Conformational properties of *Bacillus subtilis* RNA polymerase σ^A factor during transcription initiation

Ban-Yang CHANG* and Roy H. DOI†

*Agricultural Biotechnology Laboratories, National Chung-Hsin University, Taichung, Taiwan 40227, Republic of China and †Department of Biochemistry and Biophysics, University of California, Davis, CA 95616, U.S.A.

By the use of a partial proteolysis method and Western-blot analysis, the conformational properties of *Bacillus subtilis* σ^A factor in the transcription initiation stage were studied. From a comparison of the trypsin-digestion patterns of free σ^A and of σ^A associated with core enzyme, it was found that the production of 45 kDa σ^A tryptic-derived fragment was enhanced when σ^A was associated with the core enzyme. More importantly, a 40 kDa σ^A tryptic-derived fragment was found exclusively in this associated state. Based on the change of the digestion kinetics when producing the 45 kDa tryptic fragment and the generation of this new 40 kDa tryptic fragment from σ^A, it was apparent that a conformation change of σ^A occurred during the association of σ^A with the core enzyme. Also, similar patterns were found for the σ^A present in the holoenzyme–promoter DNA complex. These findings suggest that no further distinctive conformational change of σ^A occurs at the step of RNA polymerase holoenzyme and promoter DNA complex formation. Trypsin-digestion patterns of σ^A in different RNA polymerase holoenzyme and promoter DNA complexes were also studied. The presence of similar trypsin digestion-patterns of σ^A in those complexes strongly supports the idea that a similar σ^A conformation is used in the recognition of different σ^A-type promoters and the formation of different open complexes.

INTRODUCTION

The 2.4 and 4.2 regions of σ factors have been shown to interact directly with the promoter −10 and −35 regions respectively [1–11]. This means that these two regions should be exposed during recognition of promoter DNA by the RNA polymerase holoenzyme. However, our previous conformational studies of free σ^A using a partial proteolysis method showed that the promoter −10-binding region of σ^A was not exposed on the surface, since it was not readily accessible to proteases [12]. Moreover, gel-retardation and footprinting assays of free σ^A on two different σ^A-type promoters showed that free σ^A was unable to bind promoters by itself [13]. Although some other factors might be responsible for the inability of free σ^A to bind promoters, the 'buried' nature of the promoter −10-binding region of free σ^A might be the most important and direct one. This observation suggested to us that a conformational change of σ^A was required before binding of σ^A and promoter during the transcription-initiation stage. This conformational change might occur during association of σ^A and core enzyme, or during RNA polymerase holoenzyme and promoter recognition, or at both steps.

The precise and direct way of studying the σ-protein conformation, or its conformational change, is to do X-ray crystallographic investigations of the three-dimensional structure of the σ protein itself, of the σ protein associated with core enzyme, and of the σ protein in the holoenzyme–promoter complex. Unfortunately, there are difficulties in obtaining all the necessary crystals. Alternative physical methods might be feasible for the study of the conformational properties of the σ protein itself, but they are unfeasible for the study of the conformational properties of σ factor in the RNA polymerase holoenzyme or in the RNA polymerase–promoter complex, since it is difficult to avoid the effects arising from core-enzyme subunits. Thus the partial proteolysis method, in combination with Western-blot analysis, was adopted to investigate the conformational properties of σ^A factor in the transcription-initiation stage. Here we report the differences in the conformational properties of free σ^A, σ^A associated with core enzyme and σ^A in holoenzyme–promoter complexes as investigated by the above-mentioned methods.

MATERIALS AND METHODS

Materials

Taq polymerase was ordered from Amersham International. DEAE-Affi-Gel Blue, goat anti-(rabbit Ig) secondary antibody conjugated with horseradish peroxidase, and materials for PAGE were from Bio-Rad Laboratories. Cyanogen bromide-activated Sepharose-4B gel was purchased from Sigma. Centricon-10 concentrators were from Amicon. Other chemicals used in this study were from Fisher.

Preparation of anti-σ^A antibody

σ^A protein from the T7 promoter and T7 RNA polymerase overproduction system was purified by using the same method mentioned in a previous paper [12], except that the Sephadex G-75 molecular-sieving column used in that method was replaced by a Superose 12 column and f.p.l.c. The purity of σ^A protein obtained by this protocol was above 99%. To prepare the polyclonal anti-(σ^A) antibody, a 300 μl portion of purified σ^A protein (200 μg) in PBS (1.5 mM K_2HPO_4, 6.5 mM Na_2HPO_4 and 140 mM NaCl) was mixed uniformly with 300 μl of Freund's complete adjuvant and injected into a New Zealand White rabbit. After 2 and 4 weeks the rabbit was boosted with a 300 μl portion of the purified σ^A protein (100 μg), which was emulsified with 300 μl of Freund's incomplete adjuvant. The immunized rabbit was bled 1 week after the last booster shot. The collected blood sample (150 ml) was incubated in a 37 °C incubator for 2 h and centrifuged to collect the antiserum (80 ml). A 2.4 ml portion of the antiserum was further purified by passing it through a

Abbreviations used: PMSF, phenylmethanesulphonyl fluoride; Eσ^A, RNA polymerase holoenzyme of *Bacillus subtilis*; Eσ^{70}, *Escherichia coli* RNA polymerase holoenzyme.

DEAE-Affi-Gel Blue affinity column to remove proteases and albumin. The flow-through antiserum, after being dialysed against PBS, was than concentrated using a Centricon-10 concentrator to a volume of about 2 ml. The last step of antiserum purification was to pass the concentrated, partially purified antiserum through another affinity column which was packed with Sepharose 4B gel cross-linked with *Escherichia coli* BL 21 cellular protein extract. The flow-through anti-(σ^A) antiserum was collected and stored in small aliquots at −20 °C.

Preparation of the promoter DNA fragments

The ϕ29 phage G3aG3b promoter DNA template was synthesized by using a PCR method. The primers for its synthesis were a 22-mer oligonucleotide (5′-GGATTTGTGGGCGTTCTTGTCG-3′) starting from −87 of the G3a promoter and a 23-mer oligonucleotide with the DNA sequence 5′-CCTGAACCTCTCCGTCCACCATC-3′, which started from +92 of the G3b promoter. The length of this synthesized G3aG3b promoter DNA was 315 bp. The P2 promoter DNA fragment (202 bp in length) was also prepared by the PCR method. One primer necessary for its synthesis was a 20-mer oligonucleotide with the sequence 5′-GGCTGCCAGGTTGTTGATGT-3′, starting from the *Xba*I site of the pWT plasmid, into which the *Sau*3A fragment (from the σ^A operon) containing the P2 promoter was cloned. The other primer was a 22-mer oligonucleotide with the DNA sequence 5′-TTAAACCTAAAATTAATCATTT-3′ and started from +25 of the P2 promoter. In addition, a 224 bp polylinker DNA fragment from the pKS$^-$ plasmid was also PCR-synthesized and used as a promoterless control DNA template in these experiment. The two primers for the synthesis of this promoterless control DNA template were a 16-mer oligonucleotide (5′-AACAGCTATGACCATG-3′) and a 17-mer oligonucleotide (5′-GTAAAACGACGGCCAGT-3′). All the synthesized DNA fragments were dissolved in high-salt binding buffer [40 mM Tris/HCl, pH 7.9, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 160 mM KCl and 10% (v/v) glycerol] before use.

Preparation of *Bacillus subtilis* RNA polymerase holoenzyme ($E\sigma^A$) and free σ^A

$E\sigma^A$ and free σ^A were purified as described in a previous paper [12]. Their concentrations were determined with a Bio-Rad protein determination kit. $E\sigma^A$ stored in storage buffer [20 mM Tris/HCl, pH 7.9, 10 mM $MgCl_2$, 1 mM EDTA, 0.1 mM dithiothreitol, 1 mM phenylmethanesulphonyl fluoride (PMSF), 0.2 M KCl and 50% (v/v) glycerol] was washed with a high-salt binding buffer [40 mM Tris/HCl, pH 7.9, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 160 mM KCl and 10% (v/v) glycerol] using Centricon-10 concentrators and was kept in the same buffer before use. The free σ^A protein in its storage buffer [20 mM Tris/HCl, pH 7.9, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol and 30% (v/v) glycerol] was also washed with the same high-salt buffer before use.

Western-blot analysis of the tryptic fragments generated from free σ^A, σ^A associated with core enzyme, and σ^A in $E\sigma^A$–promoter DNA complexes

The method for Western-blot analysis is the same as that reported in the previous study [12]. To prepare samples for these analyses, free σ^A and σ^A associated with core enzyme were incubated in the high-salt binding buffer at 37 °C for 5 min before the addition of trypsin. Samples for the analysis of the trypsin-digestion patterns of σ^A in the RNA polymerase and promoter DNA complexes were prepared in the following way. $E\sigma^A$ (unsaturated with σ^A) and promoter DNA at a molar ratio of 1:4 were mixed and incubated at 37 °C for 5 min before trypsin was added. After the addition of trypsin, all digestion mixtures (free σ^A, $E\sigma^A$, and σ^A–promoter DNA complexes) were further incubated at 37 °C for 30 min. Following this digestion, 10 mM PMSF (to give a final concentration of 1.0 mM) and an equal volume of 2× sample application buffer [0.125 M Tris/HCl, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 0.002% Bromophenol Blue and 10% (v/v) 2-mercaptoethanol] were added to stop the reactions. The samples were subjected to SDS/PAGE and transferred to nitrocellulose paper for Western-blot analyses

N-terminal sequence of peptide fragments from σ^A

The N-terminal amino acid sequencing method was the same as that described in [14].

RESULTS

The native σ^A protein conformation is important in determining the partial tryptic digestion patterns of free σ^A

There are 53 potential tryptic sites (Figure 1) in the *B. subtilis* σ^A protein. Thus in order to use the partial proteolysis method as a probe for studying the conformation properties of σ^A protein, the prerequisite is that the native σ^A protein conformation (tertiary structure) should over-ride its amino acid sequence (primary structure) in determining the partial proteolysis pattern. To clarify this point, trypsin-digestion patterns of free σ^A in the presence or absence of the denaturant SDS were compared. Different partial proteolysis patterns were found in these two treatments. Several tryptic fragments, not found or only found in very small amounts in the native σ^A digestion, were observed more readily when σ^A was treated with 0.2% (w/v) SDS (compare lanes 2–6 with lanes 8–12 in Figure 2). One of the tryptic fragments which was generated under denaturing conditions and had a molecular mass of about 40 kDa on SDS/PAGE was N-terminally sequenced. It had Ala-Glu-Glu-Glu-Phe-Asp-Leu-Asn-Asp-Leu as the first 10 N-terminal amino acids and this sequence was located at region 1 of the σ^A factor. Since no fragment of this size was found in the native σ^A digestion (12), it is clear that the accessibility of native σ^A to trypsin attack is different from that of the SDS-treated σ^A. This difference is probably due to the more 'open' nature of σ^A protein to trypsin after its interaction with SDS and it also supports the idea that the conformation of σ^A over-rides its amino acid sequence (primary structure) in the determination of the partial proteolysis patterns of σ^A. However, since the band density for each peptide was not proportional to its size even under this denaturing condition, it is still possible that the primary amino acid sequence has some effect on the trypsin-digestion patterns of σ^A protein.

The difference in the trypsin-digestion patterns among free σ^A, σ^A associated with core enzyme, and σ^A in the $E\sigma^A$–promoter DNA complex

From the examination of Figure 2 (lanes 2–6) and Figure 3 (lane 7), we found that 47 and 35 kDa tryptic fragments were present in the trypsin-digestion mixture of free σ^A; a 45 kDa tryptic fragment was also present, but in a lower amount compared with that generated in the holoenzyme digestion (Figure 3), and there was no 40 kDa fragment observed. However, these four tryptic fragments were all present in the digestion of σ^A-associated core enzyme (Figure 3, lanes 2–6). The enhancement of the generation of the 45 kDa tryptic fragment and the generation of the extra 40 kDa tryptic fragment in the digestion of σ^A associated with

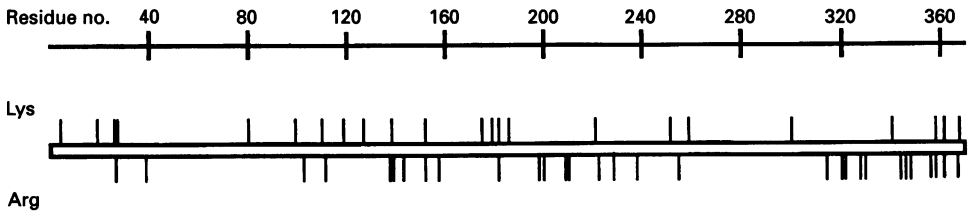

Figure 1 Potential trypsin-digestion sites in *Bacillus subtilis* σ^A protein

The top line of numbers indicates the position of amino acids in the σ^A protein sequence. The positions of lysine and arginine amino acid residues are indicated by the vertical lines above and below the bar respectively.

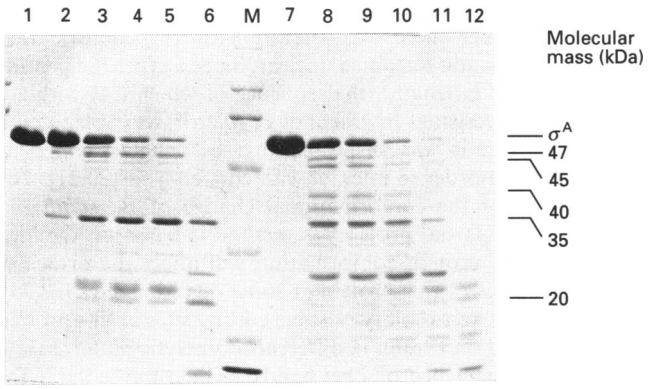

Figure 2 Comparison of the trypsin-digestion patterns of σ^A protein in the native and denatured states

Lanes 1 and 7, native σ^A protein only. Lanes 2–6 are trypsin-digestion patterns of σ^A in the native state; lanes 8–12 are trypsin-digestion patterns of σ^A protein with 0.2% (w/v) of SDS. The concentration of trypsin in each lane was as follows: 0.42 μg/ml (lanes 2 and 8), 0.84 μg/ml (lanes 3 and 9), 1.64 μg/ml (lanes 4 and 10), 3.24 μg/ml (lanes 5 and 11), 6.67 μg/ml (lanes 6 and 12). Lane M contains the protein markers (from BRL Company) with apparent molecular masses (kDa) of 200, 97.4, 68, 43, 29, 18.4 and 14 from the top to the bottom band respectively. The numbers at the right-hand margin indicate molecular masses of specific tryptic fragments of σ^A which are discussed in the text.

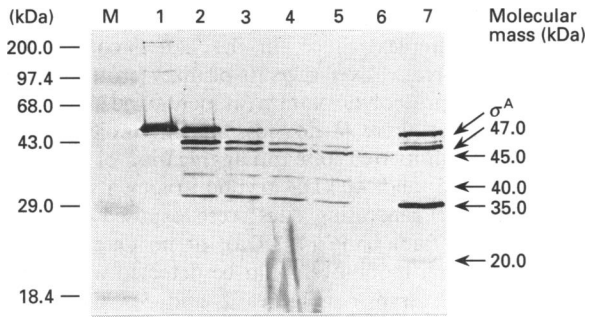

Figure 3 Trypsin-digestion patterns of σ^A in the Eσ^A as determined by Western-blot analysis

Eσ^A (20 μg) (unsaturated with σ^A) in binding buffer was digested with different amounts of trypsin. Lanes 1–6 show trypsin-digestion patterns of σ^A associated with core enzyme. The concentration of trypsin for each treatment is 0 μg/ml trypsin (lane 1), 1.92 μg/ml (lane 2), 3.84 μg/ml (lane 3), 5.76 μg/ml (lane 4), 7.68 μg/ml (lane 5), and 9.6 μg/ml (lane 6). Lane 7 is the digestion pattern of free native σ^A in the presence of 1.64 μg/ml of trypsin. The molecular mass markers are in lane M with their molecular masses (kDa) indicated in the left-hand margin. The numbers at the right-hand margin indicate the mass (kDa) of tryptic peptides of σ^A.

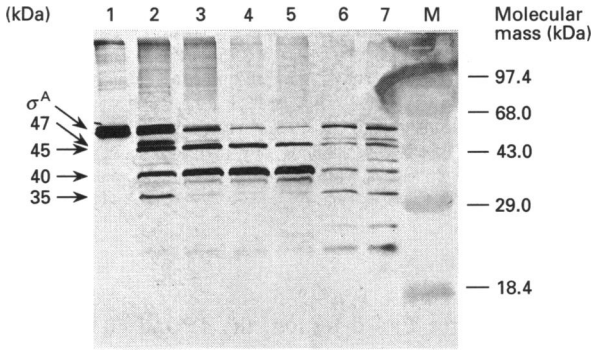

Figure 4 Trypsin-digestion patterns of the Eσ^A and G3aG3b promoter complex as determined by Western-blot analysis

Eσ^A (20 μg) (unsaturated with σ^A) was used to complex with four times the number of mol of the DNA template (promoter or promoterless); the reactions were carried out as described in the Materials and methods section. Lane 1, Eσ^A without the addition of trypsin; lanes 2–5, Eσ^A complexed with G3aG3b promoter DNA and in different concentrations of trypsin: 2.17 μg/ml, 6.51 μg/ml, 10.85 μg/ml, and 15.19 μg/ml respectively; lane 6, trypsin digestion of Eσ^A in the presence of promoterless DNA and 6.51 μg/ml trypsin; lane 7, Eσ^A digested with 6.51 μg/ml of trypsin. Lane M contained molecular-mass markers with mass (kDa) indicated in the right-hand margin. The numbers at the left-hand margin indicate the mass (kDa) of the tryptic peptides from σ^A.

core enzyme indicated that there was a definite difference in the digestion kinetics between free σ^A and σ^A in the holoenzyme. In addition, the 47 and 35 kDa tryptic fragments generated in the holoenzyme digestion come from the digestion of σ^A dissociated from the core enzyme (free σ^A), since σ factor and core-enzyme subunits of Eσ^A have a low overall binding constant [15] and usually RNA polymerase core enzymes are not saturated with σ factor after purification [16].

The trypsin-digestion pattern of σ^A in the Eσ^A–promoter complex was also studied. In order to have a better binding efficiency between Eσ^A and promoter, a strong Φ29 phage G3aG3b tandem promoter was PCR-synthesized. The activity of this promoter was shown to be the same as previously reported [17]. Besides the choice of the G3aG3b strong promoter, an excess of promoter DNA template was added to the binding mixture to ensure that all RNA polymerase holoenzymes were complexed with promoter DNA. The molar ratio of promoter DNA to the Eσ^A was 4:1. These strategies enabled us to detect the correct digestion patterns of σ^A in the Eσ^A–G3aG3b promoter DNA complex. The digestion patterns of σ^A in the Eσ^A–G3aG3b promoter complex are shown in Figure 4. Several major bands corresponding to molecular masses of 47, 45, 40 and 35 kDa

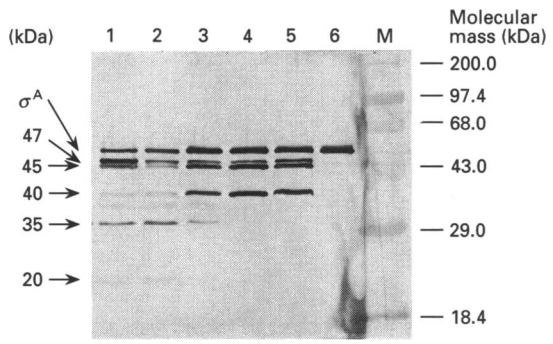

Figure 5 Trypsin-digestion patterns of Eσ^A complexed with different promoters

Eσ^A (20 μg) was complexed with P2 promoter, G3a promoter and G3aG3b promoter, in a molar ratio of promoter DNA (or promoterless DNA) to Eσ^A of 4:1. The complexes were then digested by adding trypsin to a final concentration of 3.75 μg/ml and incubated at 37 °C for 30 min. SDS/PAGE and Western-blot analysis were performed to detect bands derived from σ^A. Lane 1, digestion of Eσ^A in the absence of DNA; lane 2, in the presence of promoterless DNA; lane 3, Eσ^A complexed with P2 promoter; lane 4, Eσ^A complexed with G3a promoter; lane 5, Eσ^A complexed with G3aG3b promoter; lane 6, Eσ^A only. The numbers at the right-hand margin indicate the mass (kDa) of molecular-mass markers. The numbers at the left-hand margin indicate the mass of tryptic fragments from σ^A.

were detected (Figure 4, lanes 2–5). However, in contrast with the result with free Eσ^A digestion, the 45 and 40 kDa tryptic fragments were much more resistant to trypsin digestion even at higher concentrations of trypsin (Figure 4, lanes 3–5). In addition, the 40 kDa tryptic fragment seemed to accumulate at the expense of the 45 kDa fragment. Since the σ^A digestion patterns of free Eσ^A (Figure 4, lane 6) and Eσ^A in the presence of promoterless DNA (Figure 4, lane 7) were very similar, it was clear that only in the presence of promoter DNA could the relatively stable 45 and 40 kDa tryptic fragments form.

Trypsin-digestion patterns of σ^A in different Eσ^A–promoter complexes

The binding of Eσ^A to promoter DNA results in both a change in DNA conformation and in the structural properties of Eσ^A [10]. But it is not known whether σ^A maintains the same conformational properties in its recognition of different σ^A-type promoters. In order to understand this point, trypsin digestion patterns of σ^A in different Eσ^A–promoter complexes were studied. The tested promoters were the P2 promoter from the σ^A operon of *B. subtilis*, the G3aG3b tandem promoters and the G3b promoter from phage Φ29. The P2 promoter has a very different DNA sequence and a very different RNA polymerase footprint from those of the G3b promoter [13]. Under the binding conditions where all the Eσ^A was bound to promoter DNA (4 mol of promoter DNA:1 mol of Eσ^A) (results not shown), both 45 and 40 kDa tryptic fragments originating from σ^A were found in Eσ^A complexed with P2 promoter (Figure 5, lane 3), G3b promoter (Figure 5, lane 4) and G3aG3b promoter (Figure 5, lane 5) after being digested by the same amount of trypsin. From these results we reasoned that σ^A used the same, or very similar, structural properties for the recognition of P2 promoter, G3aG3b tandem promoter, and G3b promoter and the formation of their open complexes. Since the 35 kDa tryptic fragment was found to be present in the digestion of the Eσ^A and P2 promoter complex (Figure 5, lane 3) and not in the other two holoenzyme and promoter complexes (Figure 5, lanes 4 and 5), it was clear that under these experimental conditions the Eσ^A and P2 promoter complex was not as stable as the Eσ^A–G3b promoter complex. This conclusion was further supported by the weaker binding of Eσ^A on the P2 promoter, which was observed in our previous footprinting assays [13]. Since different holoenzyme–promoter complexes gave different band densities for the 45, 40 and 35 kDa σ^A-derived tryptic fragments, we suggest that the band-density analyses of these peptides might be a potential method for understanding the binding strength of different promoters with Eσ^A.

DISCUSSION

The transition of *E. coli* RNA polymerase holoenzyme (Eσ^{70}) conformation, induced by the interaction of σ^{70} with core enzyme, has been studied by fluorescence spectroscopy [15]. During this transition a small change in the shape of core enzyme was detected using a small-angle X-ray diffraction method [18,19]. However, whether there was alteration of σ^{70} conformation during this transition remained unclear. Since a synthetic peptide (30 amino acids in length), the sequence of which was within a 40 kDa trypsin-resistant fragment of *E. coli* σ^{70}, was able to bind to core enzyme, it was suspected that σ^{70} might change its conformation in order to interact with core enzyme [20,21]. The present study of the conformational change of *B. subtilis* σ^A protein using a partial proteolysis method is based on the idea that changes of protein conformation will affect the protease-digestion kinetics of this protein at some digestion sites [22,23], or will affect the accessibility of some cutting sites in this protein to proteases and thus result in different proteolytic patterns (loss of bands or emergence of new bands). Thus, our criteria for demonstrating the conformational changes of σ^A protein during transcription initiation would be the presence of detectable differences in the proteolytic patterns of σ^A at different steps during transcription initiation, and therefore it does not matter whether or not σ^A protein or its proteolytic fragments have normal mobilities on SDS/PAGE.

Because σ^A might be surrounded by other subunits of RNA polymerase and promoter DNA, the detection of the change in the partial proteolysis patterns of σ^A at different transcription-initiation steps could be due to either a conformational change of σ^A or to steric hindrance from the core enzyme and the promoter DNA. In order to rule out the possibility of steric hindrance, we looked for new tryptic fragments which were only generated in the digestion of σ^A associated with core enzyme, or in the Eσ^A–promoter complex. Since, in the native condition, the conformation of free σ^A over-rides its primary protein structure in determining its proteolytic pattern (as mentioned in the Results section), and since there is no possible intermolecular steric hindrance for σ^A in its free state (no aggregation of free σ^A was observed), the 45 and 40 kDa tryptic fragments would be detectable if their generating sites were exposed in free σ^A. However, only a faint band (45 kDa), or no detectable band (40 kDa), with such mobilities could be detected when free σ^A was digested with trypsin (Figures 2 and 3). These results indicated either that the generating sites for those two peptide fragments were not fully exposed or that they were not exposed at all when σ^A protein was in the free and native state. Contrarily, the easy visualization of these two peptides during the digestion of σ^A, when it is associated with core enzyme or when it is in the Eσ^A–promoter DNA complex, must indicate a digestion enhancement at some trypsin-digestion sites of σ^A in those two complexes. The best explanation for those differences must be the occurrence of a conformational change of σ^A. Actually, the nature of the generation of the 45 and 40 kDa tryptic fragments, when σ^A is in association with core enzyme, or when it is in the

RNA polymerase and promoter DNA complex, is very similar to that of the appearance of the 40 kDa tryptic fragment when σ^A is treated with SDS and digested with trypsin (Figure 2, lanes 8–10); i.e. they are all a function of the conformational change of σ^A protein. This similarity supports our idea that greater accessibility of trypsin to new or existing sites in the σ^A protein occurs and that σ^A changes its conformation when it interacts with core enzyme.

Another interesting question is 'How many distinct conformational states of σ^A occur during transcription initiation?' From the generation of the same set of 45 and 40 kDa σ^A-derived tryptic fragments in the trypsin digestion of Eσ^A and Eσ^A promoter complexes (Figures 3 and 4), we suggest that there is no distinctive conformational difference between σ^A in these two steps; i.e. only one major distinct conformational change of σ^A occurs in transcription initiation and it is in the step of σ^A and core enzyme association.

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