits in vitro (as shown in Figure S7) and performed a single round transcription assay (Figure 6). RNAP reconstituted with ω6 was used as a negative control for the experiment. Transcription was carried out in the presence of 0.2 μg/μL heparin essentially to establish the pattern of single round runoff transcription. RNA polymerase isolated from *Escherichia coli* RL916 acting as a positive control. Reconstituted RNA polymerase with native ω showed single round transcription, which was inhibited by rifampicin. RNA polymerase reconstituted with silent mutant ω7 was transcriptionally inactive, but single round transcription with ω7 (CodonPlus) was positive. We carried out the structural analysis that shows the interaction between ω7 and β′ in the RNA polymerase complex, as shown in Figure S5B. It shows that the mutation site and β′ residues are present near (5 Å) to each other. A multiround transcription assay was performed with RNA polymerase reconstituted with all the mutants, as depicted in

Figure 5. Structural transition of ω in *Escherichia coli* CodonPlus strain. (A) Secondary structure prediction for ω by PONDR and the amount of secondary structure present in ω in RNA polymerase crystal structure (PDB ID 4JKR). Free state vs bound state for ω. (B) CD profile of ω7 purified from BL21 and CodonPlus.
Figure 6. Single round transcription assay by in vitro reconstituted RNA polymerase. In all cases, ΔΔT7 DNA promoter generating 83 nucleotide long transcript was used. T-A DNA template (0.4 pmol) and 4 pmol of enzymes were used. The concentration of rifampicin is 10 μM. Lane 1. RNA polymerase from RL916. Lane 2. Reconstituted RNA polymerase with αββ'ω. Lane 3. Reconstituted RNA polymerase with αββ'ω. Lane 4. Reconstituted RNA polymerase with αββ'ω. Lane 5. Reconstituted RNA polymerase with αββ'ω. Lane 6. Reconstituted RNA polymerase with αββ'ω. Lane 7. Reconstituted RNA polymerase with αββ'ω. Lane 8. Reconstituted RNA polymerase with αββ'ω.

Figure S7, indicating almost 5 times reduced activity for RNA polymerase reconstituted with structured αββ'ω. Partial Proteolysis of ω and Its Silent Mutant by Trypsin. Limited or partial proteolysis is a classic biochemical technique employed to get information regarding the change in protein structure and conformation. Since native ω is an intrinsically disordered protein and its silent mutants are structured, they are expected to indicate the differential rate of digestion by protease. ω has 12 trypsin sites as shown in Figure 7A. We learned from this experiment that, in αββ'ω, the C-terminal is getting structured as opposed to that of wild type ω. The differential rate of digestion by trypsin can be noted in Figure 7B. Mass spectrometric analysis of the assay mixture informed us that there is delayed cutting for intermediate and C-terminal regions of the protein.

ppGpp Interaction with RNAS Reconstituted with ω and Its Silent Mutant. Results on the single round transcription assay presented in a previous section prompted us to assess the binding of ppGpp to RNA polymerase. ppGpp is a nucleotide second messenger, which is known to bind at the ω and β' interface as shown in Figure 8A, which is located at 28 Å distance from the active site of the polymerase complex. ppGpp binding to RNA polymerase possesses a profound effect in regulating Escherichia coli transcription. We examined this interaction to understand the influence of ω variants in ppGpp recognition of the enzyme. ppGpp binding to RNA polymerase was titrated by measuring the change in heat capacity of the reaction upon binding. We studied binding affinity of ppGpp to RNA polymerase (αββ'ω), RNA polymerase reconstituted with αω (αββ'ω), and RNA polymerase reconstituted with αω (αββ'ω). We observed that there was a reduced binding affinity for ppGpp to RNApolymerase ω and RNApolymerase ω. It indicates that RNA polymerase assembly with mutant ω is defective and that could result in inactive RNA polymerase.

DISCUSSION

In an open system, Gibb’s free energy (ΔG = ΔH − TΔS) describes the equilibrium. The conversion of unstructured ω to structured ω is accompanied by a negative entropy change, which is compensated by the change in enthalpy. As a result, the overall change in free energy is predominantly negative. By a series of measurements using different techniques, it has been shown that the mutant ω has a stronger binding with the rest of the core enzyme in comparison to that of native ω. 

Structured ω does bind to αββ'ω assembly, which is evident from in vitro reconstitution, but they were defective in transcription. This again hints at the importance of disorder- edness of ω. It is well recognized that β' F (bridge)-helix and β' G-loop encompasses the active centers of RNA polymerase and they work in a coordinated fashion to warrant the proper placement of NTPs at the active site and their incorporation into the elongating transcripts. We argue that, due to the binding of the structured ω, the mobility of the flexible segments at the active site is affected, which manifests into the abrogation of initiation of transcription.

It appears that the paradigm that describes a direct correlation between structure and function of a protein is not always true with the discovery of the intrinsically disordered proteins. In the last decade, there is a growing amount of evidence for intrinsically disordered proteins, which marks the above assumption. The smallest subunit of RNA polymerase, ω falls in the category of intrinsically disordered protein. Previous reports have shown that the flexibility of ω is critically important for its function and interaction with β'.

Silent mutations were considered inconsequential in terms of protein folding for an extensive period since the encoded protein sequence remains unaltered. However, in eukaryotes, they are appreciated to have an impact on a specialized process like gene splicing and expression levels. However, they are not entirely silent mutants. Although the protein sequence remains the same, the DNA and mRNA sequences are altered due to introduction of the silent mutation. While considering the role of silent mutants, two levels of control need to be considered: (a) rate of translation and (b) mRNA stability. Both may vary as a function of the frequency of codon usage, which would reflect on the protein folding. In the recent past, few observations elegantly demonstrated this point. It would be a bonus for the researchers if differential folding due to single silent mutation exhibits an altered phenotype.

Any study with such a system will direct us to observe the utility of the frequent codon or rare codons, as well as the influence of their positions on the reading frame.

In the present study, the reconstituted RNA polymerase with different ω-subunits shows a lethal dominant-negative phenotype where transcription was inhibited by the same ω-sequence but with an altered folded structure. This unique observation was supported by mainly two experiments, CD studies and single round transcription assay. The former shows a clear folding pattern, whereas the latter demonstrates the functionality. Both experiments are definitive but unfortunately cannot infer various stages of structural alteration or lethality during the path of folding. Our assay mainly focuses on defects in transcription initiation, and thus, we believe that the structure of the active site occupies a critical role.

What represents the nature of this different folded state as they have the same sequence of amino acids? We would like to define them as “metastable” state emanated from an altered
rate of translation or mRNA stability. That they are metastable and Anfinsen principle ultimately guides their destiny was shown by in vitro urea denaturation–renaturation experiments. As the timescales for protein synthesis and folding are often similar, it is quite evident that the rate of translation can be used to tune the assembly of the growing polypeptide. In this respect, the position of the mutation plays a dominant role. We mentioned before that ω is an intrinsically disordered protein and plasticity of the disordered region during assembly of the functional enzyme is significant. It is interesting to note that all dominant-negative mutants map at the C-terminal region. Thus, it would be worthwhile to investigate which factor-like translation by ribosome or the mRNA structure is playing a significant role here.

- MATERIAL AND METHODS

Protein Purification of Wild Type ω and Its Silent Mutants. DNA sequence coding for ω was cloned into vector pET-28b (Novagen) using Ncol/Hind III restriction sites and was termed as pET-ω. Recombinant plasmids were transformed in Escherichia coli BL21 (DE3) grown on LB agar plates containing 50 μg/mL kanamycin. ω and its mutant were purified from the Escherichia coli BL21 (DE3) strain transformed unless mentioned otherwise with plasmid pET-ω. The cells were grown at 37 °C to an OD600 of 0.6, induced with a final concentration of 1 mM IPTG, and grown further for 3 h. Cells were harvested, and the pellet was dissolved in lysis buffer (50 mM Tris–HCl, pH 7.9; 100 mM NaCl; 1 mM PMSF; 2 mM EDTA; 10 mM benzamidine). Cells were lysed by sonication. The soluble fraction containing the protein was loaded onto a Ni-NTA column pre-equilibrated with the lysis buffer. The column was washed with wash buffer (50 mM Tris–HCl, pH 7.9; 1 mM PMSF, 100 mM NaCl, 10 mM benzamidine, 20 mM imidazole). The protein was eluted out with an elution buffer (50 mM Tris–HCl, pH 7.9, 1 mM PMSF, 100 mM NaCl, 10 mM benzamidine, 0.5 M imidazole). The fractions containing the pure protein were pooled together and dialyzed against storage buffer (20 mM Tris–HCl, pH 7.9; 100 mM NaCl; 0.1 mM DTT; 0.1 mM EDTA, and 50% glycerol). Dialysis was performed at 4 °C for 16 h. The purity
of protein was confirmed by analysis on 15% SDS-PAGE, and the concentrations were estimated by the Bradford assay. Circular Dichroism Spectroscopy. Far UV spectra of \( \omega \) and its mutants were recorded on a JASCO J-715 CD
spectropolarimeter. Buffer used was 50 mM Tris–HCl and 100 mM NaCl, pH 7.9. Mean residue ellipticity was estimated using the formula \[ \theta = (1000 \times \theta \times m) / L \times C \], where \( \theta \) is the measured ellipticity in degrees, \( m \) is the mean residue weight in g/d mol, \( C \) is the concentration in g/L, and \( L \) is the path length in cm. The percent \( \alpha \)-helicity was calculated using K2D2 software.\(^{58}\) Protein concentrations used were in the range of 0.2–0.6 mg/mL.

**Generation of Silent Mutants of \( \omega \) at Different Positions.** Mutations were carried out in pET-\( \omega \) vector bearing wild type \( \omega \) gene. In order to generate silent mutants of \( \omega \), site-directed mutagenesis was performed. Gradient PCR was performed using the primers presented in the primer list in Table S1. Following PCR amplification of the vector, amplified products were checked on 1% agarose gel and gel extraction was performed from the band having the desired product. The gel-extracted product was subjected to ligation using T4 DNA ligase provided by NEB followed by DpnI digestion and transformation in the DH5\( \alpha \) strain of *Escherichia coli*. DpnI digestion was performed to remove the parent plasmid. Cloning results were confirmed by sequencing, and the protein sequence was confirmed by mass analysis.

**Refolding Experiment.** Purified \( \omega_{11}, \omega_{12}, \) and \( \omega_{15} \) proteins were subjected to denaturation by incubating them with 7 M urea overnight. Urea was dialyzed out in a stepwise manner against the renaturing buffer (50 mM Tris–HCl, 100 mM NaCl), and aliquots were collected every 2 h. Protein refolding was followed by CD spectroscopy.

**Reconstitution of RNA Polymerase from Purified Subunits.** Purified subunits HexA\(-\alpha, \beta, \beta', \) and \( \omega \) and its silent mutants were mixed in a molar ratio of 2:8:4:1 in a mild denaturation buffer (50 mM Tris–HCl, pH 7.9 at 4 °C; 200 mM KCl; 10 mM MgCl\(_2\); 10% glycerol; 1 mM EDTA, 10 \( \mu \)M ZnCl\(_2\), and 0.25% N-lauryl sarcosine with 10 mM DTT) and kept for an hour at 4 °C. The volume of the reconstitution mixture was kept at 10 mL with a total protein concentration of 1.5 mg/mL. The reconstitution mixture was next dialyzed for 16 h at 4 °C, against 1 L of renaturation buffer (50 mM Tris–HCl, pH 7.9 at 4 °C; 200 mM KCl; 10 mM MgCl\(_2\); 20% glycerol; 0.1 mM EDTA, 10 \( \mu \)M ZnCl\(_2\) with 1 mM DTT). The dialyzed sample was centrifuged at 32000g for 30 min, and the clear supernatant was collected. The supernatant was loaded onto the Ni-NTA column matrix, which was pre-equilibrated with the same refolding buffer. The column was washed with 5 column volumes wash buffer (50 mM Tris–HCl, pH 7.9 at 4 °C; 200 mM NaCl; 5% glycerol; 0.1 mM EDTA; 0.1 mM DTT; 20 mM imidazole), and the protein was eluted out with an elution buffer (50 mM Tris–HCl, pH 7.9 at 4 °C; 400 mM NaCl; 5% glycerol; 0.1 mM EDTA; 0.1 mM DTT; 250 mM imidazole). The elutions were checked on an 8–15% gradient SDS-PAGE gel for the presence of the subunits. Eluents from the Ni-NTA column were pooled and concentrated with the help of 100 kDa Centricon. Concentrated RNAP was loaded onto a Heparin-Sepharose column pre-equilibrated with the refolding buffer. The protein was eluted with the elution buffer (10 mM Tris–HCl, pH 7.9 at 4 °C; 800 mM NaCl; 5% glycerol; 0.1 mM EDTA; 0.1 mM DTT). The fractions were checked for the presence of core RNAP on a 10% SDS-PAGE gel, and the purity of the protein was found to be >99%. Fractions containing reconstituted core RNAP were pooled together and used for the single round transcription assay immediately.

**Single Round Transcription Assay.** Activity of reconstituted RNAP using wild type \( \omega \) and its silent mutants was checked by the promoter specific transcription assay. Four picomoles of reconstituted RNAP was preincubated with purified \( \sigma \) in a 1:2 molar ratio at 37 °C for 10 min in the transcription buffer (40 mM Tris–HCl pH 7.9 at 4 °C, 10 mM MgCl\(_2\), 75 mM KCl, 0.1 mM EDTA, 0.1 mM DTT, 14 mM \( \beta \)-mercaptoethanol, 0.025 mg/mL nuclease free BSA) to allow formation of the holoenzyme. T-A1 promoter DNA was added (0.4 pmol) and incubated for 20 min at 37 °C to allow for specific promoter-RNAP complex formation. Next, the substrate-heparin mixture, containing 0.15 mM ATP, 0.15 mM GTP, 0.15 mM CTP, 0.05 mM UTP, and 2 \( \mu \)Ci [\( \alpha \)-\( P_32 \)]-UTP, was added. The mixture was then incubated for 15 min at 37 °C to allow for the RNA chain elongation reaction. The reaction was terminated with a Stop Solution (5 mM EDTA and 100 \( \mu \)g/mL *Escherichia coli* tRNA). The transcript RNA was precipitated by ethanol, washed, and then separated on a 10% denaturing polyacrylamide gel containing 6 M urea. The gel was put in a fixative solution and exposed to imaging plates, and then the plates were analyzed by a phosphorimager (FLA2000; Fujifilm).

**Mass Spectrometry for Protein Analysis.** To verify for the protein of interest, in-gel trypsin digestion was performed alongside ESI analysis. The band corresponding to a molecular marker of 14.4 kDa was cut, destained, and subjected to in-gel tryptic digestion, at pH 7.5 (50 mM ammonium bicarbonate, \( NH_4HCO_3 \)), 37 °C overnight. The resulting mixture of tryptic peptides extracted from the gel was passed through a reverse phase column (Zorbax RX C18, 4.6 mm × 250 mm, 5 mm). A gradient elution using \( H_2O/ACN/0.1\% \) formic acid was followed at a flow rate of 0.2 mL min\(^{-1}\), and the eluting peptides were characterized by conventional MS (LC-ESI-MS) and MS/MS (LC-ESI-MS/MS). An HCT Ultra PTM Discovery mass spectrometer (Bruker Daltonics) has an ESI source and houses a classic ion trap (Paul-type), using which both MS (LC-ESI-MS) and tandem MS (LC-ESI-MS/MS) data were acquired. Both CID and ETD can be carried out within this ion trap in an alternate manner, and thus, the LC-ESI-MS/MS data generated are composed of those resulting from both CID and ETD. While CID is carried out using helium, ETD (ion/ion reaction) is accomplished using fluoranthene radical anions. The fluoranthene radical anions are generated by means of negative chemical ionization utilizing methane as the reagent gas. The ETD (ion/ion) reaction time was set as 100 ms, and the supplemental collisional activation (i.e., smart decomposition) was ON during ETD. The data were processed using Data Analysis Version 4.0 (Bruker Daltonics).

**Size-Exclusion Chromatography.** Purified protein with a concentration of 1 mg/mL (25 mM Tris–HCl and 100 mM NaCl) was loaded on a Superdex S75 column. Size-exclusion chromatography was performed using GE Healthcare FPLC. The flow rate of the buffer was maintained at 0.3 mL/min. 100 \( \mu \)L volume of protein was injected for the chromatography. Protein elution was monitored at 280 nm.

**Checking for Lethality upon Protein Induction.** Single colonies were inoculated into LB broth and incubated at 37 °C temperature under shaking conditions. They were recultured in fresh LB tubes with starting 0.1% culture and monitored until OD\(_{600}\) reached the value of 0.6. Dilutions were made using LB, and 10 \( \mu \)L was spotted on LB agar plates. For control, plates...
were not infused with IPTG; however, the test plates had 1 mM IPTG.

**Partial Proteolysis by Trypsin.** 1 mg of protein was incubated with trypsin (Promega) for 2, 5, and 10 min in a ratio of trypsin/protein at 1:50. Trypsin was heat-inactivated, and the reaction mixture was loaded onto 20% SDS-PAGE and subjected to mass spectroscopic analysis. All mass spectrometric analysis was performed using a Bruker Ultraflextreme machine.

**Isothermal Calorimetry.** An ITC-200 microcalorimeter (GE Healthcare) was used for calorimetry studies. In brief, RNAP reconstituted with and without α/ωα/ω was taken in the concentration range of 20–50 μM, and ppGpp concentration was varied from 0.1 to 5 mM in all the experiments. Both the ligand and protein were dissolved in a buffer consisting of 20 mM Tris-HCl pH 7.9, 150 mM NaCl and 0.5 mM β-mercaptoethanol. ORIGIN software Version 7.0 (Microcal, Malvern, Worcestershire, U.K.) was used to calculate the binding affinity (K_b). At the time of plotting, the heat of dilution for ppGpp was subtracted and the first data point was removed from the experimental curve as suggested by the analysis software.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.9b02103.

Mass spectroscopy, denaturation curves for the proteins, CFU assay to check for protein lethality, and CD spectra (PDF)

**Accession Codes**

β'-subunit of RNA polymerase: P0A8V2. ω'-subunit of RNA polymerase: P0A800.

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**Author Contributions**

D.C. and U.R.P. designed the research, performed the research, and analyzed the data. S.G. performed the research (single round transcription assay). D.C. and U.R.P. wrote the paper. D.C. consolidated the work.

**Funding**

This work is funded by a Center for Excellence (CoE) grant from the Department of Biotechnology (DBT), Government of India. U.R.P. and S.G. are University Grant Commission fellows.

**Notes**

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

We acknowledge the Proteomics Facility, Molecular Biophysics Unit, Indian Institute of Science, Bangalore. We acknowledge Mrs. Sunita Prakash for helping us with mass spectroscopy. We acknowledge Prof. Raghavan Varadarajan, Molecular Biophysics Unit, Indian Institute of Science, Bangalore, for all valuable suggestions. Shivender Yadav, Organic Chemistry, Indian Institute of Science, Bangalore, is acknowledged for his help in ITC experiments. We would like to thank the reviewers for their critical suggestions.

**ABBREVIATIONS**

IPTG Isopropyl β-D-1-thiogalactopyranoside
CD Circular dichroism
RNAP RNA polymerase
CFU Colony forming unit

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