Characterization of the Interactions between the Bacteriophage T4 AsiA Protein and RNA Polymerase†

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ABSTRACT: The anti-σ factor AsiA effects a change in promoter specificity of the *Escherichia coli* RNA polymerase via interactions with two conserved regions of the σ^{70} subunit, denoted 4.1 and 4.2. Free AsiA is a symmetrical homodimer. Here, we show that AsiA is monomeric when bound to σ^{70} and that a subset of the residues that contribute to the homodimer interface also contributes to the interface with σ^{70}. AsiA interacts primarily with C-terminal sections of regions 4.1 and 4.2, which show remarkable sequence similarity. An AsiA monomer can simultaneously, and apparently cooperatively, bind both isolated regions 4.1 and 4.2 at preferred, distinct subsites, whereas region 4.1 alone or region 4.2 alone can interact with either subsite. These results suggest structural and functional plasticity in the interaction of AsiA with σ^{70} and support the notion of discrete roles for regions 4.1 and 4.2 in transcription regulation by AsiA. Furthermore, we show that AsiA inhibits recognition of the −35 consensus promoter element by region 4 of σ^{70} indirectly, as the residues on region 4 responsible for AsiA binding are distinct from those involved in DNA binding. Finally, we show that AsiA must directly disrupt the interaction of region 4 with the RNA polymerase β subunit flap domain, resulting in a distance change between region 2 and region 4 of σ^{70}. Thus, a new paradigm for transcription regulation by AsiA is emerging, whereby the distance between the DNA binding domains in σ^{70} is regulated, and promoter recognition specificity is modulated, by mediating the interactions of the σ region 4 with the β subunit flap domain.

DNA recognition by the prokaryotic RNA polymerase holoenzyme is mediated by the dissociable σ (sigma) subunit. The elegant, recent X-ray crystallography studies of the prokaryotic RNA polymerase (from *Thermus aquaticus*) and its components (*1*−*3*) confirm the expectations from many previous studies (for instance, see refs *4* and *5*) that the sequence-specific interactions between the RNA polymerase holoenzyme ($\alpha_2\beta\beta'\omega\sigma^A$) and the principal promoter elements of the DNA are via the primary sigma factor, σ^A (a member of the σ^{70} family, see ref *4*). In the complex between the *T. aquaticus* RNA polymerase holoenzyme and a fork-junction promoter DNA fragment (*3*), highly conserved sequences in regions of σ denoted 2 and 4 (*4*) interact with the −10 and −35 elements, respectively, of the promoter. The results of the structural studies also suggest that the specificity of promoter recognition is managed by adjusting the relative orientation and spacing of the participant σ regions. This adjustment is realized by interactions between σ and the core subunits. These interactions, for instance, are responsible in part for establishing the proper orientation of region 4 of σ with respect to the DNA and for maintaining the requisite spacing between regions 2 and 4 for optimal interactions with the respective promoter elements (*3*).

Prokaryotic transcription initiation can be regulated by the interaction of anti-σ factors with their cognate σ factors. In *Escherichia coli*, the T4 phage-encoded anti-σ factor AsiA, the first anti-σ factor to be discovered (*6*−*10*), inhibits transcription at both σ^{70}-dependent early phage promoters and host promoters (*8*, *9*, *11*, *12*). AsiA additionally functions as a transcriptional co-activator, supporting efficient transcription at middle phage promoters in concert with the phage protein MotA (*12*−*16*). AsiA is an all-helical (*17*) symmetrical homodimer in solution (*18*−*20*) with a novel helical fold and a helix−turn−helix DNA binding element (*19*). AsiA has no sequence homologues (with the exception of those homologues from the T4-like phages, http://phage.bioc.tulane.edu). AsiA binds tightly to σ^{70} (*6*−*10*), and its effects on transcription presumably stem entirely from this interaction. This tight interaction has been localized to the highly conserved sequence within region 4 of σ^{70} referred

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to as 4.2 (*21*−*23*), and more recently a high affinity interaction between AsiA and region 4.1 has also been demonstrated (*18*).

The highly conserved sequence 4.2 of region 4 of σ^{70} forms a helix−turn−helix DNA binding motif that recognizes the −35 element of the promoter DNA (*1*, *3*, *24*). AsiA binding to region 4 inhibits transcription at −10/−35 promoters and thus interferes with this recognition (*22*, *23*, *25*−*27*). Whether this interference is direct (AsiA and DNA compete for interactions with a specific set of residues in 4.2) or somehow indirect is still a matter of concern (*22*, *23*, *26*). Likewise, the physical/structural basis for the stimulation of transcription from middle phage promoters by AsiA is unknown.

On the basis of our recent results, we have suggested that transcription regulation by AsiA is multifaceted and somewhat more intricate than previously suspected and that both regions 4.1 and 4.2 of σ^{70} are important for the regulatory functions that AsiA performs and might be functionally discrete in this context (*18*, *19*). Very recent results lend substantial support to this view (*28*). AsiA might also function to interact directly with DNA (*19*). Herein, we present results that bear directly on the structural underpinnings of these aspects of the interactions between AsiA and RNA polymerase and the mechanism of transcription regulation by AsiA. Additionally, our results suggest that AsiA inhibits transcription at −10/−35 promoters indirectly and that AsiA interferes directly with the interactions between region 4 of σ^{70} and the β flap region of the polymerase and thereby alters the spacing between the −10 and −35 promoter recognition elements.

EXPERIMENTAL PROCEDURES

Production and Isotopic Labeling of AsiA. The *asiA* gene (*11*) was subcloned into the pET-24b expression vector (Novagen, Madison, WI) and transformed into *E. coli* BL21-(DE3) for AsiA overproduction. Isotopically labeled (^{13}C, ^{15}N) AsiA was produced using minimal medium (M9) with ^{13}C-glucose (Cambridge Isotope Laboratories, Inc., Andover, MA) and $^{15}NH_4Cl$ (Isotec Inc., Miamisburg, OH) as the sole carbon and nitrogen sources, respectively. Purification of AsiA was accomplished as described previously (*12*) with minor modifications. For the luminescence resonance energy transfer experiments and related experiments, AsiA was prepared as described previously (*23*).

Production of σ^{70}-Derived Peptides. Peptides corresponding to conserved regions 4.1 ($\sigma^{70}_{4.1}$, LRAATHDVLAGLTAREAKVLRMRFGI, residues 540−565 of *E. coli* σ^{70}) and 4.2 ($\sigma^{70}_{4.2}$, DYTLEEVGKQFDVTRERIRQIEAKALRKLR, residues 570−599) of *E. coli* σ^{70} were synthesized at the Biochemical Research Service Laboratory at the University of Kansas by standard FMOC[1] methods and purified using reversed-phase HPLC with acetonitrile (HPLC grade, Fisher Scientific, Pittsburgh, PA) gradient elution (0.1% trifluoroacetic acid (Aldrich, Milwaukee, WI) in all eluants). Purities of the peptides were confirmed by analytical HPLC, and the masses were confirmed using MALDI-TOF mass spectrometry (using a Voyager-DE MALDI STR instrument, PerSeptive Biosystems Inc., Framingham, MA) and electrospray ionization (ESI) mass spectrometry (using a Q-Tof quadrupole time-of-flight hybrid instrument, Micromass Ltd., Manchester, UK). The sequences of the peptides were also confirmed by tandem mass spectrometry.

Isotopically labeled $\sigma^{70}_{4.1}$ and $\sigma^{70}_{4.2}$ were produced recombinantly as fusions to calmodulin. The fusion proteins, from the N-terminus, consisted of vertebrate calmodulin, followed by a 13 amino acid linker harboring an enterokinase cleavage site, followed by either $\sigma^{70}_{4.1}$ or $\sigma^{70}_{4.2}$. The genes encoding the fusions were cloned into modified pET-15b vectors, and the vectors were transformed into the *E. coli* BL21(DE3) cell strain for protein overproduction. Uniformly isotopically labeled protein was produced as described above for isotopically labeled AsiA. The fusion protein was isolated from crude cellular extracts and purified by affinity chromatography on phenyl Sepharose CL-4B (Pharmacia, Piscataway, NJ) using the usual procedure for calmodulin purification (*29*). The isotopically labeled $\sigma^{70}_{4.1}$ and $\sigma^{70}_{4.2}$ peptides were cleaved from the fusion proteins with recombinant enterokinase (Novagen, Madison, WI) using 1.0 unit per 0.5−0.75 mg of fusion protein in solutions containing 5−6 mg/mL fusion protein. Cleavage was assessed by SDS−PAGE. After cleavage, the solutions were passed over a *p*-aminobenzamidine (Sigma Aldrich, St. Louis, MO) column (1.8 × 2 cm) to remove the enterokinase. PMSF was added to the eluant to a final concentration of 0.5 mM. The peptides were purified (and calmodulin removed) by reversed phase HPLC as described above for the synthetic peptides. The yield of either peptide was ∼3−4 mg per liter of culture. Purities/identities/sequences were confirmed as described above for the synthetic peptides.

AsiA Samples and Complexes of AsiA with σ^{70}-Derived Peptides. Samples of AsiA required to characterize interprotomer contacts consisted of equimolar quantities of uniformly ^{13}C-, ^{15}N-labeled AsiA and unlabeled AsiA. Samples of AsiA for NMR studies contained 1.0−1.5 mM AsiA, 50 mM d_4-acetic acid, 50 mM KCl, 0.02% sodium azide, and 10% D_2O, pH 6.2−6.3 (meter reading). Complexes of isotopically labeled AsiA with unlabeled σ^{70}-derived peptides for NMR analyses were prepared by incremental addition of the peptides in the buffer described above to AsiA in the same solution. Excess peptide was added to ensure that all of the AsiA was bound (*18*). Complexes were concentrated with minimal peptide loss using Millipore Centricon ultrafiltration units (YM-3, nominal 3 kDa cutoff) to final volumes of ∼700 μL. For complexes of unlabeled AsiA with isotopically labeled peptides, AsiA was added incrementally to the peptides and in excess to ensure all of the peptides were bound.

NMR Spectroscopy. All NMR spectra were recorded with a Varian INOVA spectrometer operating at 600 MHz (^{1}H) as described recently (*17*−*19*). In all cases, the sample temperature was 25 °C. The ^{1}H chemical shifts were referenced to external Na^+DSS^- in D_2O (0.00 ppm), while ^{13}C and ^{15}N chemical shifts were referenced indirectly assuming the absolute frequency ratios $^{13}C/^1H = 0.251449530$ and $^{15}N/^1H = 0.101329118$ (*30*). Data processing and analysis were accomplished using Felix (Accelrys, San Diego, CA).

[1] Abbreviations: FMOC, 9-fluorenylmethoxycarbonyl; HPLC, high performance liquid chromatography; MALDI, matrix assisted laser desorption ionization; TOF, time-of-flight; NMR, nuclear magnetic resonance; HSQC, heteronuclear single quantum coherence; NOE, nuclear Overhauser effect; PMSF, phenylmethylsulfonylfluoride.

NMR Resonance Assignments and NOE Measurements. Gradient sensitivity enhanced ^{1}H, ^{15}N-HSQC (*31*) spectra were recorded with 1024 and 128 complex points in the ^{1}H (*t*2) and ^{15}N (*t*1) dimensions, respectively (spectral widths, 7692.31 and 1609.98 Hz, respectively). Constant time ^{1}H, ^{13}C-HSQC (*32*) spectra were recorded with 512 and 115 complex points in the ^{1}H and ^{13}C dimensions (spectral widths, 7692.35 and 4000 Hz). The following gradient sensitivity enhanced triple resonance experiments, for resonance assignment of the main chain (and $^{13}C^{\beta}$) of the AsiA component of the complexes of AsiA with the σ^{70}-derived peptides, for assignment of the isotopically labeled peptides free in solution, and for assignment of the resonances of labeled peptides bound to AsiA, were recorded with the indicated number of complex points and spectral widths: HNCA/HN(CO)CA (512 (^{1}H, t_3, 7692.31 Hz) × 56 (^{13}C, t_2, 4000.00 Hz) × 32 (^{15}N, t_1, 1609.98 Hz)), CBCA(CO)NH (512 (^{1}H, t_3, 7692.31 Hz) × 47 (^{13}C, t_2, 8000 Hz) × 32 (^{15}N, t_1, 1609.98 Hz)), HBHA(CBCACO)NH (512 (^{1}H, t_3, 7692.31 Hz) × 64 (^{1}H, t_2, 2800 Hz) × 32 (^{15}N, t_1, 1609.98 Hz)), HNCO (512 (^{1}H, t_3, 7692.31 Hz) × 60 (^{13}C, t_2, 1800.00 Hz) × 32 (^{15}N, t_1, 1609.98 Hz)) (*33*−*35*). Side chain resonances have also been assigned, in order that the intermolecular contacts could be established for the complexes. For these assignments, HCCH-TOCSY (*36*, *37*) experiments were used (512 (^{1}H, t_3, 7692.31 Hz) × 100 (^{1}H, t_2, 3600.04 Hz) × 44 (^{13}C, t_1, 4000.00 Hz)). For intermolecular contacts/NOEs, ^{13}C F_1-filtered, F_3-edited NOESY-HSQC (*38*) spectra were recorded (512 (^{1}H, t_3, 7692.31 Hz) × 96 (^{1}H, t_2, 7692.31 Hz) × 40 (^{13}C, t_1, 4000 Hz)). Control spectra (all polypeptides either isotopically labeled or unlabeled) were acquired to confirm the integrity of the filtering/editing experiments.

Donor−*Acceptor-Labeled Cysteine Mutants of σ^{70}.* The σ^{70} double-cysteine mutants, [T440C, D581C] and [A59C, R596C], were overexpressed and labeled as described previously (*39*, *40*). The fluorescence donor and acceptor were (Eu^{3+})DTPA-AMCA-maleimide and CY5-maleimide, respectively (*40*). Modified σ^{70} was purified further by size exclusion FPLC using a Superdex 200 HR column (Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated and eluted with a solution containing 50 mM HEPES (pH 7.9), 100 mM KCl, 10 μM EDTA, and 0.5 mM DTT.

RNA Polymerase Holoenzyme Reconstituted with Labeled σ^{70} Mutants. RNA polymerase holoenzyme containing labeled σ^{70} mutants was reconstituted from core and either excess labeled [T440C, D581C] or [A59C, R596C] σ^{70} as described previously (*39*, *41*). The reconstituted holoenzyme was purified using size exclusion FPLC as described for the modified σ^{70} (above). In abortive transcription initiation assays, the RNA polymerase holoenzyme containing the donor−acceptor labeled [T440C, D581C] σ^{70} displayed 80 (±8%) of the activity of holoenzyme containing wild type σ^{70} (71 ± 3% for the [A59C, R596C] mutant).

Fluorescence Measurements and Analyses. Luminescence resonance energy transfer experiments were performed with a laboratory-built two-channel spectrofluorometer as described previously (*42*). Decays of donor in the presence of acceptor and sensitized acceptor decay curves were fitted using nonlinear regression with SCIENTIST (Micromath Scientific Software, Salt Lake City, UT), essentially as described previously (*40*, *42*). The best fits of sensitized acceptor decay curves were obtained using a three exponential decay model. The obtained decay lifetimes were further analyzed as described elsewhere (*42*), and the distances between a donor and an acceptor were calculated from the energy transfer as described (*40*).

Measurements were performed on samples in a 120 μL cuvette using 30 nM labeled σ^{70} or RNA polymerase holoenzyme containing the labeled mutant σ^{70} (45 nM for holoenzyme containing the labeled [T440C, D581C] mutant), 50 mM HEPES (pH 7.9), 100 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 10 μM EDTA, 0.1 mg/mL BSA, and 2.8% (w/v) PEG (8000) at 25 °C. The effects of AsiA were assessed by incremental AsiA additions to the solution, followed by a 5 min equilibration period (25 °C), and subsequent acquisition of donor and acceptor emission. After three consecutive cycles of AsiA addition and measurement (the total incubation time of σ^{70} with AsiA was 15 min), the solution in the cuvette was replaced with a fresh mixture. The measurements at each AsiA concentration were repeated 2−3 times. Error bars represent standard deviations from the mean values.

AsiA Inhibition of DNA Binding to RNA Polymerase. Binding of AsiA to RNA polymerase holoenzyme containing the donor−acceptor labeled [T440C, D581C] mutant σ^{70} or wild type σ^{70} was studied by measuring the inhibition of DNA binding to the holoenzyme by AsiA. RNA polymerase holoenzyme (4 nM) containing either wild type or modified σ^{70} was incubated with increasing amounts of AsiA for 10 min at 25 °C in the same buffer used for the fluorescence experiments (above). Following the incubation, 0.1 nM ^{32}P-labeled λ PR promoter DNA (109 base pair fragment containing the λ PR promoter with consensus sequences in the −10 and −35 hexamers and a 17 base pair spacer) and 60 ng of poly (dI-dC) (Amersham Pharmacia Biotech, Uppsala, Sweden) was added, and the mixture was incubated for 10 min. Heparin (500 μg/mL) was then added followed by an additional 3 min incubation. Protein−DNA complexes were separated from free DNA by electrophoresis using a nondenaturing 4% polyacrylamide gel in 1 × TBE (1 h 20 min, 80 V, ambient temperature). The bands on the dried gel were exposed and analyzed using phosphor imaging and ImageQuant software (Amersham Biosciences, Piscataway, NJ).

AsiA Binding to Donor−*Acceptor Labeled σ^{70}.* AsiA binding to the donor−acceptor labeled [A59C, R596C] σ^{70} mutant was confirmed by a nondenaturing gel mobility shift assay. The modified σ^{70} (3.6 pmol) was incubated with or without AsiA (20 pmol) in 10 μL of 20 mM Tris, 30 mM HEPES (pH 7.9), 100 mM NaCl, 10 μM EDTA, 1 mM DTT, and 20% glycerol for 15 min at 25 °C. Samples were then subjected to electrophoresis using a 6% (37.5:1, acrylamide/bisacrylamide) nondenaturing polyacrylamide gel in 25 mM Tris, 180 mM glycine buffer (pH 8.3, 1 h 15 min, 120 V). Bands containing the modified σ^{70} were detected using fluorescence imaging (detecting the CY5 label).

RESULTS

Sequence Similarity of Regions 4.1 and 4.2 of σ^{70} and the β Flap-Tip Helix. The C-terminal region of σ^{70} is comprised of two adjacent conserved regions denoted region 4.1 and region 4.2 (*4*). Conserved region 4.2 of σ^{70} forms a helix−turn−helix motif (Figure 1) that interacts in a sequence specific manner with the −35 promoter consensus element

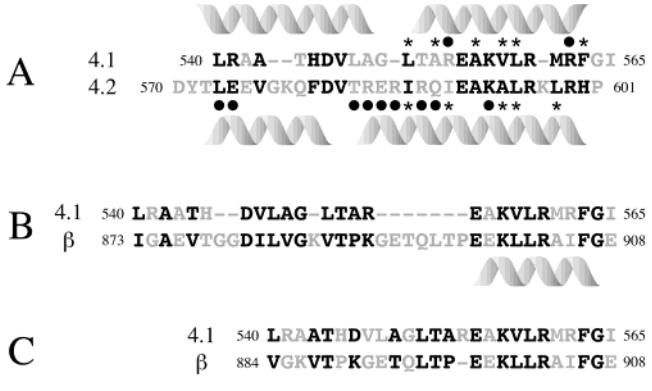

FIGURE 1: Sequence similarity between conserved regions 4.1 and 4.2 of the σ^{70} subunit and the β flap region of the β subunit of the *E. coli* RNA polymerase. Alignments were performed using CLUSTALW (*50*). Bolded residues indicate identity, high similarity, and weak similarity. (A) Alignment of regions 4.1 and 4.2 of σ^{70} showing the high sequence identity/similarity of the C-terminal sections of these regions. Secondary structural elements are indicated. Filled circles (●) indicate residues involved in binding to the −35 region of the promoter according to the structure of Campbell et al. (*1*), while asterisks (*) indicate residues involved in binding to AsiA. (B and C) Alignments of region 4.1 of σ^{70} and sections of the β flap region including the β flap-tip helix (helix indicated in panel B). The synthetic $\sigma^{70}_{4.1}$ peptide used in our studies corresponds to residues 540−565, and the $\sigma^{70}_{4.2}$ peptide used in our studies corresponds to residues 570−599.

(*1*−*4*, *43*−*45*). Region 4.2 has also been shown to interact tightly with AsiA (*21*, *25*, *26*, *46*, *47*). We have shown recently that a peptide corresponding to region 4.2 ($\sigma^{70}_{4.2}$, 30 amino acids) binds tightly to AsiA (*18*). The role of conserved region 4.1 has recently been clarified from X-ray crystallographic studies that suggest that it interacts with region 4.2 and with the β flap-tip helix (of the β subunit of the RNA polymerase core) to aid in proper positioning of region 4.2 with respect to the −35 region of the promoter (*2*, *3*). With respect to AsiA function, we have shown recently that a peptide corresponding to region 4.1 ($\sigma^{70}_{4.1}$, 26 amino acids) also interacts tightly with AsiA. In addition, $\sigma^{70}_{4.1}$ and $\sigma^{70}_{4.2}$ can bind simultaneously (and tightly) to AsiA (*18*).

It is important that regions 4.1 and 4.2 of σ^{70}, in particular the C-terminal sections, exhibit considerable sequence similarity (Figure 1A). This is significant, in terms of the interaction with AsiA, since it is these C-terminal sections that contact AsiA (see below). Furthermore, a region of the β subunit of the RNA polymerase core known as the β flap-tip helix, which interacts with these C-terminal sections of regions 4.1 and 4.2 of σ^{70} (*2*, *3*, *48*), also shares this sequence similarity (Figure 1B,C).

Dissociation of the AsiA Dimer upon Binding to σ^{70}-Derived Peptides. Consistent with our previous suggestions (*18*, *19*), binding of the σ^{70}-derived peptides to AsiA in solution dissociates the AsiA dimer (Figure 2). Previously, we have used isotope edited, filtered NMR experiments (3-D ^{13}C F_1-filtered, F_3-edited NOESY-HSQC, ref *38*) to identify the residues that form the hydrophobic core of the AsiA homodimer interface (*18*, *19*). These experiments allow selective observation of contacts between a uniformly isotopically (^{13}C, ^{15}N) labeled protein or polypeptide and an unlabeled one. Isotope edited, filtered NMR experiments on a 1:1 mixture of uniformly ^{15}N-, ^{13}C-labeled AsiA and

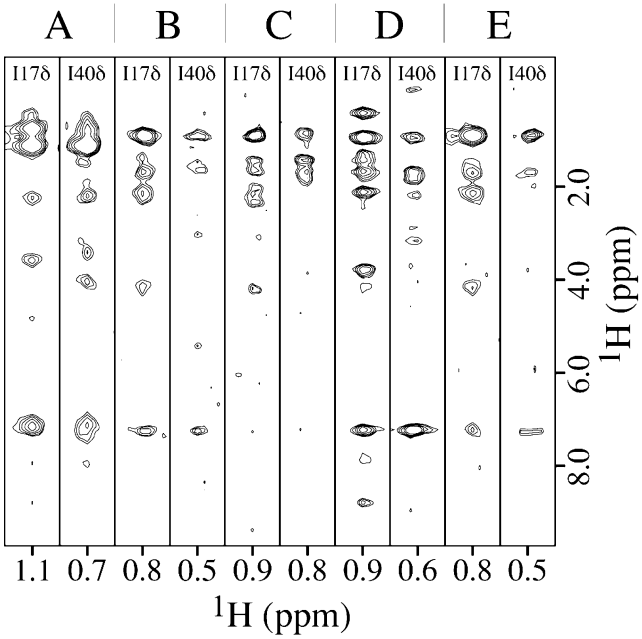

FIGURE 2: Intermolecular NOE contacts between the protomers of the AsiA dimer and between AsiA and conserved regions 4.1 and 4.2 of σ^{70} from 3-D ^{13}C F_1-filtered, F_3-edited NOESY-HSQC spectra. Shown are the NOE cross-peaks involving the δ methyl groups of I17 and I40 on an AsiA protomer and protons of either the other protomer in the dimer or of the 4.1/4.2 peptides. (A) NOE cross-peaks between protomers in AsiA dimers of a 1:1 mixture of ^{13}C, ^{15}N-labeled AsiA and unlabeled AsiA. (B−D) NOE cross-peaks between ^{13}C, ^{15}N-labeled AsiA and unlabeled peptides corresponding to regions 4.1 (B), 4.2 (C), or both 4.1 and 4.2 (D) of σ^{70}. (E) Same as panel B, except the AsiA present consisted of a 1:1 mixture of ^{13}C, ^{15}N-labeled and unlabeled AsiA.

unlabeled AsiA (Figure 2A) selectively reveal intermolecular contacts between the protomers of the AsiA dimer that allowed proper positioning of the protomers in the dimer structure (*19*). In Figure 2A are shown interprotomer NOEs indicating close contacts between the δ methyl groups of I17 and I40 on one protomer of the AsiA dimer with protons on the other protomer. The side chains of I17 and I40 are at the AsiA dimer interface (*19*). When the same experiment is performed with a complex of uniformly labeled AsiA and unlabeled $\sigma^{70}_{4.1}$ (Figure 2B), unlabeled $\sigma^{70}_{4.2}$ (Figure 2C), or unlabeled $\sigma^{70}_{4.1}$ and unlabeled $\sigma^{70}_{4.2}$ (Figure 2D), close contacts between the peptides and these same methyl groups on AsiA are observed. A number of amino acid side chains at the AsiA dimer interface other than I17 and I40 are also involved in interacting with the σ^{70}-derived peptides (Figure 3), indicating that the regions of AsiA that participate in the homodimer interface overlap significantly with those regions involved in the heterodimer interface (with σ^{70}), consistent with our previous suggestion that σ^{70} interacts with AsiA by displacing one of the AsiA protomers (*19*). However, it is important to note that the AsiA dimer interface is comprised of not only the core hydrophobic residues (Figure 3) but also includes a substantial peripheral, mostly polar component (see below). Finally, it is evident that no AsiA dimer is present in the complexes of AsiA with the σ^{70}-derived peptides (Figure 2E). For example, the isotope edited, filtered NMR spectrum of a complex of unlabeled $\sigma^{70}_{4.1}$ with a 1:1 mixture of uniformly ^{13}C-, ^{15}N-labeled and unlabeled AsiA is identical to that of the complex of $\sigma^{70}_{4.1}$

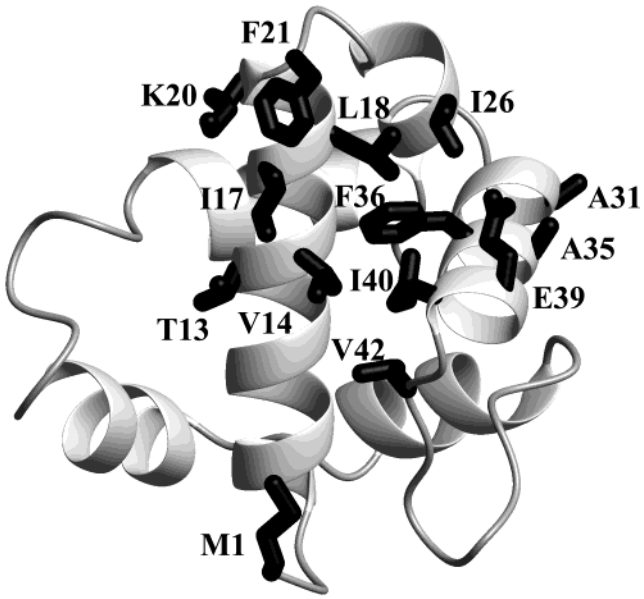

FIGURE 3: Key interfacial residues in AsiA. Shown are the side chains of residues comprising the mostly hydrophobic core of the AsiA homodimer interface (excluding A31 and A35) and the heterodimer interface with σ region 4 (excluding M1).

with all labeled AsiA (compare Figure 2E and B). If the AsiA dimer were present in this complex, then NOE cross-peaks corresponding to the interprotomer contacts would be present in the spectrum acquired with the complex containing the mixture of labeled and unlabeled AsiA. Although only small regions of these 3-D spectra are shown in Figure 2, the spectra in Figure 2B,E are identical, indicating no AsiA dimer exists in this complex. Results of recent studies using disulfide-tethered AsiA dimers and fluorescence resonance energy transfer studies using AsiA labeled with fluorescence chromophores support these conclusions (*28*). The complex of an AsiA monomer with σ region 4 is also sufficiently stable to be detected by MALDI-TOF mass spectrometry (*20*).

Chemical Shift Perturbation in AsiA upon Binding to σ^{70}-Derived Peptides. Previously, we have assigned the chemical shifts for AsiA (*17*, *19*). To determine the site-specific chemical shift perturbations in AsiA upon binding to σ^{70}-derived peptides, we have assigned the main chain chemical shifts of AsiA bound to $\sigma^{70}_{4.1}$, of AsiA bound to $\sigma^{70}_{4.2}$, and of AsiA bound simultaneously to both $\sigma^{70}_{4.1}$ and $\sigma^{70}_{4.2}$ (Figure 4). In Figure 4 are shown contour plots of the ^{1}H, ^{15}N-HSQC spectra for these complexes (Figure 4A−C, red contours) superimposed on the spectrum of free AsiA (black contours) and plots of the chemical shift changes that occur in AsiA when these peptides bind (Figure 4D−F). In all three cases, the most substantial changes occur at the N-terminal end of the N-terminal helix (helix 1) of AsiA, in residues 44 and 45 (loop between helices 3 and 4), and in residues 70−75 (turn region between helices 5 and 6; in the case of the complex with $\sigma^{70}_{4.1}$, the peaks corresponding to these residues are no longer observable). These residues comprise the mostly polar, peripheral component of the AsiA dimer interface (Figure 5), and their chemical shift changes reflect structural and solvation changes that occur upon dissociation of the AsiA dimer as a result of binding to the peptides. Because these regions are not involved in direct interactions with the σ peptides, their environments change dramatically upon dissociation (substantial solvation changes), leading to the large chemical shift changes. Conversely, the chemical shift changes of the hydrophobic residues that comprise the core of the AsiA dimer interface (and that interact with the σ peptides) are not as dramatic because for these residues the loss of interprotomer contacts when the σ peptides bind are compensated for by contacts with the peptides.

It is also important that the chemical shift changes in AsiA associated with binding of $\sigma^{70}_{4.1}$ are very similar to those produced when $\sigma^{70}_{4.2}$ binds, and the residues on AsiA that

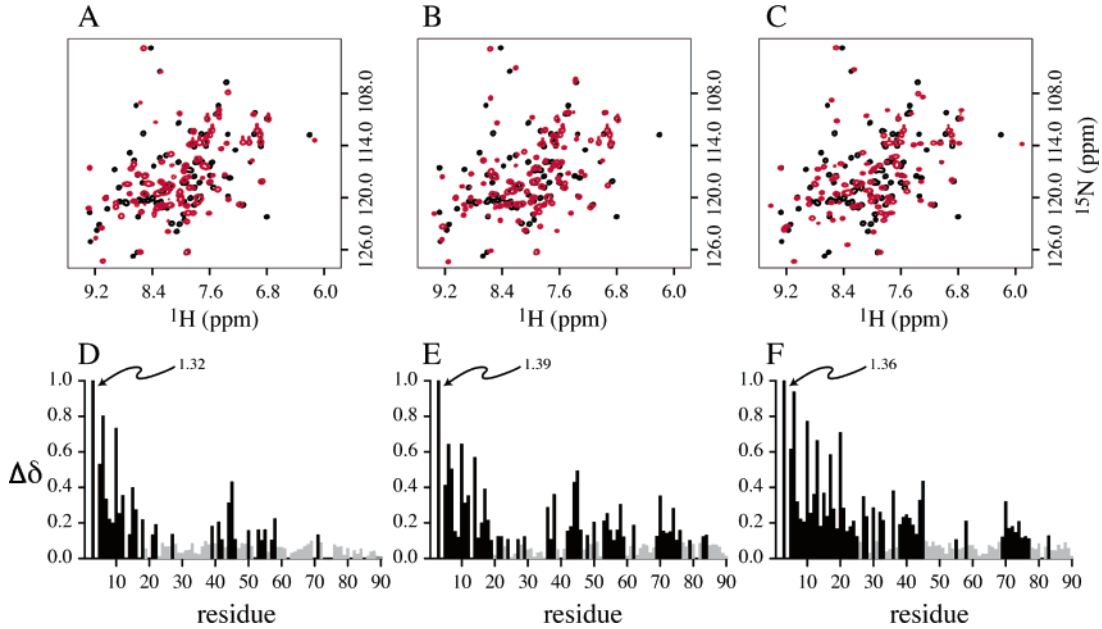

FIGURE 4: Chemical shift changes in AsiA upon binding to conserved regions 4.1 and 4.2 of *E. coli* σ^{70}. (A−C) ^{1}H, ^{15}N-HSQC spectra of free ^{13}C, ^{15}N-labeled AsiA (black contours) and AsiA bound to peptides (unlabeled) corresponding to region 4.1 (A, red contours), 4.2 (B, red contours), and both 4.1 and 4.2 (C, red contours) of σ^{70}. (D−F) Weighted average ^{1}H and ^{15}N chemical shift changes (*51*) in AsiA upon binding to the 4.1 (D), 4.2 (E), and both 4.1 and 4.2 (F) peptides. Light colored bars indicate changes of <0.1.

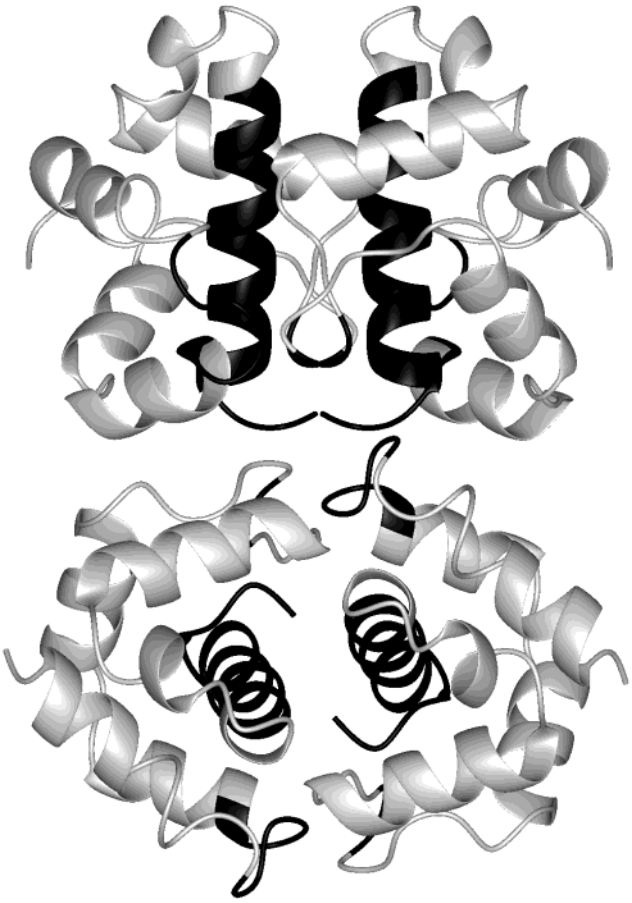

FIGURE 5: Location of chemical shift changes in AsiA accompanying binding to conserved regions of σ^{70}. Mapped onto the AsiA dimer ribbon (dark) are the residues in AsiA whose ^{1}H, ^{15}N chemical shifts change significantly regardless of whether the 4.1 peptide, the 4.2 peptide, or both peptides bind to AsiA. These residues are at the periphery of the AsiA dimer interface, and most of these chemical shift changes are the result of structural/solvation changes accompanying dimer dissociation and not because of interaction with the peptide.

interact with $\sigma^{70}_{4.1}$ are also those that interact with $\sigma^{70}_{4.2}$ (as shown from the ^{13}C F_1-filtered, F_3-edited experiments described above). These results indicate that the structure of AsiA with $\sigma^{70}_{4.1}$ bound is very similar to that with $\sigma^{70}_{4.2}$ bound and suggests that $\sigma^{70}_{4.1}$ and $\sigma^{70}_{4.2}$ individually bind to AsiA in a similar manner and at a similar site or sites (see below). This is perhaps not surprising, given the sequence similarity of these peptides, especially at their C-terminal ends, and given the results indicating that only the C-terminal ends of these peptides interact with AsiA (see below). Moreover, when $\sigma^{70}_{4.1}$ and $\sigma^{70}_{4.2}$ bind simultaneously to AsiA, the chemical shift changes at the C-terminal end of the N-terminal helix of AsiA are somewhat larger (than those observed with either $\sigma^{70}_{4.1}$ and $\sigma^{70}_{4.2}$ alone), additional significant chemical shift changes are observed (most notably in helix 2, helix 3, and the intervening turn, residues 25−41), and some of the changes (that were observed when only either $\sigma^{70}_{4.1}$ or $\sigma^{70}_{4.2}$ was bound) are no longer observed (for instance, in helix 4, residues 50−59). New intermolecular contacts are also observed. Clearly, accommodating both peptides requires an adjustment of the AsiA structure that is somewhat different than that necessary to accommodate only one of the peptides.

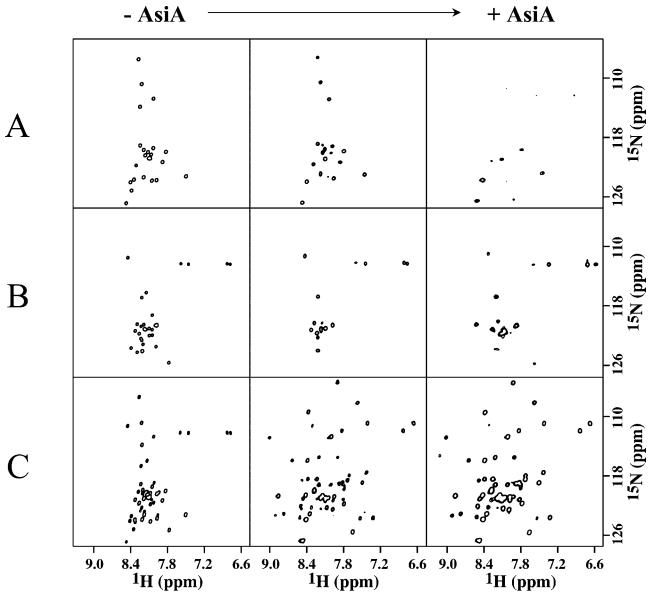

FIGURE 6: Conformational heterogeneity of regions 4.1 and 4.2 of σ^{70} when bound to AsiA. (A−C) ^{1}H, ^{15}N-HSQC NMR spectra monitoring the titration of ^{13}C, ^{15}N-labeled peptides corresponding to regions 4.1 (A), 4.2 (B), and both 4.1 and 4.2 (C) of σ^{70} with AsiA. In panels A and B, the remaining peaks after addition of excess AsiA correspond mostly to those at the N-terminal ends of these peptides, and their chemical shifts change very little as AsiA is added. The C-terminal sections of these peptides are conformationally averaged. No conformational averaging is observed when both peptides are present, as shown in panel C. Addition of a large excess of AsiA with both peptides present does not cause further changes in the spectrum of the complex in panel C.

Plasticity in the AsiA−σ^{70} Interaction. The $\sigma^{70}_{4.1}$ and $\sigma^{70}_{4.2}$ peptides, when bound individually to AsiA, exhibit significant conformational heterogeneity on the NMR time scale (Figure 6). When uniformly ^{13}C-, ^{15}N-labeled $\sigma^{70}_{4.1}$ (Figure 6A) or $\sigma^{70}_{4.2}$ (Figure 6B) is titrated with unlabeled AsiA, most of the peaks in the NMR spectrum of the labeled peptide broaden substantially to the point that they are no longer observable as the complex is formed. In each case, most all of the remaining peaks correspond to residues in the N-terminal halves of the peptides and their chemical shifts change very little or not at all as compared to free peptide. These results indicate, first of all, that only the C-terminal sections of these peptides are interacting with AsiA. This is consistent with results of mutational studies suggesting that amino acids at the C-terminal end of region 4.2 contribute most to the affinity of the interaction with AsiA (*23*, *28*). Second, the results indicate that each peptide exhibits heterogeneity (on the NMR time scale) in its interaction with AsiA. Considering that the sequences of the C-terminal ends of the $\sigma^{70}_{4.1}$ and $\sigma^{70}_{4.2}$ peptides are highly similar (Figure 1), this suggests that either of these peptides can occupy either of (at least) two sites on monomeric AsiA and that exchange between these sites and concomitant conformational exchange are occurring (on the NMR time scale).

The $\sigma^{70}_{4.1}$ and $\sigma^{70}_{4.2}$ peptides can bind simultaneously to AsiA forming a tight, slow exchange ternary complex that mimics precisely the complex of AsiA with the entire region 4 of σ^{70} (*18*). As shown in Figure 6C, in such a complex each peptide adopts a single, well-defined bound conformation, and each is apparently restricted to occupation of a single site. Although the $\sigma^{70}_{4.1}$ and $\sigma^{70}_{4.2}$ peptides do not

interact with each other free in solution (the spectrum of the mixture of the peptides free in solution in Figure 6C is the sum of the spectra of each peptide free in solution, Figure 6A,B), when both are bound to AsiA they limit the site occupancy and conformational heterogeneity of one another. Furthermore, addition of a large excess of AsiA (not shown) does not cause observable changes in the spectrum of the bound peptides in the ternary complex, indicating apparent cooperativity in binding of these peptides to AsiA.

DNA and AsiA Binding Sites on σ^{70} Region 4. The X-ray crystal structure of region 4 of σ^{A} of *T. aquaticus* bound to DNA (*1*) clearly reveals the details of the interaction between DNA and region 4. Whereas side chains of residues in the helix−turn−helix DNA binding motif of conserved region 4.2 provide most of the contacts to the DNA, the structure also suggests two contacts between the DNA and the side chains in conserved region 4.1 (Figures 1, 7). We have assigned the chemical shifts for atoms in the $\sigma^{70}_{4.1}$ and $\sigma^{70}_{4.2}$ peptides bound simultaneously to AsiA, which combined with the results of the edited/filtered experiments (Figure 2) allow us to identify side chains of amino acids in region 4 that interact with AsiA (Figures 1 and 7). First of all, these results clearly support the contentions presented above, as only residues in the C-terminal sections of regions 4.1 and 4.2 are observed to form contacts with AsiA. In addition, it is apparent that the surfaces on region 4 that interact with DNA and AsiA are discrete. Thus, although AsiA disrupts the interaction between region 4 and DNA, this effect appears to be indirect in that AsiA apparently does not interact directly with the side chains from region 4 responsible for binding to the DNA.

AsiA Disrupts the Interaction Between the β Flap-Tip Helix and the σ^{70} Regions 4.1 and 4.2. Results of recent structural studies show an intimate interaction between the helix at the tip of the flap region of the β subunit of the RNA polymerase core (the β flap-tip helix) and region 4 of σ^{70} (*2*, *3*). This interaction is essential for establishing and maintaining proper spacing between promoter elements (*48*) and ultimately for permitting formation of the appropriate contacts between the σ^{70} subunit and the DNA (*3*). Importantly, the β flap-tip helix interacts with residues at the C-terminal end of region 4.1 and with residues at the C-terminal end of 4.2. These are the same sections of regions 4.1 and 4.2 that interact with AsiA (Figure 7). This strongly suggests that one consequence of AsiA binding to region 4 of σ^{70} must be the disruption of the interactions of the β flap-tip helix with regions 4.1 and 4.2 and that the AsiA-dependent switch from early to middle promoters is realized in part because of this disruption. It is perhaps most likely that AsiA simply displaces the β flap-tip helix completely, which in turn initiates events that alter the spacing between the promoter elements.

AsiA Alters the Spacing Between Regions 2.4 and 4 of σ^{70} in RNA Polymerase Holoenzyme But Not of Free σ^{70}. The effect of AsiA binding to σ^{70} on the distance between regions 2.4 and 4 in both free σ^{70} and σ^{70} in the RNA polymerase holoenzyme was investigated using luminescence resonance energy transfer. Cysteine residues introduced into regions 2.4 and 4 of σ^{70} (T440C, D581C) were modified with fluorescence donor and acceptor probes, and the energy transfer between the probes was monitored as a function of

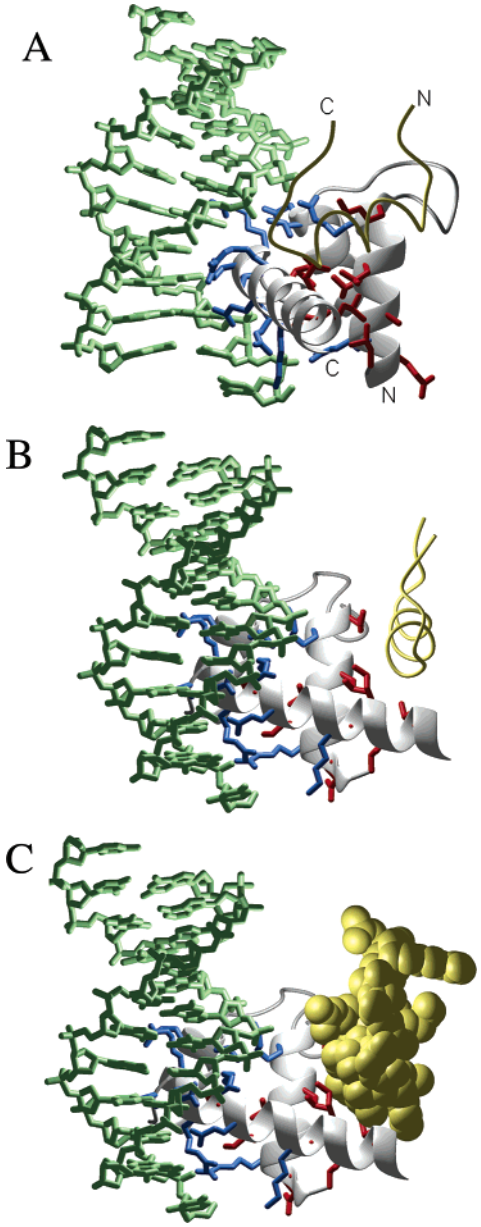

FIGURE 7: Binding sites for DNA and AsiA on region 4 of σ^{A} (σ^{70}). (A) Depicted are residues 376−426 of σ^{A} of *Thermus aquaticus* (light gray, corresponding to the C-terminal half of region 4.1, most of 4.2, and the intervening residues) bound to the −35 region of promoter DNA (light green) from the X-ray crystal structure (*1*). Side chains colored blue are those that contact the DNA and presumably maintain the affinity and specificity of the interaction. In *T. aquaticus*/*E. coli*, these are R379/R554, R387/R562, L398/L573, E399/E574, T408/T583, R409/R584, E410/E585, R411/R586, R413/R588, Q414/Q589, and K418/K593 (*2*). The side chains colored red are those that, in the complex of σ^{70} from *E. coli* with AsiA, form the contacts with AsiA (the residue types shown are those from *T. aquaticus*, and the *T. acquaticus*/*E. coli* residues involved are L376/L551, E378/A553, A381/A556, V383/V558, L384/L559, K388/F563, I412/I587, I415/I590, A419/A594, L420/L595, and L423/L598). Also shown is part of the flap region of the β subunit (yellow, residues 766−783), including the β flap-tip helix. This polypeptide was positioned by superposition of the main chain carbon and nitrogen atoms of the residues of region 4 shown with the corresponding atoms from the crystal structure of the *T. aquaticus* RNA polymerase holoenzyme bound to promoter DNA (*3*). The N- and C-termini of the polypeptides are indicated. (B) Same as panel A from a different angle. (C) Same as panel A, but including a space-filling model of the β flap-tip region including side chains. The side chains are from the high-resolution structure of the *T. aquaticus* core RNA polymerase (*52*, *53*).

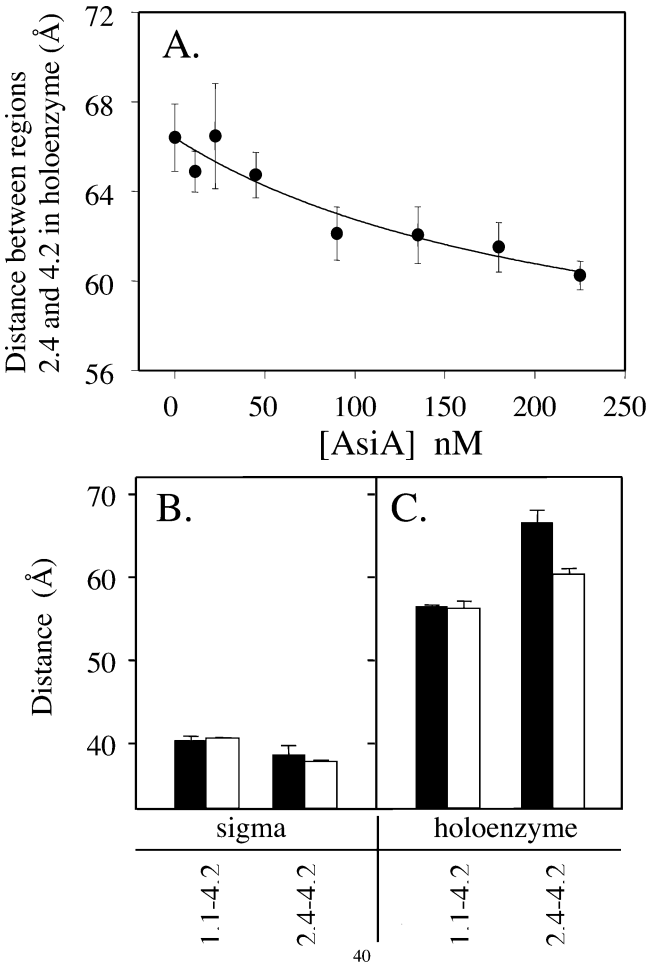

FIGURE 8: AsiA induced interdomain distance changes in σ^{70}. (A) Distances between the donor and the acceptor groups of the donor−acceptor labeled [T440C, D581C] mutant σ^{70} (corresponding to the distance between regions 2.4 and 4.2) in the RNA polymerase holoenzyme as a function of the concentration of added AsiA. (B, C) Distances between the donor and the acceptor groups of the donor−acceptor labeled [T440C, D581C] (distance between regions 2.4 and 4.2) and [A59C, R596C] (distance between regions 1.1 and 4.2) mutant σ^{70} molecules in free σ^{70} (B) and σ^{70} in the RNA polymerase holoenzyme (C) in the absence (dark bars) and presence (light bars) of excess AsiA.

added AsiA. This mutant σ^{70} with attached fluorescence probes, in the RNA polymerase holoenzyme, binds to AsiA with an affinity only slightly less than its affinity for holoenzyme with wild type σ^{70}, as assessed by the ability of AsiA to interfere with DNA binding to the holoenzyme (not shown). In the holoenzyme, AsiA binding induces a substantial decrease in the distance between the fluorescence probes and hence between regions 2.4 and 4 of σ^{70} (Figure 8A,C). The distance between regions 2.4 and 4 in free σ^{70}, however, does not change when AsiA binds (Figure 8B). These results indicate that AsiA binding decreases the distance between the regions of σ^{70} in the RNA polymerase holoenzyme responsible for interacting with the −10 and −35 promoter elements, thus hindering DNA binding.

AsiA Does Not Alter the Spacing Between Regions 1.1 and 4 of Free σ^{70} or σ^{70} in RNA Polymerase Holoenzyme. The N-terminal region of σ^{70} of *E. coli* has been shown to be autoinhibitory with respect to promoter recognition by σ^{70} (*44*, *45*), putatively via an indirect mechanism (*49*). Large conformational changes in σ^{70} accompanying binding to the RNA polymerase core alleviate this inhibition (*40*). The effect of AsiA binding to σ^{70} on the distance between regions 1.1 and 4 in both free σ^{70} and σ^{70} in the RNA polymerase holoenzyme was also investigated. For these measurements, cysteine residues introduced into regions 1.1 and 4 of σ^{70} (A59C, R596C) were modified with fluorescence donor and acceptor probes, and the energy transfer between the probes was again monitored as a function of added AsiA. AsiA binds to this modified σ^{70} (not shown); however, AsiA binding does not produce a significant change in the distance between regions 1.1 and 4 in either the free σ^{70} or the σ^{70} in the RNA polymerase holoenzyme (Figure 8B,C), indicating that AsiA inhibition of early promoter recognition does not occur by modulation of autoinhibition by region 1.

DISCUSSION

Previously, we proposed a model for the interaction of AsiA with σ^{70} from *E. coli* whereby σ^{70} binds to AsiA by displacing one of the protomers of the AsiA dimer (*18*, *19*). In this model, the function of the dimer is to shield the active hydrophobic surface from solvent until it interacts with σ^{70}. Herein, we have shown that the AsiA homodimer interface and the heterodimer interface (with σ^{70}) overlap significantly and that no AsiA dimer is present in solution when AsiA is bound to σ^{70}, both of which are consistent with the model that we (*18*, *19*, *28*) and others (*20*) have proposed.

The predominantly hydrophobic cleft formed by AsiA residues 13−42 (the C-terminal end of helices 1, 2, 3, and intervening residues) serves as the AsiA contribution to the interface with σ^{70}. Whereas this surface also provides much of the homodimer interface, the homodimer interface also includes regions not involved directly in binding to σ^{70}, such as the N-terminus, portions of the loop between helices 3 and 4, and the turn region between helices 5 and 6. The substantial structural changes occurring in these regions of AsiA when the σ^{70}-derived peptides bind (as judged from the chemical shift changes) result from dimer dissociation and increased exposure to solvent and thus are largely independent of the identity of the peptide that induces the dissociation.

The chemical shift perturbation data and the results of the isotope filtered/edited experiments indicate that, when bound individually, the $\sigma^{70}_{4.1}$ and $\sigma^{70}_{4.2}$ peptides bind to a similar site or sites on AsiA. Thus, the structure of AsiA with $\sigma^{70}_{4.1}$ bound is similar to that with $\sigma^{70}_{4.2}$ bound. This is perhaps not unexpected, given that only the C-terminal sections of these peptides interact directly with AsiA and that the amino acid sequences of these sections are highly similar.

The conformational heterogeneity observed in the $\sigma^{70}_{4.1}$ and $\sigma^{70}_{4.2}$ peptides when they bind (individually) to AsiA also likely reflects the sequence similarity of the sections of these peptides that interact directly with AsiA and the fact that more than one site on an AsiA monomer is available to bind them. When $\sigma^{70}_{4.1}$ and $\sigma^{70}_{4.2}$ bind to an AsiA monomer simultaneously, a single complex devoid of significant conformational heterogeneity is observed, indicating most likely that each peptide preferentially occupies one of the two distinct sites on AsiA and limits the site occupancy of the other (these contentions are also supported by our previous studies (*18*)). Only the C-terminal sections of these peptides, which exhibit very high sequence similarity, interact

directly with AsiA. When only a single peptide is present, it can occupy either of these two sites and does so on a time-averaged basis. The time scale for the structural transitions that the peptide undergoes as it changes binding sites is approximately the NMR time scale, resulting in broadening of the peaks corresponding to those residues that are changing conformation/binding sites. Simultaneous binding of the peptides to AsiA appears cooperative. Although the peptides do not observably interact with one another in solution in the absence of AsiA, it is not known if the apparent cooperativity in binding to AsiA results from direct interactions between the peptides when bound.

Our results also suggest two very important conclusions with respect to the mechanism of AsiA function. First of all, our results suggest that AsiA does not inhibit transcription from early promoters by directly occluding the DNA binding site on the 4.2 region of σ^{70}. Although AsiA clearly interferes with the interaction of region 4 of σ^{70} and the −35 consensus promoter element (*22*, *23*, *25*−*27*), it is not known if this interference is direct or indirect. Our results indicate that AsiA binds in the cleft between the C-terminal ends of regions 4.1 and 4.2, described as a hydrophobic pocket (*1*), which is distinct from the surface that binds the DNA (Figure 7). The residues in this pocket do not interact with the DNA, and the residues that interact with DNA do not interact with AsiA (Figures 1 and 7). Thus, the mechanism of interference seems to be indirect, most likely resulting from structural adjustments in region 4 accompanying AsiA binding that prevent optimal interactions with the DNA. Second, our results indicate that binding of AsiA to σ^{70} does however directly occlude the sites of interaction of regions 4.1 and 4.2 with the β flap-tip helix. The crystallographic evidence (*2*, *3*) clearly shows intimate interactions between the β flap-tip helix and the same sections of regions 4.1 and 4.2 that interact with AsiA (the hydrophobic pocket). With AsiA bound, it is very unlikely that the interactions of these sections of 4.1 and 4.2 with the β flap-tip remain unperturbed, and in all likelihood the interaction of 4.1 and 4.2 with AsiA and the β flap-tip are mutually exclusive. It has been postulated that the role of the β flap-tip is to establish or maintain proper spacing between regions in σ^{70} that interact with consensus promoter elements, for instance between the −10 and −35 elements of −10/−35 promoters (*2*, *3*, *48*), and that some anti-σ factors might alter the interaction of the σ factor with the β flap. Thus, AsiA binding would be postulated to alter directly the interaction between region 4 of σ^{70} and the β flap-tip helix, or preclude it, and thus change the spacing between regions 2.4 and 4.2 of σ^{70}, thereby altering transcription. Displacing the β flap-tip helix is almost certainly an integral component of the switch from early to middle promoters effected by AsiA. It should also be mentioned that, because of the sequence similarity between the β flap-tip helix and the sections of regions 4.1 and 4.2 that interact with AsiA, the potential exists for a direct interaction between the AsiA and the β flap-tip helix. We are currently pursuing this possibility.

The results of the luminescence resonance energy transfer experiments support the above postulates. Previous studies have shown that substantial conformational changes in σ^{70} accompany binding to core polymerase and that the distance between the regions responsible for interaction with the −10 and −35 promoter elements (2.4 and 4.2) increases dramatically (∼15 Å) (*40*). This conformational change relieves autoinhibition and also provides correct spacing of the promoter binding elements for productive DNA recognition and binding (*5*, *40*). The consequence of AsiA binding to the RNA polymerase holoenzyme is a significant decrease in the distance between regions 2.4 and 4.2 that interferes with DNA binding. Together with the results discussed above, these results indicate that altering the interaction between the β flap-tip helix and the region 4 of σ^{70} by AsiA binding results in a change in conformation inconsistent with that necessary for binding to −10/−35 promoters, thus effectively inhibiting transcription from these promoter types. Presumably, this change upon AsiA binding also facilitates interactions with phage middle promoters and other extended −10 promoters.

The observed conformational heterogeneity noted above, and the sequence similarity between the C-terminal ends of σ regions 4.1 and 4.2, reflect a structural and functional plasticity in at the AsiA−σ^{70} interface that permits multiple, distinct interactions of σ^{70} with AsiA. We have proposed recently that regions 4.1 and 4.2 of σ^{70} might function discretely with respect to their interactions with AsiA (*18*, *19*) because each can bind tightly to AsiA (in the absence of the other) and because of the fact that AsiA performs at least two discernible functions—inhibition of transcription from early promoters and facilitation of transcription from middle promoters—that need not involve both regions. In this respect, the conformational heterogeneity and sequence similarity permit either region 4.1 or 4.2 to serve as the primary contact with AsiA, for instance, for a given promoter type or under a given set of conditions. In support of this proposition, we have shown that the interaction of AsiA with region 4.1 of σ^{70} stimulates transcription from certain promoters (*28*). AsiA stimulates transcription at phage middle promoters and extended −10 promoters when a mutant σ^{70}, lacking its C-terminus including all of region 4.2, is substituted for full-length σ^{70}. Interestingly, the MotA protein is no longer necessary as a co-activator in this instance. Under normal circumstances, MotA binds to the C-terminal end of σ^{70} (*16*), just adjacent to region 4.2, and it is reasonable to expect, therefore, that binding of MotA perturbs the interaction of region 4.2 with AsiA. Consequently, the plasticity in the AsiA−σ^{70} interface could permit adjustment of the interaction to maintain high affinity in the absence of region 4.2. In this scenario, region 4.1 alone maintains the interaction. This plasticity may also play an important role in maintaining the fidelity of the AsiA−σ^{70} interaction while tolerating sequence variation among middle promoters.

It is likewise reasonable to propose a similar scenario for the interaction of the β flap-tip helix with regions 4.1 and 4.2 of σ^{70}. The sequence similarity noted in Figure 1, and the largely hydrophobic pocket formed by these regions of high sequence similarity, suggest that promoter sequence variations or adjustments for promoter element spacing can be accommodated by facile compensatory adjustments in the interactions between the β flap-tip helix and the regions 4.1 and 4.2. For instance, the recent crystal structures (*2*, *3*) suggest that region 4.1 and the β flap-tip helix serve to position region 4.2 with respect to the promoter. Repositioning, or optimization for a particular promoter sequence, would require adjustment of the interactions of region 4.1 and the β flap-tip helix with 4.2. It is likely that this type of

plasticity is necessary to enable the essential functional flexibility. A larger scale flexibility in the RNA polymerase holoenzyme has been suggested, based on the recent crystal structures (*2*, *3*), which apparently accounts for major rearrangements of the β flap and region 4 of σ^{70} with respect to the bound DNA, allowing adjustment for variations in −10 and −35 promoter element spacing.

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