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Stabilization of the primary sigma factor of *Staphylococcus aureus* by core RNA polymerase

Rajkrishna Mondal[1], Tridib Ganguly[2], Palas K Chanda[1], Amitava Bandhu[1], Biswanath Jana[1], Keya Sau[3], Chia Y Lee[4] & Subrata Sau[1,]*

[1]Department of Biochemistry, Bose Institute, [2]School of Life Sciences, IISER, Kolkata, [3]School of Biotechnology and Life Sciences, Haldia Institute of Technology, Haldia, India, [4]Department of Microbiology and Immunology, University of Arkansas for Medical Sciences, Little Rock, USA

The primary sigma factor (σ^A) of *Staphylococcus aureus*, a potential drug target, was little investigated at the structural level. Using an N-terminal histidine-tagged σ^A (His-σ^A), here we have demonstrated that it exits as a monomer in solution, possesses multiple domains, harbors primarily α-helix and efficiently binds to a *S. aureus* promoter DNA in the presence of core RNA polymerase. While both N- and C-terminal ends of His-σ^A are flexible in nature, two Trp residues in its DNA binding region are buried. Upon increasing the incubation temperature from 25° to 40°C, ~60% of the input His-σ^A was cleaved by thermolysin. Aggregation of His-σ^A was also initiated rapidly at 45°C. From the equilibrium unfolding experiment, the Gibbs free energy of stabilization of His-σ^A was estimated to be +0.70 kcal mol^{-1}. The data together suggest that primary sigma factor of *S. aureus* is an unstable protein. Core RNA polymerase however stabilized σ^A appreciably. [BMB reports 2010; 43(3): 176-181]

INTRODUCTION

Bacterial sigma factors bind to RNA polymerase (RNAP) and direct transcription initiation from the promoter DNAs. Studies on several sigma factors reveal that these proteins are composed of four conserved regions/domains, which are linked by regions of less significance (1-4). Regions are further divided by multiple sub-regions, which perform specific functions including binding to the -10 and -35 elements of bacterial promoters. Subunits of RNAP have long been considered as potential targets of antibacterial drug discovery (5). Small molecule inhibitors for most RNAP subunits including sigma factors are not available to date (6).

Staphylococcus aureus causes a variety of human and animal diseases (7). It produces multiple virulence factors as well as global regulators for controlling the expression of virulence factors (7, 8). Prevention of staphylococcal infections is becoming problematic primarily because of the emergence and dissemination of the multiple-drug-resistant *S. aureus* strains across the world (7-9). Novel antistaphylococcal drugs are required to eradicate all the *S. aureus* strains including antibiotic-resistant strains. One of the potential avenues of antistaphylococcal drug discovery would be the in depth structural characterization of the alternative and primary sigma factors of *S. aureus* as they may be different from the mammalian ortholog (10) and be involved in the transcription of most *S. aureus* genes (8, 11, 12).

The primary sigma factor (σ^A) of *Staphylococcus aureus*, indispensable for *S. aureus* growth, was purified to homogeneity and shown to react with the antibody raised against the *B. subtilis* or *E. coli*-specific primary sigma subunit (11, 12). It also recognized several *S. aureus* promoters that are homologous to the promoters transcribed by the primary sigma factors of *E. coli* and *B. subtilis*. A mutation in σ^A-encoding gene was complemented by the *B. subtilis* primary sigma factor-encoding gene (12). Except region 1, all other regions of *B. subtilis*/*E. coli*-specific primary sigma factor share significant sequence identity with *S. aureus* σ^A (1, 2). Interestingly, the alternative sigma factor of *S. aureus* that transcribes many virulence genes also exhibited notable homology with σ^A (8). Despite the apparent differences from other bacterial primary sigma factors and potentiality as drug target, σ^A was not studied at the structural level. Also very little is known about the stability of any primary sigma subunit while in free form. Using a recombinant *S. aureus* σ^A, here we have reported its physicochemical properties and also demonstrated that it is a thermodynamically unstable protein, which is stabilized by binding with core RNA polymerase.

RESULTS AND DISCUSSION

Physicochemical properties of recombinant *S. aureus* σ^A

To investigate the structure, function and stability of *S. aureus* σ^A, an N-terminal histidine-tagged variant of this protein (His-

*Corresponding author. Tel: 91-33-2355-9416; Fax: 91-33-2355-3886; E-mail: subratasau@gmail.com or subratasau@yahoo.co.in

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σ^A) was purified to homogeneity by single step affinity chromatography (see Materials and Methods for details and Fig. 1A). To check whether His-σ^A is biologically active, we investigated its promoter DNA binding activity by gel shift assay using a ^{32}P-labeled DNA fragment harboring the promoter region of *S. aureus groES* gene (13). As evident from Fig. 1B, His-σ^A (lane 3) or core RNAP (lane 1) alone did not bind to groES promoter DNA. His-σ^A however exhibited efficient binding to the above promoter DNA in the presence of core RNAP (lane 2). Taken together, the data suggest that core RNAP is needed for the promoter DNA binding activity of His-σ^A and addition of histidine tag to *S. aureus* σ^A did not abolish its DNA binding activity. As observed in other bacterial primary sigma factors (1, 2), the DNA binding domain of *S. aureus* σ^A possibly remains buried while in free state and appears upon core RNAP binding.

To determine the oligomeric state of His-σ^A in solution, we performed both analytical gel filtration chromatography and glutaraldehyde crosslinking by standard procedures. As evident from Fig. 1C, passage of 5 μM His-σ^A through a gel filtration column produced a single peak that corresponded to monomeric His-σ^A when compared with the elution profiles of standard proteins. Glutaraldehyde-mediated crosslinking also revealed that His-σ^A exists as a monomer in solution (data not shown).

His-σ^A harbors two tryptophan residues at positions 225 and 226. The intrinsic tryptophan fluorescence spectrum of His-σ^A displayed an emission maximum at 336.6 nm upon excitation at 295 nm (data not shown), suggesting that Trp residues in His-σ^A may be buried. The Stern-Volmer plot (14), derived from the acrylamide quenching data of Trp fluorescence of His-σ^A, was found linear (Fig. 1D), further indicating that both the Trp residues are equally accessible to quencher.

To gather information about the secondary structures in σ^A, the CD-spectrum (200-260 nm) of His-σ^A was recorded by a standard procedure. As presented in Fig. 1E, the CD-spectrum of His-σ^A exhibited two peaks of large negative ellipticity at 208 and 222 nm, indicating the presence of α-helix in this protein. The peak obtained at 208 nm is more pointed than that recorded at 222 nm. Analysis of the above CD-spectrum by a software program CDNN (15) revealed nearly 42% α-helix and 15% β-sheet in His-σ^A at room temperature. The putative three dimensional structure, developed with 124-367 amino acid residues of σ^A, also showed that it is primarily composed of α-helix (Fig. 1F).

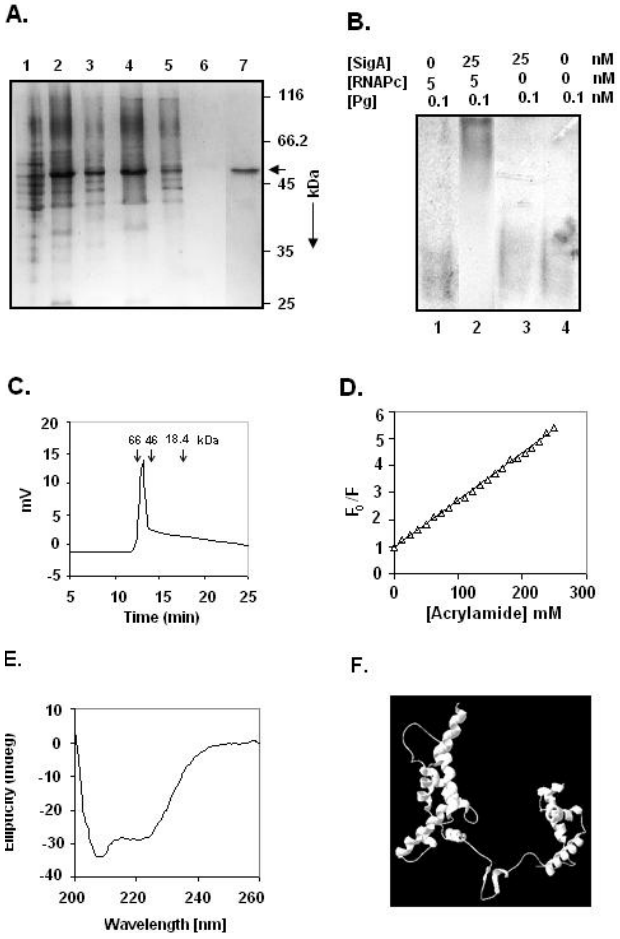

Fig. 1. Purification and characterization of His-σ^A. (A) Analysis of protein fractions of Ni-NTA affinity chromatography by 12% SDS-PAGE. Five microgram protein was loaded in each lane. Lanes 1-7 contain uninduced *E. coli* (p1283) cell extract; induced *E. coli* (p1283) cell extract, cell debris fraction, cell supernatant fraction, flow-through, wash fraction and elution fraction, respectively. Masses (in kDa) of marker proteins are shown at the right side of gel. Arrow indicates His-σ^A. (B) Autoradiogram of gel shift assay shows the binding of indicated amount of His-σ^A and/or *E. coli* core RNAP (RNAPc) to a ^{32}P-labeled *Pg* DNA (13). (C) The elution profile of His-σ^A from analytical gel filtration chromatography. The peak positions of BSA (66 kDa), Ovalbumin (46 kDa) and β-Lactoglobulin (18.4 kDa) are shown by arrows. (D) The Stern-Volmer plot for quenching of tryptophan fluorescence of His-σ^A with ~0-250 mM acrylamide. (E) CD-spectrum (200-260 nm) of His-σ^A. (F) Schematic representation of the model structure of truncated σ^A harboring α-helices (ribbons), β-sheet (arrow) and loops (tubes).

Flexible regions/domains in His-σ^A

By partial proteolysis, multiple flexible regions/domains in both *B. subtilis* and *E. coli*-specific primary sigma factors were detected previously (3, 4). Bioinformatic analyses including homology modeling indicate that *S. aureus* σ^A also harbors 3-4 conserved regions/domains. To confirm this observation, we performed a proteolysis of His-σ^A by chymotrypsin and found one minor (I) and three major (II, III and IV) protein fragments from His-σ^A at the early stage of the enzymatic cleavage (Fig. 2A). Several faint bands were also noticed between III and IV. Intensities of all major fragments (except III) were decreased as the digestion progressed. The fragment I and most possibly fragment II interacted with anti-his antibody (Fig. 2B), indicat-

ing the loss of the C-terminal end of His-σ^A immediately after its exposure to the enzyme. Digestion with trypsin also yielded ~3 stable protein fragments from His-σ^A (data not shown).

Contrary to chymotrypsin/trypsin digestion, five fragments (V-IX) were generated from His-σ^A at the early period of digestion with thermolysin (Fig. 2C). Of the fragments, fragments VI and VIII remained nearly undigested over the entire period of digestion. Fragment V, which is ~2 kDa less in molecular mass than His-σ^A, interacted with anti-his antibody (Fig. 2D) suggesting again that σ^A carries a flexible C-terminal end. Nineteen chymotrypsin, 58 trypsin and 107 thermolysin cleavage sites were detected in His-σ^A by a bioinformatics analysis (data not shown). As evident from the above proteolysis data, majority of the potential cleavage sites are however buried within the interior folds of His-σ^A.

Our MALDI-TOF analysis reveals that the molecular masses of the protein fragments, derived from the 4 min digestion of His-σ^A with chymotrypsin, are 33,000, 32,233.3, 27,900.11, 26,633.4, 24,500, 24,233.31 and 23,000 Da, respectively. The first six fragments might harbor Met37-Tyr329, Asp85-Phe372, Asp85- Tyr329, Asp96-Tyr329 and Asp96-Phe314 residues of His-σ^A. It is not clear now how 23,000 dal fragment was originated from His-σ^A or why fragment I was not recognized by MALDI- TOF. Possibly, N-terminal end of fragment I was cleaved during making of sample for MALDI-TOF analysis. The data however suggest that the N-terminal end of *S. aureus* σ^A is also flexible in nature.

Stability of recombinant *S. aureus* σ^A

A primary sigma factor must be a stable protein as it is involved in the transcription initiation of a large number of genes. To see whether *S. aureus* σ^A is indeed a stable protein, we studied thermolysin-mediated proteolysis at different temperatures. Nearly 60% of total His-σ^A was digested by thermolysin when incubation temperature of proteolysis was raised from 25° to 40°C (Fig. 3A). Thermal aggregation of His-σ^A also revealed that this protein starts aggregating at 45°C and above (Fig. 3B). Together the data suggest that *S. aureus* σ^A may be an unstable protein. To confirm the above observation, we further investigated the guanidium hydrochloride (GdmCl)-induced unfolding of His-σ^A by recording the tryptophan fluorescence emission of the protein by a standard procedure. As demonstrated in Fig. 3C, fluorescence intensity of His-σ^A decreases rapidly at ~0-2 M GdmCl concentrations, then it diminishes very slowly up to 6 M GdmCl (Fig. 3C). To determine the Gibbs free energy of stabilization of His-σ^A at 0 M GdmCl, the above equilibrium unfolding data was analyzed according to the standard two-state model (16). From the resulting plot (Fig. 3D) of free energy values versus GdmCl concentrations at the transition region, the ΔG^{H2O} (the Gibbs free energy of stabilization of His-σ^A at 0 M GdmCl), *m*-value (cooperativity parameter) and $[GdmCl]_{1/2}$ (concentration at the midpoint of transition) were determined to be about +0.70 kcal mol^{-1}, -1.6 kcal $(mol.M)^{-1}$ and 440 mM, respectively. The data indicate that σ^A is truly an unstable protein in the absence of core RNAP. Currently, the exact reason of poor stability of

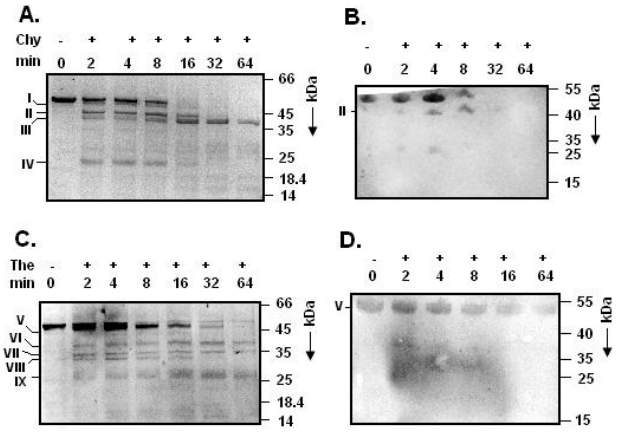

Fig. 2. Analyses of (A) chymotrypsin (Chy) and (C) thermolysin (The)-digested His-σ^A fragments by 12% SDS-PAGE. Masses (in kDa) of marker proteins are shown at the right side of gel. I-IX denote different digested fragments of His-σ^A. Western blotting analyses of chymotrypsin (B) and thermolysin (D) digested His-σ^A fragments.

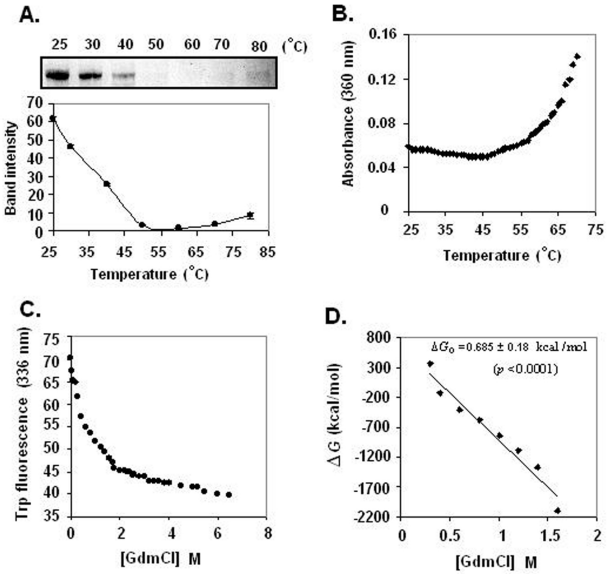

Fig. 3. Stability of His-σ^A. (A) SDS-PAGE analysis of thermolysin-mediated cleavage of His-σ^A at indicated temperatures. Intensity of remaining His-σ^A (obtained by densitometric scanning) was plotted against digestion temperature. (B) Aggregation of His-σ^A at different temperatures. (C) Intrinsic tryptophan fluorescence emission of His-σ^A at 0-6.5 M GdmCl concentrations. (D) Free energy for denaturation of His-σ^A as the function of GdmCl concentration. GdmCl-induced unfolding data (shown in panel C) was analyzed by a standard method (16) for determining ΔG^{H2O} and other parameters.

S. aureus σ^A is not known with certainty. We however noticed that thermostability of primary sigma factor of *B. subtilis* (3) is nearly similar to that of *S. aureus* σ^A. Taken together, we speculate that the primary sigma subunits, which share extensive sequence identity with those of *B. subtilis* and *S. aureus*, might be unstable while in free form.

Stabilization of recombinant *S. aureus* σ^A

An indispensable protein like *S. aureus* σ^A must be stabilized *in vivo* in order to avoid degradation by cellular proteases. Previous reports (11, 12) and our gel shift assay (Fig. 2B) indicate that core RNAP may stabilize *S. aureus* σ^A as the latter binds to the promoter DNA in the presence of former protein complex. To test this possibility, we investigated the chymotrypsin-mediated proteolysis of His-σ^A in the presence of sub-stoichiometric amounts of core RNAP and/or Pg DNA. In comparison with the digestion of His-σ^A alone (Fig. 4A, lane 2), the digestion of His-σ^A in fact was prevented substantially when reaction mixture contained either RNAP core and *Pg* DNA (lane 5) or core RNA alone (lane 3). As expected from the gel shift assay (see above), *Pg* DNA could not stabilize His-σ^A appreciably (lane 4). Non-specific protein like repressor of mycobacteriophage L1 (17) only marginally protected His-σ^A from degradation with chymotrypsin (lane 6/7). Similar pattern of protection was observed when proteolysis was performed with thermolysin (Fig. 4A, lanes 8-12). Estimation reveals (Fig. 4B) that ~20-40% of the total input His-σ^A was protected in the presence of core RNAP alone, whereas, ~10% of the total recombinant σ^A remained undigested when RNAP core was replaced with the ~15 molar excess of L1 repressor, suggesting the greater stabilization of *S. aureus* σ^A by the former protein. The exact mechanism of stabilization of σ^A or the biological significance of such stabilization is not known clearly. All exposed chymotrypsin/thermolysin -cleavage sites on σ^A are possibly not masked upon association with core RNAP as most regions of sigma subunit reside on the surface of RNAP holoenzyme (18). Like the primary sigma factors of *B. subtilis* (19) and *E. coli* (20), σ^A may however undergo conformational change during interaction with core RNAP and it in turn puts most cleavage sites into the interior of the protein. Our data however indicate that primary sigma subunit of *S. aureus* needs to be assembled with core RNAP immediately after synthesis.

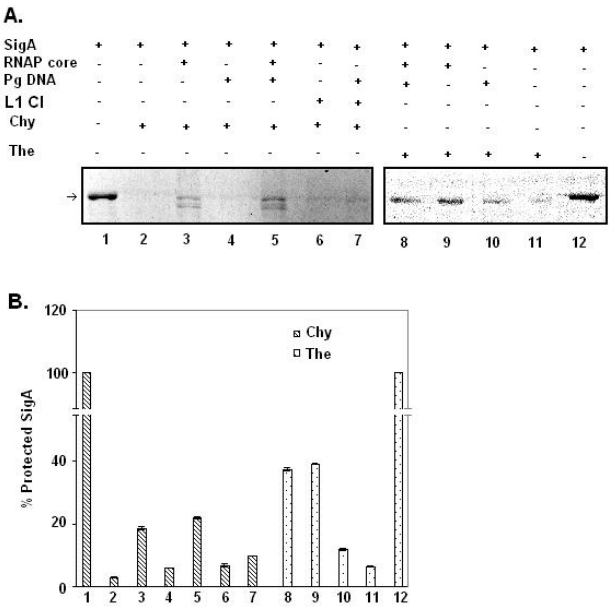

Fig. 4. Stabilization of His-σ^A. (A) 12% SDS-PAGE analysis of chymotrypsin/thermolysin-mediated cleavage of His-σ^A in the presence (+) and absence (−) of *E. coli* core RNAP or L1 repressor (17) alone or along with *Pg* DNA (13). Arrow denotes His-σ^A. Intensity of undigested His-σ^A in each lane was determined by densitometric scanning and presented by bar diagram (B).

MATERIALS AND METHODS

Bacteria and growth conditions

S. aureus and *E. coli* cells were routinely grown in Trypticase soy broth (21) and Luria broth (22), respectively. Growth media were supplemented with appropriate antibiotics whenever needed.

DNA and protein techniques

Plasmid DNA isolation, DNA estimation, digestion/modification of DNA by restriction/modifying enzymes, polymerase chain reaction (PCR), purification of DNA fragments, cloning of the DNA fragments into plasmids, labeling of DNA fragments with [α-^{32}P] dATP (BARC, India) and agarose gel electrophoresis were carried out following standard procedures (21, 22) or according to the manufacturer's kits. Protein estimation, native and SDS-PAGE, staining of polyacrylamide gel, western blotting, chemical crosslinking were performed by standard methods (21, 23). *S. aureus* Newman DNA was isolated according to Chanda *et al*. (15). All PCR amplified DNA inserts were sequenced at Bose Institute (India).

Overexpression and purification of *S. aureus* His-σ^A

To overexpress *S. aureus* His-σ^A, a ~1,120 bp DNA fragment was PCR amplified from *S. aureus* Newman genomic DNA using primers sigA1 (5'CCGGAATTCATGTCTGATAACACAG) and sigA2 (5'CCCAAGCTTTTAATCC ATAAAGTCTTTC) and cloned into *Hinc*II-digested pUC18 DNA. The Newman DNA insert (sequence verified) from one recombinant pUC18 clone was subcloned into an *E. coli* vector pET28a (Novagen, USA) to generate p1283. Subcloning attached 36 additional amino acid residues (including a stretch of six His residues) at the N-terminal end of *S. aureus* σ^A. Cells of *E. coli* BL21 (DE3) [Novagen, USA] harboring p1283 [namely, *E. coli* (p1283)] were grown to log phase followed by induction with 0.2 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) for 3 h at 30°C.

The induced cells were sequentially harvested, washed with 0.9% NaCl and resuspended in 1/10th volume of buffer A [50 mM phosphate buffer (pH 8.0), 300 mM NaCl, 20 mM imidazole, 5% glycerol] containing 10 μg/ml of PMSF (phenylmethane sulfonylfluoride). After disrupting the cells, His-σ^A was purified from the crude extract by Ni-NTA column chromatography (Qiagen, Germany) according to the manufacturer's protocol. The yield of purified His-σ^A was ~500 μg per liter of induced *E. coli* culture. The eluted protein was dialyzed against the buffer B [50 mM phosphate buffer (pH 8.0), 200 mM NaCl, 5% glycerol and 1 mM EDTA] before *in vitro* experiments were performed. The concentration of His-σ^A was calculated using the molecular mass of monomeric His-σ^A.

Biochemical and radioactive procedures

Analytical gel filtration chromatography of 6 μM His-σ^A was performed in a HPLC system (Waters) according to Das *et al*. (24) with minor modifications. Briefly, ~100 μl of 6 μM His-σ^A in buffer B was loaded onto a ~14 ml gel filtration column (pre-equilibrated in buffer B) and the absorbance of the eluted fractions, determined at 220 nm, was expressed as Volts. The elution profiles of BSA (bovine serum albumin), ovalbumin and β-lactoglobulin were also determined similarly.

To perform the gel shift assay, 25 nM His-σ^AA and 5 nM *E. coli* core RNAP (Epicenter, India) in 20 μl of modified buffer B [buffer B plus 100 μg/ml of BSA] were incubated with 0.1 nM ^{32}P labelled *Pg* DNA (13) either separately or together for 20 min on ice followed by analysis of all samples using standard native PAGE (17).

MALDI-TOF analysis of the chymotrypsin-digested fragments was performed according to the standard methods (21, 25).

Spectrophotometric methods

Circular Dichroism (CD) spectrum (200-260 nm) and intrinsic tryptophan fluorescence spectrum (λ_{em} = 300-400 nm; λ_{ex} = 295 nm) of 6 μM His-σ^A in buffer B were recorded according to the standard methods (14, 17) using a JASCO J815 spectrophotometer and a Hitachi F-3000 spectrofluorimeter, respectively.

Equilibrium unfolding of His-σ^A was investigated by recording the tryptophan fluorescence emission of 5 μM His-σ^A at 336 nm (λ_{ex} = 295 nm) in the presence of 0-6 M GdmCl. Assuming that unfolding follows two-state model, free energy of unfolding (ΔG) was calculated according to the following standard equation (16) with minor modification:

$$\Delta G = -RT\ln K = -RT\ln (fn - f)/(f - fu) \quad (1)$$

where R, T, f, fn, and fu denote universal gas constant, absolute temperature in Kelvin, observed fluorescence, fluorescence at native state, and fluorescence at completely unfolded state, respectively. From the straight lines (not shown) developed using the low and high GdmCl concentrations in Fig. 3C, the values of fn and fu were determined. Considering a linear relationship between free energy change of unfolding and GdmCl concentrations, free energy change at 0 M GdmCl concentration (ΔG^{H2O}) was determined from the following equation:

$$\Delta G = \Delta G^{H2O} - m\,[\mathrm{GdmCl}] \quad (2)$$

Thermal aggregation of 5 μM His-σ^A in buffer B was monitored using light scattering at 360 nm in a spectrophotometer (Shimadzu 3000) connected to a temperature-regulated water bath (24).

The Stern-Volmer plot was generated by a standard procedure (14) using the acrylamide quenching data of Trp fluorescence of His-σ^A (5 μM in buffer B) monitored with a Hitachi F-3000 spectrofluorimeter (λ_{em} = 337 nm; λ_{ex} = 295 nm).

Partial proteolysis

Partial proteolysis of His-σ^A by chymotrypsin and thermolysin were performed by standard procedures (21, 24). To study the stabilization of His-σ^A, 20 μl reaction mixture containing 1 μg of His-σ^A and 0.1 μg of *E. coli* core RNAP or L1 repressor (17) or *Pg* DNA (13) was incubated for 10 min at 25°C. Chymotrypsin or thermolysin was added to each reaction mixture to the final concentration of ~3.3 ng. After incubation for additional 25 min at 25°C, gel loading buffer was added to all reaction mixtures followed by their analysis using a 12% SDS-PAGE. Similar chymotrypsin/thermolysin-mediated digestion of His-σ^A was also performed in the presence of both 0.1 μg of *E. coli* RNAP core or L1 repressor and 0.1 μg of *Pg* DNA.

Bioinformatic analysis

S. aureus σ^A and related sequences, downloaded from NCBI (USA), were analyzed by the standard procedures (26). Using the crystal structure of *T. thermophilus* sigma subunit (pdb code: 3DXJ, 18) as a template, the three-dimensional model structure of *S. aureus* σ^A was developed by the First Approach Mode of Swiss-Model (ExPasy homepage). The resulting model structure was visualized by Swiss-Pdb Viewer (ExPasy homepage).

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