Conformational Flexibility in $\sigma^{70}$ Region 2 during Transcription Initiation*

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Prokaryotic RNA polymerase holoenzyme is composed of core subunits ($\alpha_2\beta\beta'/\omega$) plus a $\sigma$ factor that confers promoter specificity allowing for regulation of gene expression. Holoenzyme is known to undergo several conformational changes during the multiple steps of transcription initiation. However, the effects of these changes on the functions of specific regions have not been well characterized. In this work, we addressed the role of possible conformational change in region 2 of Escherichia coli $\sigma^{70}$ by engineering disulfide bonds that “lock” region 2.1 with region 2.2 and region 2.2 with region 2.3. When these mutant holoenzymes were characterized for gross defects in multiple-round transcription, we found that insertion of either disulfide bond did not result in a fundamental block, indicating that the disulfide-containing holoenzymes are active. However, both disulfide-containing holoenzymes exhibited defects in formation and stability of the open complex. Our results suggest that conformational flexibility within $\sigma^{70}$ region 2 facilitates open complex formation and transcription initiation.

RNA polymerase, a multi-subunit enzyme that is made up of five polypeptide chains ($\alpha_2\beta\beta'/\omega$), is solely responsible for the synthesis of messenger, transfer, and ribosomal RNA in the bacterial cell. This enzyme has two distinct functional forms: core enzyme and holoenzyme. Although core enzyme is catalytically active and capable of transcription elongation, it is unable to initiate transcription at specific promoter sequences. Promoter recognition is accomplished by binding of the specificity factor $\sigma$, which positions holoenzyme on the promoter sequence (1, 2). Through the use of seven $\sigma$ factors, Escherichia coli is able to direct transcription from multiple sets of promoter sequences, which confers the ability to regulate gene expression (3, 4). Amino acid sequence alignment of $\sigma$ factors within the $\sigma^{70}$ family identified four regions of sequence homology (regions 1–4) (5). Region 2, which contains core binding and −10 promoter recognition determinants, accounts for the most sequence similarity among $\sigma^{70}$ family members. This suggests that this region plays an important role in transcription. Because $\sigma^{70}$ region 2 was shown to be a major interaction domain with the $\beta'$ coiled-coil (6–8), this region may be the site of functionally necessary conformational changes upon interaction with core enzyme.

Based upon the sum of prior mechanistic, x-ray diffraction, electron microscopy, and luminescence resonance energy transfer studies, RNA polymerase core enzyme and $\sigma^{70}$ are thought to undergo multiple conformational changes through the process of transcription initiation: from $\sigma$ binding, to formation of the closed complex, to stable open complex formation, to transcription initiation, and finally to $\sigma$ factor release (3, 7, 9–13). Evidence for intermediates during this process has been identified using the well studied lacUV5 promoter (12, 14–16). The currently accepted pathway for open complex formation based on this promoter is shown below (15).

\[
R + P \rightarrow R_P \rightarrow R_P \rightarrow R_P \rightarrow R_P.
\] (Pathway 1)

R is RNA polymerase holoenzyme, and P is the lacUV5 promoter. R$_P$ is the closed complex where contacts with double-stranded promoter DNA are established. R$_P$ is a kinetically significant intermediate on the pathway to the open complex (R$_P$), where melting of the promoter DNA and formation of the transcription bubble occurs. Although conformational changes are thought to accompany many if not all of these steps, the nature and importance of these changes at each sequential step have not been characterized.

Identification of conformational changes accompanying formation of protein-DNA complexes necessitates alternative means of analysis, primarily because traditional methods such as NMR are not suitable for larger multi-subunit protein complexes such as RNA polymerase. Both circular dichroism and NMR require high protein concentrations (micromolar to millimolar) at which RNA polymerase has been shown to aggregate (17, 18), making these spectroscopic approaches not useful for analyzing this system. Methods previously utilized to address conformational changes in RNA polymerase holoenzyme, such as fluorescence resonance energy transfer (FRET) and thermodynamic measurements (9, 13), allowed the observation of gross conformational changes but were not able to examine specific regions of the proteins or changes during the sequential steps of transcription. Information from the recently available crystal structures of Thermus aquaticus RNA polymerase (19–21) provided static snapshots of different conformational states of the enzyme; however, transcription is likely to be a continuous and dynamic process. Additionally, the flexibility of accessible mobile regions of a large multi-subunit enzyme may be extremely sensitive to crystal packing interactions or the solution environment, preventing small conformational changes from being seen.

To address these issues, we have utilized a freely available disulfide bond recognition algorithm “Disulfide by Design” (22) to strategically place disulfide bonds within a protein, restricting the conformational flexibility of that region. If no functional consequence arises at a particular mechanistic step as a result of the inserted disulfide bond, one can argue that no conformational change is required in that region of the protein at that
time. However, if function of the protein is inhibited, then it strongly argues that some conformational flexibility is essential during that step. Although the introduction of non-native disulfide bonds has been used in the past to examine conformational change and protein stability (23–26), success rates were often low because of the lack of an algorithm to accurately predict the location at which disulfide bond was most likely to form. The Dusulfide by Design algorithm correctly predicted the location of amino acid residues that form disulfide bonds with over 80% accuracy when tested against proteins of known structure (22). This algorithm was successfully used to engineer a disulfide bond within the coiled-coil domain of the β’ subunit of RNA polymerase to examine whether conformational change was required for σ70 binding and binding of a non-template strand oligonucleotide by σ70 (27).

To further our knowledge of the gross conformational changes in σ70 previously analyzed by luminescence resonance energy transfer and thermodynamic studies, we investigated whether the α-helices in region 2 of E. coli σ70 undergo conformational changes during transcription initiation. Using the Dusulfide by Design algorithm, we have engineered two disulfide bonds within the essential region 2 of σ70 and assayed for the functional consequences of restricting conformational change during transcription initiation. Both mutant holoenzymes exhibit defects in open complex formation and in the stability of the open complex, suggesting that some conformational flexibility is necessary to facilitate transcription initiation.

**EXPERIMENTAL PROCEDURES**

All of the reagents were purchased from Sigma unless otherwise indicated. Spectrophotometric grade glycerol was purchased from Fisher. Ni-NTA-agarose was purchased from Qiagen. E. coli LB culture medium contained 1.0% Bacto Tryptone, 0.5% Bacto Yeast Extract, and 0.5% NaCl. Ni-NTA buffer contained 25 mM Tris-HCl (pH 7.9), 50 mM NaCl, 5 mM imidazole, 0.1% Tween 20, and 5% glycerol. TE buffer contained 50 mM Tris-HCl (pH 7.9) and 0.5 mM EDTA. Storage buffer contained 50 mM Tris-HCl (pH 7.9), 100 mM NaCl, 0.1 mM EDTA and 50% glycerol.

**Dusulfide Bond Engineering**—The amino acid sequence of E. coli σ70 region 2 (amino acids 360–440) was analyzed using a disulfide recognition algorithm (22) designed to recognize cysteine pairs that are properly oriented to favor disulfide bond formation. This algorithm is part of a Windows-based program called Dusulfide by Design that is freely available by e-mailing the author of the program at domski@wayne.edu. This analysis revealed two potential amino acid pairs in region 2, Val387/Gly408 and Ala415/Trp434, that when substituted with cysteines have a strong potential for forming a disulfide bond (Xα torsion angles of 91.6 and 96.7°, respectively).

**Plasmid Construction**—Plasmid characteristics are described in Table I. A cysteine-less variant of σ70 was made by the addition of C442S through site-directed mutagenesis using plasmid pSL442C(σ70) as template (28). The region containing the substituted serines was subcloned by transferring the PstI-HindIII fragment into pHMKHis-σ70 (29) to produce plasmid pLA55. Cysteine and serine substitutions at positions Val387, Gly408, Ala415, and Trp434 of σ70 were generated by site-directed mutagenesis of pLA55 using standard QuickChange mutagenesis (Stratagene). Oligonucleotide sequences are available upon request. All of the plasmids generated by PCR were sequenced to ensure that spurious secondary mutations had not been incorporated.

**Expression and Purification of Mutant Holoenzymes**—Plasmids pHMKσ70 and pLA55 through pLA81 were transformed into BL21(DE3)pLysS (Novagen) for overexpression. The cells were grown to an A600 of 0.4–0.6 in 2-liter flasks at 30°C in LB supplemented with 50 μg/ml kanamycin and 10 μg/ml chloramphenicol. Isopropyl-β-D-thiogalactoside was used for induction at a final concentration of 0.5 mM. The cells were harvested 3 h after induction by centrifugation at 8,000 × g for 15 min and frozen at -80°C until use.

All of the RNA polymerase holoenzymes were purified by the protocol below. The cell pellets were lysed in Ni-NTA buffer, sonicated for two 1-min intervals, and centrifuged at 15,000 × g for 20 min. The resulting pellets contained mostly σ70 inclusion bodies, which were discarded. The supernatants were diluted to 35 ml with Ni-NTA buffer and incubated with 3 ml of pre-equilibrated Ni-NTA-agarose (Qiagen) for 1 h at 4°C. Supernatant-agarose suspensions were poured into 15-mL Econo- pak columns (Bio-Rad) and washed extensively with 20 column volumes of Ni-NTA buffer. Low salt conditions (50 mM NaCl) were used to avoid possible dissociation of the holoenzyme subunits. RNA polymerase holoenzymes were eluted with ten 1-ml fractions of Ni-NTA buffer with 300 mM imidazole.

Because the holoenzyme preparations were still heavily contaminated with cell protein, immunofluorescence chromatography was used to further purify the preparation. The pooled Ni-NTA fractions were diluted 3-fold with TE buffer and loaded onto 0.5-ml immunofluorescence columns containing the polyclonal anti-β’ monoclonal antibody NT73 (30). The columns were washed with 10 column volumes of TE buffer with 0.15 mM NaCl, and eluted with 5 ml of TE buffer containing 0.75 mM NaCl with 30% propylene glycol. Fractions containing holoenzyme were pooled, concentrated in 4 ml of 100-kDa cut-off Ultrafree-4 concentrations (Millipore), and dialyzed into storage buffer. Final holoenzyme concentrations in storage buffer were in the range of 100–200 μg/ml and assumed 100% active protein. Stoichiometry of the purified holoenzymes was determined by densiometry comparing the ratios of α and α subunits using the software package Un-Scan-It Gel (Silk Scientific). Each holoenzyme contained a ratio of 1 α subunit to 2 α subunits (<10% deviation), indicating that all purified holoenzymes are in proper stoichiometry.

**IC5-PE-maleimide Labeling**—To verify disulfide bond formation within the α subunit of the purified holoenzymes, each holoenzyme was labeled with the fluorescent dye IC5-PE-maleimide (Dojindo). Each holoenzyme (0.7 μg) was added to buffer containing 50 mM Tris-CHI (pH 7.9), 50 mM NaCl, and 10 pmol of IC5-PE-maleimide. The samples were incubated at 37°C for 30 min and subjected to electrophoresis along with unlabeled control samples on a 4–12% NuPAGE Bis-Tris gel with MES buffer. The gel was then visualized on a Molecular Dynamics Typhoon system (Cy5 filter) and later stained with Gel-Code Blue reagent (Fierce).

**DNA Purification**—DNA concentrations in all of the experiments were determined by absorbance at 260 nm where 1 μg/ml DNA = 50 μg/ml of double-stranded DNA. Supercored pRLG593 template (31) for the multiple-round transcription experiments was purified by Mid-prep (Promega), followed by two phenol/chloroform extractions, and finally an ethanol precipitation.

Linear templates (from lacUV5 to +39) used for the closed complex gel shifts, DNase I foot-printing, and filter binding experiments were prepared by digestion of pRLG593 DNA with HindIII, labeling of

| Plasmid | Characteristics | Source |
|---------|-----------------|--------|
| pLA55   | p[C442S]σ^70 with N-terminal HMK-His<sub>α</sub> | This work |
| pLA57   | pLA55 with V387SC G408C | This work |
| pLA60   | pLA55 with A415C and W434C | This work |
| pLA80   | pLA55 with V387S and G408C | This work |
| pLA81   | pLA55 with A415S and W434C | This work |

1 The abbreviations used are: Ni-NTA, nickel-nitritolactric acid; MES, 4-morpholineethanesulfonic acid; BSA, bovine serum albumin.
In Vitro Transcription—Multiple-round transcription was started by the addition of 12 nM of each holoenzyme to 30 nM supercoiled pRLG593 template. Transcription assays were performed at 37 °C in 40 mM Tris-HCl (pH 7.9), 150 mM KCl, 0.1 mM EDTA, 5 mM MgCl2, 10% (v/v) Hepes, and treated with 3 nM supercoiled pRLG593 in buffer containing 40 mM Tris-HCl (pH 7.9), 5 mM MgCl2, 150 mM KCl, and 0.1 \( \mu \text{g} / \text{ml} \) BSA for 30 min at 37 °C. Heparin (final concentration, 50 \( \mu \text{g} / \text{ml} \)) was added at time 0, and the aliquots (10 \( \mu \text{L} \)) were removed at various time points after heparin addition and placed into tubes containing 2.5 \( \mu \text{L} \) of NTP mix (final concentrations: 250 \( \mu \text{M} \) ATP, 100 \( \mu \text{M} \) CTP, 100 \( \mu \text{M} \) GTP, 10 \( \mu \text{M} \) UTP, 13 \( \mu \text{C} / \text{\muL} \) of \( [\alpha-32\text{P}]\text{UTP} \)). The transcription reactions were stopped with formamide loading buffer after 10 min and analyzed on 5% polyacrylamide, 8 \( \mu \text{m} \) urea gels. Open complex stability assays on linearized templates were performed as described above except for the use of 3 \( \mu \text{M} \) PCR-generated pRLG593 linear template (−60 to +39) instead of supercoiled DNA. Quantitation with background correction was performed using ImageQuant software (Molecular Dynamics). Fractions of open complexes remaining at the different time points after heparin addition were plotted using Origin 5.0 software (MicroCal).

RESULTS

RNA polymerase core enzyme and \( \sigma^{70} \) are known to undergo several conformational changes throughout the process of transcription initiation (3, 7, 10); however, the large size of these multisubunit protein-DNA complexes precludes traditional ways of probing for conformational changes, such as NMR analysis. In this work, we used a previously successful disulfide recognition algorithm (22, 27) to probe for possible conformational changes in \( \sigma^{70} \) region 2 during the steps of transcription initiation.

This algorithm threads a given amino acid sequence onto the Cα backbone of a known crystal structure and ranks each pair of amino acid residues for the likelihood of disulfide bond formation. Two pairs of residues within \( \sigma^{70} \) region 2, Val387/Gly408 (2.1C-2.2C) and Ala415/Trp434 (2.2C-2.3C), were shown to have strong potential for disulfide bond formation when substituted with cysteines (estimated \( \chi_3 \) angles of 91.6 and 96.7°, respec-
tively, where 90° is optimal). The positions of these residues within the x-ray crystal structure of the protease-resistant domain of E. coli σ70 (32) are presented in Fig. 1. An example of modeling the Cys387–Cys408 disulfide bond within σ70 region 2 shows the orientation of the cysteine side chains and the 92° X3 torsion angle (Fig. 1B). Secondary structure predictions (PHD, Swiss model) indicated that substitution of all four residues with cysteine was unlikely to perturb the α-helical structure of this region.

**Purification and Verification of Disulfide Bond Formation—**
All of the cysteine substitutions were introduced via site-directed mutagenesis into pLA55, a pET-derived vector containing a His6-tagged cysteine-less variant of σ70. Overexpression of both disulfide-containing σ70 variants yielded both large inclusion bodies, which were unable to be refolded into active monomeric protein and a small amount of soluble σ70 in the cell supernatants. The majority of the soluble σ70 was present as RNA polymerase holoenzyme (data not shown). Because of the difficulty in refolding these σ factors, holoenzyme instead was isolated using two chromatographic steps. First, holoenzyme in the cell supernatants was purified by native Ni-NTA chromatography, which resulted in eluates containing free disulfide-containing σ70 and disulfide-containing σ70 holoenzyme (Fig. 2, lanes 1 and 3). It is important to note that any wild type σ70 present in the cell was removed during this step, because wild type σ70 did not have a His6-tag. The lack of wild type σ70 in the isolated holoenzyme preparations was also confirmed by fluorescent labeling, as discussed below. The Ni-NTA eluates had a significant amount of protein contamination, primarily because the column was run at relatively low salt to ensure that the mutant holoenzymes did not dissociate into core enzyme and σ70. To further purify holoenzyme, the Ni-NTA eluates were passed over an immunoaffinity column containing a monoclonal antibody to the C terminus of the β′ subunit of RNA polymerase (30). Because this step is highly specific, the eluates contained pure disulfide-containing σ70 RNA polymerase holoenzyme free of contaminants, with the disulfide-containing σ70 in proper stoichiometry with the core subunits (Fig. 2, lanes 2 and 4) as determined by densitometry.

Once purified holoenzyme was obtained, a method was needed to verify that disulfide bond formation occurred within the mutant σ70 subunit of the holoenzymes. NMR and mass spectrometry, although extremely useful, were not practical because of the large size of the multisubunit holoenzyme complex. Instead, labeling with the fluorescent dye IC5-PE-maleimide was utilized to visualize free cysteines present in the denatured holoenzyme preparations. This method is advantageous because the IC5-maleimide dye is highly fluorescent, so labeled complexes can be subjected to SDS-PAGE and visualized with the use of a Typhoon imaging instrument. Wild type σ70 holoenzyme that contains three cysteines in σ70 was used as a positive control for labeling. To verify that the engineered cysteines were accessible to the IC5-maleimide dye, a single-cysteine σ70 holoenzyme (2.1S-2.2C) was also used as a control. Note that in all holoenzyme preparations, the β′, β, and α subunits of RNA polymerase contain multiple cysteines that are inaccessible to the IC5-maleimide dye, indicating the presence of free cysteines. Lanes 1 and 2, wild type σ70 holoenzyme; lanes 3, σ70 (2.1C-2.2C) holoenzyme; lane 4, σ70 (2.2C-2.3C) holoenzyme; lane 5, σ70 (2.1S-2.2C) holoenzyme. The positions of holoenzyme subunits are indicated on the right. B, SDS-PAGE of IC5-PE-maleimide labeled and unlabeled holoenzymes. Note the mobility shift of subunits that are labeled with the IC5-maleimide dye, indicating the presence of free cysteines. Lanes 1 and 2, wild type σ70 holoenzyme; lanes 3 and 4, cysteine-less σ70 holoenzyme; lanes 5 and 6, σ70 (2.1C-2.2C) holoenzyme; lanes 7 and 8, σ70 (2.2C-2.3C) holoenzyme. The positions of holoenzyme subunits are indicated on the right. Lane M shows broad range molecular mass markers (Novagen) with sizes (in kilodaltons) indicated on the left.

cysteines and did not nonspecifically label other amino acid residues. No detectable fluorescence was observed for the σ70 (2.1C-2.2C) or σ70 (2.2C-2.3C) subunits of holoenzyme, which contain cysteine substitutions in optimal positions for disulfide

![Fig. 3. Verification of disulfide-bond formation by IC5-PE-maleimide labeling and gel band shift.](http://www.jbc.org/)

A. labeling of denatured purified holoenzymes by the cysteine-reactive fluorescent dye IC5-PE-maleimide. The samples were denatured, labeled, analyzed by SDS-PAGE under nonreducing conditions, and visualized on a Typhoon system using the Cy5-filter set. Lane 1, wild type σ70 holoenzyme; lane 2, cysteine-less holoenzyme; lane 3, σ70 (2.1C-2.2C) holoenzyme; lane 4, σ70 (2.2C-2.3C) holoenzyme; lane 5, σ70 (2.1S-2.2C) holoenzyme. The mobility shift of subunits that are labeled with the IC5-maleimide dye, indicating the presence of free cysteines. Lanes 1 and 2, wild type σ70 holoenzyme; lanes 3 and 4, cysteine-less σ70 holoenzyme; lanes 5 and 6, σ70 (2.1C-2.2C) holoenzyme; lanes 7 and 8, σ70 (2.2C-2.3C) holoenzyme. The positions of holoenzyme subunits are indicated on the right. Lane M shows broad range molecular mass markers (Novagen) with sizes (in kilodaltons) indicated on the left.
The wild type \( \sigma^{70} \) thereby altering their mobility on the SDS-PAGE gel (Fig. 3). All contain multiple cysteines that bind IC5-maleimide, UV5 promoter. As a control, the cysteine-less \( \sigma^{70} \) lac transcription from a supercoiled template containing the vitro \( \sigma^{70} \) enzyme was shown to have a specific activity similar to that of wild type \( \sigma^{70} \) holoenzyme. These results suggest that no major block in transcription initiation from a supercoiled template occurs because of the presence of the engineered disulfide bonds within \( \sigma^{70} \) and that the disulfide-containing holoenzymes have similar specific activities compared with wild type \( \sigma^{70} \) holoenzyme. To confirm that the transcription phenotype of the cysteine-substituted \( \sigma^{70} \) holoenzymes was due to the presence of the disulfide bond in \( \sigma^{70} \) and not the amino acid substitutions, positions Val^{397} (2.1S-2.2C) and Ala^{145} (2.2S-2.3C) were substituted with serines. Because the cysteine and serine side chains are similar, the serine substitutions should mimic the engineered cysteine, except for the ability to form a disulfide bond. Both the \( \sigma^{70} \) (2.1S-2.2C) and \( \sigma^{70} \) (2.2S-2.3C) holoenzymes were greatly defective in the multiple-round transcription assay, probably because of an alteration in secondary structure, resulting from a disruption in the hydrophobic packing surface between the \( \alpha \)-helices of \( \sigma^{70} \) region 2. This result confirms that the wild type level of activity observed for the disulfide-containing holoenzymes is due to the presence of the disulfide bond and that maintenance of \( \alpha \)-helical structure is essential for transcription.

**Closed Complex Formation**—Conformational changes in RNA polymerase and \( \sigma^{70} \) may occur during the series of transitions that occur in a multiple-round transcription assay. However, multiple-round assays involve many post-initiation events, such as \( \sigma \) release (34–36) and \( \sigma \)-dependent pausing (37, 38), that may obscure defects in the role of \( \sigma^{70} \) during transcription initiation. A small defect in one process might be missed if it is not part of a rate-determining step. To address this concern, a more detailed analysis of the transcription pathway was performed beginning with examining the ability of the holoenzymes to form closed complexes on linear lacUV5 templates (−60 to +39). At low temperatures, normal holoenzymes are trapped in the closed complex form and are unable to complete the transition to the open complex. Closed complex formation was analyzed by native gel shift assays at 0 °C (Fig. 5). The cysteine-less holoenzyme control forms a closed complex readily, shifting all of the labeled DNA (Fig. 5, lane 2). Likewise, the disulfide-containing holoenzymes were able to form stable closed complexes (Fig. 5, lanes 3 and 4). This indicates

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**Fig. 4.** Multiple-round in vitro transcription assay using supercoiled lacUV5 template. The activity of the purified mutant holoenzymes was measured by multiple-round transcription using a supercoiled lacUV5 promoter as described under “Experimental Procedures.” The results are the averages of three different experiments. The error bars represent standard deviations.

**Fig. 5.** Gel mobility shift assay for closed complex formation. The reactions were assembled on ice and electrophoresed on a 5% native polyacrylamide gel at 0–2 °C prior to phosphorus imaging. Lane 1, lacUV5 promoter DNA; lane 2, cysteine-less \( \sigma^{70} \) holoenzyme; lane 3, \( \sigma^{70} \) (2.1C-2.2C) holoenzyme; lane 4, \( \sigma^{70} \) (2.2C-2.3C) holoenzyme. The positions of the closed complex (RP) and free DNA are indicated on the right.
that the presence of the disulfide bonds within these regions of \( \sigma^{70} \) does not affect closed complex formation under these conditions and that a major conformational change in \( \sigma^{70} \) region 2, such as separation of the \( \alpha \)-helices, is not required at this step.

Defects in Open Complex Formation—After formation of a closed complex, holoenzymes isomerize in one or more steps to an open complex, where the transcription bubble is formed and additional protein-DNA contacts are made (13, 15). To examine open complex formation, the holoenzyme-DNA template complexes were analyzed by DNase I footprinting at varying holoenzyme concentrations. At the lacUV5 promoter, the region of DNase I protection extends from approximately –50 upstream of the transcription start site to approximately +20, with a DNase I hypersensitivity site at –24/–25 (39). The cysteine-less holoenzyme control gave the expected protection pattern with clear DNase I protection extending to around –45 upstream, with a clear hypersensitivity site at –24/–25 (Fig. 6, lanes 1–3). Unexpectedly, neither the \( \sigma^{70} \) (2.1C-2.2C) nor the \( \sigma^{70} \) (2.2C-2.3C) holoenzyme exhibited protection of the promoter DNA from DNase I cleavage (Fig. 6, lanes 4–6 and 7–9, respectively). This result was repeated in several footprinting experiments at different temperatures (25 and 30 °C) and at holoenzyme concentrations greater than 25 nM (data not shown). The lack of DNase I protection suggests that the disulfide bonds within \( \sigma^{70} \) possibly affect a conformational change necessary for the transition from the closed complex to a stable open complex.

Because DNase I footprinting of the open complex requires holoenzyme to bind to the promoter DNA at high occupancy, the percentage of open complexes formed was determined by filter binding assays (40). Open complexes were allowed to form at 37 °C on linear lacUV5 template (–60 to +39). The reactions were challenged with heparin before filtering to remove any fast dissociating complexes (i.e. nonspecific complexes) from the population. Under these conditions, heparin binds free RNA polymerase, irreversibly preventing rebinding. The \( \sigma^{70} \) (2.1C-2.2C) holoenzyme had a severe defect in promoter occupancy during open complex formation, having only 5% of the control cysteine-less \( \sigma^{70} \) holoenzyme activity (Fig. 7). The \( \sigma^{70} \) (2.2C-2.3C) holoenzyme was also defective for promoter occupancy having only 12% of the cysteine-less \( \sigma^{70} \) holoenzyme activity. As an additional control, the serine-substituted \( \sigma^{70} \) (2.1S-2.2C) holoenzyme, which was previously found to be inactive in the in vitro transcription assay, was tested and showed no occupancy of the lacUV5 template. The low promotor occupancies observed in this experiment explain why neither disulfide-containing holoenzyme was able to protect the lacUV5 template from DNase I digestion. The results from the filter binding experiments were also confirmed by single-round in vitro transcription assays from linear lacUV5 template, with the \( \sigma^{70} \) (2.1C-2.2C) holoenzyme and \( \sigma^{70} \) (2.2C-2.3C) holoenzyme having 8 and 34% of wild type activity, respectively (data not shown). Together, these results indicate that restriction of major conformational changes in \( \sigma^{70} \) region 2, such as separation of the \( \alpha \)-helices, causes defects in the transcription initiation process.

Defects in Open Complex Stability—Although the disulfide-containing \( \sigma^{70} \) holoenzymes have no apparent defects in transcriptional activity in the multiple-round transcription assay (Fig. 4), they have significant defects in promoter occupancy during open complex formation (Fig. 7). One possibility is that the restrictions imposed by the disulfide bonds within \( \sigma^{70} \) impair the stability of the open complex, resulting in the low occupancies seen in the DNase I footprinting and filter binding experiments. To further examine these defects, the stability of the open complexes to the polyanionic competitor heparin was

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**Fig. 6.** DNase I footprinting of the mutant holoenzymes on a linear lacUV5 promoter fragment. The nontemplate strand of lacUV5 was labeled at position +40. 1–25 nM of each holoenzyme (indicated by triangles) was used in each footprinting reaction. The results are shown for experiments performed at 37 °C. Lanes 1–3, cysteine-less \( \sigma^{70} \) holoenzyme; lanes 4–6, \( \sigma^{70} \) (2.1C-2.2C) holoenzyme; lanes 7–9, \( \sigma^{70} \) (2.2C-2.3C) holoenzyme. Lane M shows the standard A + G markers. The numbers to the left indicate the relative positions of the lacUV5 promoter, and a bracket indicates the region of protection. The position of the DNase I hypersensitivity site is indicated by an asterisk.

**Fig. 7.** Filter binding assay to determine promoter occupancy of the various open complexes on linear lacUV5 promoter fragments. The reactions were assembled, incubated at 37 °C, and exposed to heparin before spotting onto nitrocellulose filter disks. \( ^3 P \)-Labeled DNA retained on the filter was quantitated by Cerenkov counting and corrected for background in the absence of holoenzyme. The values were normalized to the cysteine-less \( \sigma^{70} \) holoenzyme control, which is represented as 100%. The experiments represent the averages of three experiments with less than 5% deviation.
examined. Holoenzyme-promoter complexes were preformed on either supercoiled or linear lacUV5 templates. After addition of the heparin competitor, the fraction of complexes remaining at subsequent time intervals were measured by transcription.

It has been previously shown that open complexes on supercoiled lacUV5 templates are extremely long-lived (40). As expected, the cysteine-less $\sigma^{70}$ holoenzyme control formed very stable open complexes with half-lives greater than 40 h (Fig. 8A). The $\sigma^{70}$ (2.1C-2.2C) holoenzyme exhibited a defect in open complex stability with a half-life of $-7$ h. Likewise, the $\sigma^{70}$ (2.2C-2.3C) holoenzyme had a lesser defect with a half-life of greater than 16 h. These differences in open complex stability were not dramatic enough to alter multiple-round transcriptional activity from a supercoiled template, as seen in the results from Fig. 4.

In contrast, open complex stability on a linear template was dramatically lower than that observed with supercoiled templates (40). A wild type $\sigma^{70}$ holoenzyme open complex has been shown to have a half-life of 17 min on linear lacUV5 templates (40). This result agrees with the 16-min half-life observed with the cysteine-less $\sigma^{70}$ holoenzyme control (Fig. 8B). The $\sigma^{70}$ (2.1C-2.2C) holoenzyme did show a major defect in open complex stability on the linear template, with a half-life of only 4.5 min. Alternatively, no significant decrease in stability is observed with the $\sigma^{70}$ (2.2C-2.3C) holoenzyme, whose half-life was 14 min. These results strongly suggest that the defect of $\sigma^{70}$ (2.1C-2.2C) holoenzyme in open complex formation was caused by the instability of the open complex on linear DNA. Unfortunately, these results do not explain why the $\sigma^{70}$ (2.2C-2.3C) holoenzyme has a defect in open complex promoter occupancy on linear templates.

**DISCUSSION**

Transcription initiation in prokaryotes occurs through the multi-subunit RNA polymerase holoenzyme, consisting of core subunits $\alpha\beta\beta'\omega$ and the specificity subunit $\sigma$ (1). Because core RNA polymerase and the $\sigma$ factor are the only two proteins necessary for transcription initiation to occur, conformational changes may be required at various locations along a series of intermediary steps. These steps include: formation of holoenzyme, closed complex formation upon promoter binding, open complex formation and promoter melting, and finally formation of the processive elongation complex (3, 10). Previous experiments to identify conformational change within this process have resulted in information about individual steps but have not addressed transitions between intermediates.

Luminescence resonance energy transfer experiments have suggested that upon holoenzyme formation, the DNA-binding regions of $\sigma^{70}$ (regions 2.4 and 4.2) undergo conformational changes that place them in positions compatible with the spacing of the $-10$ and $-35$ promoter elements (9, 28). Additionally, the interaction between region 1.1 and region 4, which prevents free $\sigma^{70}$ from binding DNA, has been shown to be disrupted upon holoenzyme formation, suggesting that a major conformational change occurs within $\sigma^{70}$ (41, 42). These predicted gross conformational changes in $\sigma^{70}$ were validated upon inspection of the crystal structure from *T. aquaticus* $\alpha^h$ holoenzyme (21), suggesting that $\sigma^{70}$ is a dynamic protein capable of undergoing conformational changes during holoenzyme formation.

Along with the high resolution crystal structures of *T. aquaticus* holoenzyme, a complex of this holoenzyme bound to “fork junction” DNA (20, 21) has provided new insight into when major conformational changes may occur during transcription. Four large mobile modules, which move as relatively rigid bodies with respect to the main “core” module of holoenzyme, were identified by comparing the two holoenzyme structures to the previously known core enzyme structure (19). This comparison revealed that two of the modules required changes within the DNA-binding regions of $\sigma$ (20). The first module, which contains the flap domain of the $\beta$ subunit of core enzyme bound to $\sigma$ region 4, rotated $-4^\circ$, placing the $-35$ recognition helix of $\sigma$ in position for binding the $-35$ hexamer. The second module, consisting of the RNAP clamp domain and $\sigma$ region 2, rotated toward the RNAP channel (20). These conformational changes suggest that flexibility in the holoenzyme structure is required for proper positioning of the DNA-binding determinants of $\sigma$ so that sequence specific contacts can be established. In addition, Murakami et al. (20) state that placement of the template strand within the DNA channel must involve opening of the “jaws” of RNA polymerase during open complex formation. Reclosure of the RNA polymerase jaws is then necessary for formation of the transcription tunnel, where the template strand is completely enclosed by protein (20). These hypotheses suggest that holoenzyme is a dynamic structure and may undergo several yet uncharacterized conformational changes as it proceeds through the process of transcription initiation.

Because the large size of the multi-subunit RNA polymerase...
holoenzyme prohibits NMR analysis or other conventional methods of analyzing conformational changes, we chose to utilize a disulfide prediction algorithm (22) to probe for conformational changes in specific regions of σ70 that are required for the proteins to function. We chose to engineer disulfide bonds within σ70 region 2 that would prohibit major conformational change involving separation of α-helices and then assay for function throughout the multiple steps of transcription initiation. The first disulfide bond, involving residues Cys434–Cys408, effectively locks region 2.1 to region 2.2 by virtue of the disul- 
feral bond binding (Figs. 6 and 7), both disulfide-locked
not required for closed complex formation. However, in two
these regions of
complex lifetime for the

tional change in region 2 might be necessary to facilitate melt-
lar of the region 2.1 might be necessary to facilitate DNA binding. According to
the filter binding and heparin stability assays, the σ70 (2.2C–
2.3C) holoenzyme had slightly less of a defect than σ70 (2.1C–
2.2C) holoenzyme for open complex formation. We speculate
based upon protein modeling that the disulfide bond locking
region 2.2 to region 2.3 is not as rigid because of the large loop
connecting regions 2.2 and 2.3 that may allow for some flexi-
arity between the α-helices but allows for some rotation around the disulfide bond.

Several lines of evidence led us to speculate that the α-helical
region 2 of σ70 might undergo conformational change upon
holoenzyme formation or steps thereafter. First, mutational
analysis of the coiled-coil of the β subunit of RNA polymerase
produced several mutants that had increased or decreased
affinity for σ70 (43). However, these mutations were positioned
on the underside of the coiled-coil, away from the residues
thought to bind σ70 (44). These mutations could be explained if
the helices in σ70 region 2 separated from each other to form
new interactions with the coiled-coil during holoenzyme forma-
 tion. Second, luminescence resonance energy transfer analysis
of σ70 indicates that regions 2.4 and 4.2 underwent a signifi-
cant conformational change upon holoenzyme formation, where
these regions of σ70 became more solvent exposed upon binding
of core enzyme (45). Finally, defects in open complex formation
caused by mutations in region 2.2 of σ70 (46). We sought to examine the effect of restricting helical move-
ment in σ70 region 2 during the various steps of transcription
initiation. In multiple-round in vitro transcription assays,
which test the overall transcriptional activity of the holoen-
zyme, both the σ70 (2.1C-2.2C) and σ70 (2.2C-2.3C) holoenzyme
displayed wild type levels of activity. This result indicates that
the presence of the disulfide bonds do not disrupt a rate-
limiting step required for transcriptional activity (Fig. 4). Like-
wise, the mobility shift assay for closed complex formation on
linear template showed no defect from either disulfide-locked
σ70 holoenzyme (Fig. 5). This result suggests that a major
conformational change within the α-helices of σ70 region 2 is
not required for closed complex formation. However, in two
assays for open complex formation, DNase I footprinting,
and filter binding (Figs. 6 and 7), both disulfide-locked σ70 holoen-
zymes exhibited clear defects. The defects in occupancy of the
lacUV5 promoter were severe, suggesting that a conforma-
tional change in region 2 might be necessary to facilitate melt-
ing of promoter DNA and formation of a stable open complex.
Furthermore, heparin stability assays demonstrated that
of those open complexes that were able to form, the open
complex lifetime for the σ70 (2.1C-2.2C) holoenzyme was
greatly shortened (Fig. 8).

From the experimental results, we propose that conforma-
tional flexibility within σ70 region 2 may facilitate proper place-
ment of the α-helices during transcription initiation. We sug-
gest that the 2.1C-2.2C disulfide in σ70 causes defects in
holoenzyme open complex formation and stability because of
conformational restriction on σ70 region 2. One potential con-
facilitate interactions with the – 10 nontemplate strand during the transition between closed and open complexes. 

This work has looked at a small region of α70 to determine its role in transcription initiation, but it would be interesting to further investigate: 1) what positioning of region 2.3–2.4 is required to begin strand separation and open complex formation, 2) if the disulfide-locked α70 holoenzymes are defective for positioning the DNA within the jaws of RNA polymerase, or 3) if a conformational change in region 2 helps to open the jaws during open complex formation. The continued availability of high resolution structural information and continued changes of alternative methods such as disulfide bond engineering will help us to understand the true role of conformational changes within holoenzyme during transcription initiation.

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