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Characterization of an unusual cold shock protein from *Staphylococcus aureus*

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Abstract

Of the three cold shock proteins expressed by *Staphylococcus aureus*, CspC is induced poorly by cold but strongly by various antibiotics and toxic chemicals. Using a purified CspC, here we demonstrate that it exists as a monomer in solution, possesses primarily β-sheets, and bears substantial structural similarity with other bacterial Csps. Aggregation of CspC was initiated rapidly at temperatures above 40 °C, whereas, the Gibbs free energy of stabilization of CspC at 0 M GdmCl was estimated to be +1.6 kcal mol^{-1}, indicating a less stable protein. Surprisingly, CspC showed stable binding with ssDNA carrying a stretch of more than three thymine bases and binding with such ssDNA had not only stabilized CspC against proteolytic degradation but also quenched the fluorescence intensity from its exposed Trp residue. Analysis of quenching data indicates that each CspC molecule binds with ~5 contiguous thymine bases of the above ssDNA and binding is cooperative in nature.

Keywords

Staphylococcus aureus; Cold shock protein (Csp); CspC; Single-stranded (ss) DNA; GdmCl (guanidium hydrochloride)

Introduction

Bacteria generally express multiple cold shock proteins (Csps) to perform various biological functions [1]. However, not all Csps are induced by cold shock. Some are expressed constitutively, while others are even induced by nutrient deprivation, osmotic shock, toxic chemicals and antibiotics. These small proteins are highly homologous in sequence,

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molecular mass and structural levels and generally regulate the transcription and translation of both the cold shock and non-cold shock genes by binding to the single-stranded nucleic acids through their RNP1 and RNP2 motifs [1]. Three-dimensional structures of Csps from *E. coli*, *B. subtilis*, *T. maritima*, and *B. caldolyticus* show that they all form a typical β-barrel structure consisting of five anti-parallel β-strands (namely, β1 to β5). Of the β-strands, the β2- and β3-strands primarily constitute the nucleic acid-binding surface of RNP motifs harboring mostly the aromatic and basic amino acids. Additional studies reveal that *B. subtilis* Csps are thermodynamically less stable [2], whereas, *T. maritima* Csp is a relatively stable protein under nearly identical conditions [3]. Until recently, most bacterial Csps have not been investigated at length.

Staphylococcus aureus expresses three cold shock proteins (CspA, CspB, and CspC) from three different locations in the genome [4]. These Csps are highly identical to each other at the primary sequence level and also share significant homology with other bacterial Csps [5]. CspB, however, is the only one shown to be induced notably in response to cold shock [6], suggesting that it is a 'true' cold shock protein while others are needed for performing different biological work. CspA was indeed shown to regulate the expression of 4, 4′diaponeurosporene (a pigment) through a SigB-dependent pathway [7]. Interestingly, all three Csps in a methicillin-resistant *S. aureus* strain were expressed at higher levels than those in a methicillin-sensitive *S. aureus* strain [8] indicating their possible involvement in *S. aureus*-mediated virulence. We have recently demonstrated that CspC in *S. aureus* RN4220 is expressed appreciably at the normal growth temperature and induced primarily by various antibiotics (e.g., ciprofloxacin, rifampicin, ampicillin, chloramphenicol, etc.), hydrogen peroxide and arsenate salt [5]. Currently, no biochemical and biophysical information is available for the *S. aureus*-encoded Csps including the unusual CspC. Here we have purified a recombinant *S. aureus* CspC to homogeneity and reported that it exists as a monomer in solution, harbors mainly β-strands, binds to thymine -rich ssDNA cooperatively and possesses weak thermodynamic stability.

Materials and methods

Bacteria and growth conditions

S. aureus Newman and *E. coli* BL21 (DE3) and its derivative were routinely grown in Trypticase soy broth [9] and Luria broth [10], respectively. The *E. coli* growth medium was supplemented with kanamycin whenever needed.

Molecular biological 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 radioactive materials and agarose gel electrophoresis were performed by standard procedures [10] or according to the protocols of different kits. Protein estimation, native and SDS-PAGE, staining of polyacrylamide gel and western blotting were done by standard methods [9, 11]. Chromosomal DNA from *S. aureus* Newman was isolated by a standard method [5]. All PCR made DNA inserts were sequenced at Bose Institute (India).

Purification of *S. aureus* CspC

A~214 bp DNA fragment was PCR amplified from *S. aureus* Newman DNA using primers P8 (5′ CATGCCATGGATGAATAACGGTACAG3′) and P9 (5′ CTCGAGCATTTTAACTACGTTTG3′) and cloned into *Hinc*II-digested pUC18 DNA [10]. The Newman DNA insert from one recombinant pUC18 clone (sequence insert from one recombinant pUC18 clone (sequence verified) was subcloned into an *E. coli* vector pET28a (Novagen, USA) to generate p1281. The subcloning has attached eight additional amino acid residues (including a stretch of six His residues) at the C-terminal end of CspC. *E. coli* BL21 (DE3) cells (Novagen, USA) harboring p1281 were grown to log phage followed by the addition of IPTG (isopropyl β-D-1-thiogalactopyranoside) to a final concentration of 0.5 mM. After 4 h growth at 32 °C, the induced cells were sequentially harvested, washed with 0.9% NaCl and resuspended in $1/10^{th}$ volume of Lysis buffer I [20 mM Tris-chloride buffer (pH 8.0), 500 mM NaCl, 20 mM imidazole, and 5% glycerol] containing 10 μg/ml of PMSF (phenylmethane sulfonyl-fluoride). After disruption of cells, the *E. coli* crude extract was treated with both DNaseI and RNaseA (20 mg/ml each) for 1 h at 30 °C in the presence of 5 mM $MgCl_2$. Nucleic acid-free *E. coli* extract was subjected to Ni-NTA column chromatography (Qiagen, Germany) and different fractions were collected according to manufacturer's protocol. Analysis of the above fractions by SDS-15% PAGE revealed a single protein band of ~11 kDa in the elution fraction (data not sown). The above protein was found to react with anti-his antibody (Qiagen, Germany) (data not shown) and harbor successively Met, Asn, Asn, Gly and Thr at its N-terminal end. These residues are identical to the five N-terminal end amino acid residues of *S. aureus* CspC. Together these data confirm that the purified ~11 kDa protein entity is the His-tagged *S. aureus* CspC (designated simply CspC here). The eluted CspC was dialyzed against the Buffer D [20 mM Tris-Cl (pH 8.0), 300 mM NaCl, 1 mM EDTA, and 5% glycerol] before *in vitro* experiments were performed. The concentration of CspC was calculated using the molecular mass of monomeric CspC.

Biochemical and radioactive techniques

Chemical crosslinking [9] was performed by incubating 1–5 μM CspC with 0.1% glutaraldehyde at room temperature for 2 min followed by the analysis of samples by SDS-15% PAGE.

Several standard proteins and affinity purified-CspC were resolved through 6, 8, 10, 12, and 14% polyacrylamide gels and the resulting migration data were used to construct a Ferguson plot according to Garchow *et al.* [12].

Digestion of CspC with trypsin in the presence/ absence of ssDNA1 and analysis of digested protein aliquots were performed as described by Schindler *et al.* [2].

Binding of CspC to different ^{32}P labelled ssDNA1 (5′GTCGACTTTTTTTTTTTTTTTTT3′), ssDNA2 (′ CCA-TCTTCTCTTTCGATG3′), ssDNA3 (5′ AAGTGTATAATGAA-AG 3′) and dsDNA were investigated by standard gel shift assay [9].

Biophysical methods

Circular Dichroism (CD) spectrum (190– 320 nm) and thermal aggregation of CspC were performed by standard protocol [2, 9, 13].

Recording of intrinsic tryptophan fluorescence spectrum and tryptophan fluorescence quenching study were performed according to the standard procedures [14, 15] with minor modifications. Briefly, 0.3/3 μM CspC was incubated with varying concentrations of ssDNA½ for 20 min at room temperature followed by the recording of tryptophan fluorescence emission at λ_{max} 350 nm after excitation at 295 nm. Unfolding of CspC was investigated by recording the tryptophan fluorescence emission of 5 μM CspC at 350 nm (after excitation at 295 nm) at 0–6 M GdmCl. Assuming that unfolding follows a two-state model, free energy of unfolding (G) was calculated by 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. 2C, 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:

$$\Delta G = \Delta G^{H_2O} - m\,[\text{GdmCl}] \quad (2)$$

Homology modeling

The amino acid sequences of CspA, CspB, and CspC were exploited in developing their three-dimensional model structures by the First Approach Mode of Swiss-Model (http://ExPasy.org). While the crystal structure of *B. caldolyticus* Csp (pdb code: 1hz9A) was utilized as a template for developing the model structures of both CspA and CspC, the X-ray structure of *B. subtilis* CspB (pdb code: 2i5mX) was used as a template for generating the model structure of CspB. Using the pdb coordinates of the resulting model structures, molecular visualization and surface charge determination of the structures were performed by Swiss-Pdb Viewer (http://ExPasy.org).

Results and discussion

Physicochemical properties of *S. aureus* CspC

To study the structure, stability and mode of action of *S. aureus* CspC, the protein was overexpressed in *E. coli*, purified to near homogeneity by affinity chromatography (see Materials and methods for details) and utilized in all *in vitro* experiments. To determine the oligomeric status of CspC in solution, protein- protein cross-linking experiment was

performed in the presence of glutaraldehyde and the resulting data reveal that CspC exists mostly as a monomer (~8 kDa) at 1 – 5 μM concentrations (data not shown). To determine the molecular weight of CspC precisely, we constructed Ferguson plots [12] using the relative migration data of standard proteins as well as CspC on 6–14% native polacrylamide gels. As evident from Figs. 1A and B, the molecular weight of CspC is ~8 kDa in solution, confirming the monomeric nature of this protein.

CspC carries one Trp residue at position 8 [5]. The intrinsic tryptophan fluorescence spectrum of CspC displayed an emission maximum at ~350 nm upon excitation at 295 nm (Fig. 1C). The emission maximum of a protein with buried Trp generally lies at 335 nm or less, whereas, the emission maximum of a protein with surface exposed Trp lies at 347 nm or above [17]. These data therefore indicate that the Trp residue of CspC is exposed on its surface.

The far-UV CD spectrum of CspC shows a major peak of large ellipticity at ~195–198 nm at 25 °C, indicating the presence of β-sheet in CspC at room temperature (Fig. 1D). Analysis of the above spectrum by CDNN [18] indeed revealed that CspC consists of about 53% β-sheets. The putative tertiary structure of CspC (Fig. 1E), which forms a typical Greek-key β-barrel structure (data not shown) like that of other bacterial Csps [1], also showed that it is primarily composed of β-strands. Interestingly, all three *S. aureus* Csps, which are similar in their overall structure, possess different surface charges (data not shown).

CspC carries six Phe and one each of Tyr and Trp residues at different positions [5], indicating that it might yield a characteristic near-UV CD-spectrum. The near-UV CD-spectrum of CspC indeed shows a peak of large positive ellipticity at ~266–268 nm at 25 °C (Fig. 1F). Phe residues generally show a peak between 255 – 270 nm [2]. The above peaks of CspC therefore might be due to Phe residues in CspC. For some unknown reason, the Tyr and Trp-specific peaks are not very clear from the above near-UV CD-spectrum of CspC. These latter aromatic amino acid-specific peaks are also not prominent in the near-UV CD-spectra of *B. subtilis* Csps [2].

Stability of CspC

Bacterial Csps, characterized so far, did not exhibit identical thermal and chemical stability despite structural similarity [2, 3]. To determine the thermal stability of CspC, we studied the thermal aggregation of this protein by a light scattering method and found that there was marginal increase of CspC aggregation when the incubation temperature of CspC was raised from 30° to ~40 °C (Fig. 2A). At temperatures above 40 °C, CspC however was aggregated very rapidly, suggesting that it is a thermosensitive protein.

To determine the stability of CspC, guanidium hydrochloride (GdmCl)-induced unfolding of CspC was investigated by recording the tryptophan fluorescence emission of CspC. Fig. 2B shows that the fluorescence intensity of CspC decreases rapidly at 0 – 2 M GdmCl concentrations, then it remains almost constant up to 6 M GdmCl. To determine the Gibbs free energy of stabilization of CspC at 0 M GdmCl, the above equilibrium unfolding data was analyzed according to a standard two-state model [16]. From the resulting plot (Fig. 2C) of free energy values versus GdmCl concentrations at the transition region, the ΔG^{H2O} (the

Gibbs free energy of stabilization of CspC in the absence of GdmCl), *m*-value (cooperativity parameter) and $[GdmCl]_{½}$ (concentration at the midpoint of transition) were determined to be about +1.6 kcal mol^{-1}, –1.9 kcal $(mol\ M)^{-1}$ and 847 mM, respectively. The data indicate that CspC is also a chemically sensitive protein.

Previously, *B. caldolyticus* CspB exhibited more thermostability than *B. subtilis* CspB and *E. coli* CspA [1, 2]. The specific charged amino acid residues, identified only on the surface of the *B. caldolyticus* CspB, was suggested to be responsible for the higher stability of this protein [1]. These charged residues, however, were not detected on the surface of *S. aureus* CspC (data not shown). Besides, Glu58 of *S. aureus* CspC aligns with the Pro58 residue of *B. subtilis* CspB and CspD [2, 5]. These latter two proteins were found more stable than the *B. subtilis* CspC that carries Ala residue at position 58. Taking together, we suggest that absence of specific surface residues and Glu58 possibly contribute to the apparently less stability of *S. aureus* CspC. Interestingly, surface charges of three *S. aureus* Csps are also not identical despite their structural resemblance (data not shown). While CspC possesses excess negatively charged patches, CspB harbors additional positively charged patches on the surface, indicating that their function (particularly single-stranded nucleic acid binding activity) and stability will differ significantly.

DNA-CspC interaction

CspC harbors two highly conserved single-stranded nucleic acid binding motifs, RNP1 and RNP2 [5]. To see whether CspC binds to DNA, gel shift assays with several ^{32}P labelled double-stranded (ds) and single-stranded (ss) DNA fragments were carried out by a standard procedure. As shown in Fig. 3, migration of the ssDNA1 fragment carrying a stretch of twenty thymine bases has been retarded in the presence of 1 μg or higher amount of CspC. In contrast, ssDNA1 did not show any binding to even 5 μg L1-encoded repressor [19]. Interestingly, CspC did not show detectable binding either to a double-stranded DNA or to other ssDNAs (such as ssDNA2 and ssDNA3). Oligonucleotides ssDNA2 and ssDNA3 harbor a stretch of three thymine and three adenine bases, respectively. Taken together, we suggest that CspC stably binds to ssDNA with a stretch of more than three thymine bases.

Stabilization of CspC by ssDNA

B. subtilis Csps became partly resistant to proteolysis upon binding to ssDNA [2]. To see whether *S. aureus* CspC also behaves similarly, we performed tryptic digestion of this protein both in the presence and absence of ssDNA1. Trypsin was chosen for proteolysis as it has 15 recognition sites in CspC. As shown in Fig. 4A (inset picture), intensity of the CspC-specific band starts to decrease early during tryptic digestion (I), whereas, digestion is prevented substantially in the presence of ssDNA1 (II). The corresponding plot of CspC-specific band intensity versus time of digestion reveals that the half-time of CspC digestion alone is around 11 min, whereas, ~75% of this protein remained intact even after 60 min of digestion in the presence of ssDNA1. The data indicate that CspC is sensitive to proteolysis but protected in the presence of a specific ssDNA. The likely reason of such resistance or conformational stabilization of CspC may be due to the occlusion of proteolytic sites by ssDNA binding.

Quenching of Trp fluorescence of CspC by ssDNA

The lone Trp residue of CspC that is exposed on the surface resides in the close vicinity of its RNP1 motif and aligns with the Trp residue of *B. subtilis* CspB [5]. Intrinsic fluorescence emission from the Trp residue of *B. subtilis* CspB was quenched in the presence of ssDNA [14]. A genetic study has shown that the Trp residue of *B. subtilis* CspB helps this protein to bind to ssDNA [1]. To see whether the Trp residue also plays a similar role in CspC, we measured the intrinsic Trp fluorescence intensity of this *S. aureus* protein in the presence of varying concentrations of ssDNA1 and ssDNA2, separately. As evident from Fig. 4B, quenching of Trp fluorescence intensity of CspC increases with the increasing concentration of ssDNA1 and finally reaches saturation at ~1.2 μM and higher DNA concentrations. The maximum amount of quenching with ssDNA1 was found to be about 70%. In contrast, ssDNA2 that did not show detectable binding with CspC in the gel shift assay (Fig. 3) also quenched the Trp fluorescence of CspC to about 25%. The reason for this slightly anomalous behaviour of ssDNA2 in two experiments might be due to the fact that CspC binds to different nucleotide-rich ssDNAs with variable affinities and the less stable complexes (such as CspC-ssDNA2 complex) are lost during the gel shift assay within the electrical field. Variable quenching of Trp fluorescence by different ssDNAs was, however, reported for other bacterial Csps also. The thymine-rich ssDNA was found to quench the Trp fluorescence of both *B. subtilis* CspB and *E. coli* CspA more strongly than the cytosine-rich ssDNA [14, 15]. In contrast, guanine- and adenine-rich ssDNAs only weakly quenched their Trp fluorescence. It would be interesting to study whether cytosine-, adenine-, and guanine-rich ssDNAs also affect the Trp fluorescence of *S. aureus* CspC similarly.

To determine the stoichiometry of CspC binding to ssDNA, we also investigated the quenching of Trp fluorescence under the conditions (i.e., at higher concentrations of CspC) that strongly favor the formation of the CspC- ssDNA1 complex. As evident from Fig. 4C, the break-point of the ratio of ssDNA1 to CspC lies at 0.27, indicating that ~4 CspC monomers bind to each ssDNA1 molecule and each CspC monomer covers nearly five contiguous thymine bases. The Hill plot [19] calculated from the above Trp fluorescence quenching data yielded a best fit straight line with slope close to 2.3 further indicating the cooperative binding of CspC to ssDNA1 (Fig. 4D). Under nearly similar condition, *E. coli* CspA or *B. subtilis* CspB covers 6–7 contiguous thymine bases in ssDNA [14, 15]. The biological significance of this different binding capacity of CspC is not known at this time. We however noticed that the loop regions and surface charges of CspC did not match completely with those of *E. coli CspA* and *B. subtilis* CspB (data not shown).

Conclusions

The present investigation demonstrates that *S. aureus* CspC exists as a monomer in solution, possesses primarily β-sheet and wraps nearly five contiguous thymine bases while binding to a thymine-rich ssDNA cooperatively. The DNA binding capacity of CspC apparently differs from those of other bacterial Csps despite their structural similarity. Interestingly, CspC that seems to be a thermodynamically less stable protein was stabilized in the presence of the above thymine-rich ssDNA.

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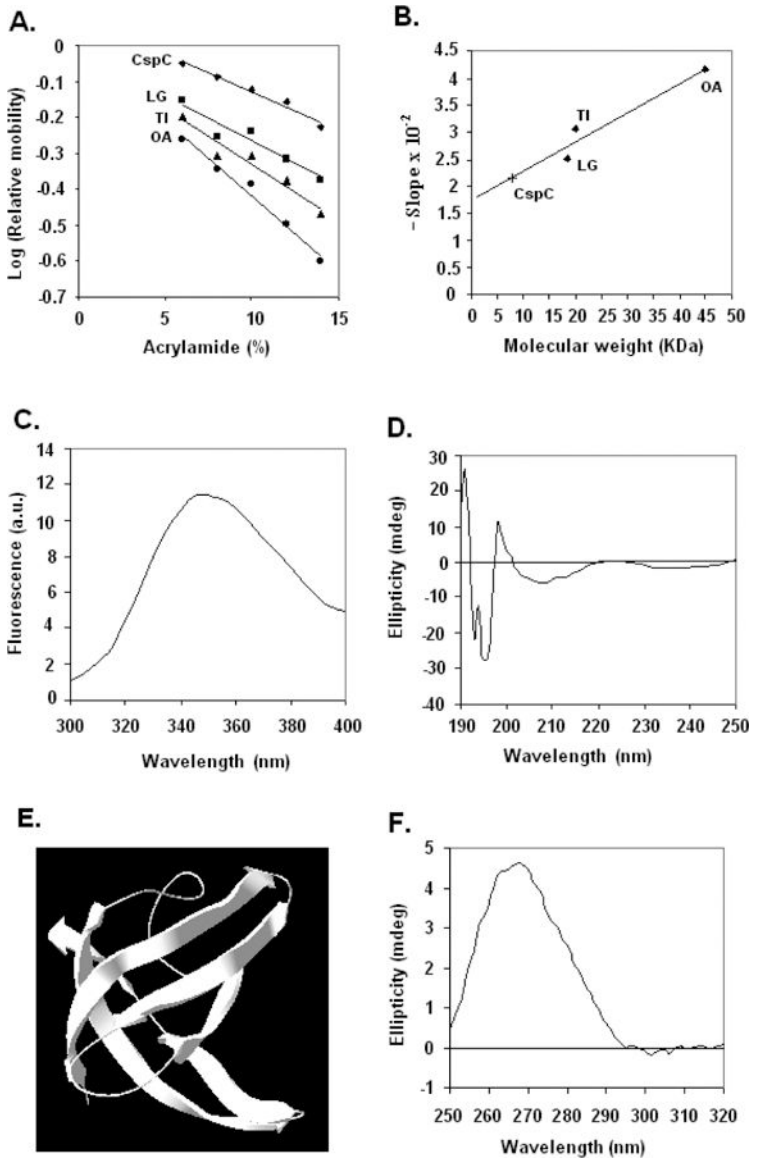

Figure 1.

Physicochemical properties and structures of *S. aureus* CspC. (A) Ferguson plot. Several standard proteins [OA, Ovalbumin (45 kDa); TI, Trypsin soyabean inhibitor (20 kDa) and LG, β-lactoglobulin (18.4 kDa)] and *S. aureus* CspC have been resolved through different native polyacrylamide gels followed by the construction of a Ferguson plot using the relative migration of protein bands and gel concentration. (B) Determination of the molecular weight of *S. aureus* CspC from the standard curve generated using the slope values of the standard proteins (from panel B) and their molecular weights. (C) Intrinsic tryptophan fluorescence emission (300 – 400 nm) of 5 μM CspC after excitation at 295 nm. Tryptophan fluorescence of CspC was recorded five times and one representative scan was presented here. (D) Far-UV CD-spectrum (190 – 250 nm) of 10 μM CspC. (E) Schematic representation of the three-dimensional model structure of CspC. All the β-strands (helices) and the connecting loops (tubes) are shown. (F) Near-UV CD-spectrum (250 – 320 nm) of CspC.

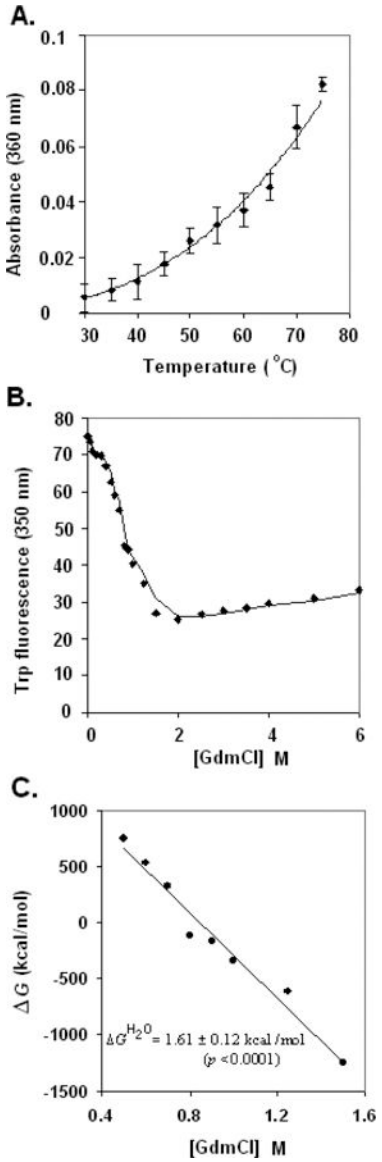

Figure 2.

Stability of CspC. (A) Aggregation of 5 μM CspC at different temperatures. (B) Intrinsic tryptophan fluorescence emission of CspC at 0 – 6 M GdmCl. Trp fluorescence of GdmCl-treated CspC was measured at 350 nm after excitation at 295 nm. (C) Free energy for denaturation of CspC as the function of GdmCl. The GdmCl-induced unfolding data (shown in panel B) was analyzed by a standard two-state method [16] in order to calculate the Gibbs free energy (ΔG_0) of stabilization of CspC at 0 M GdmCl and other parameters.

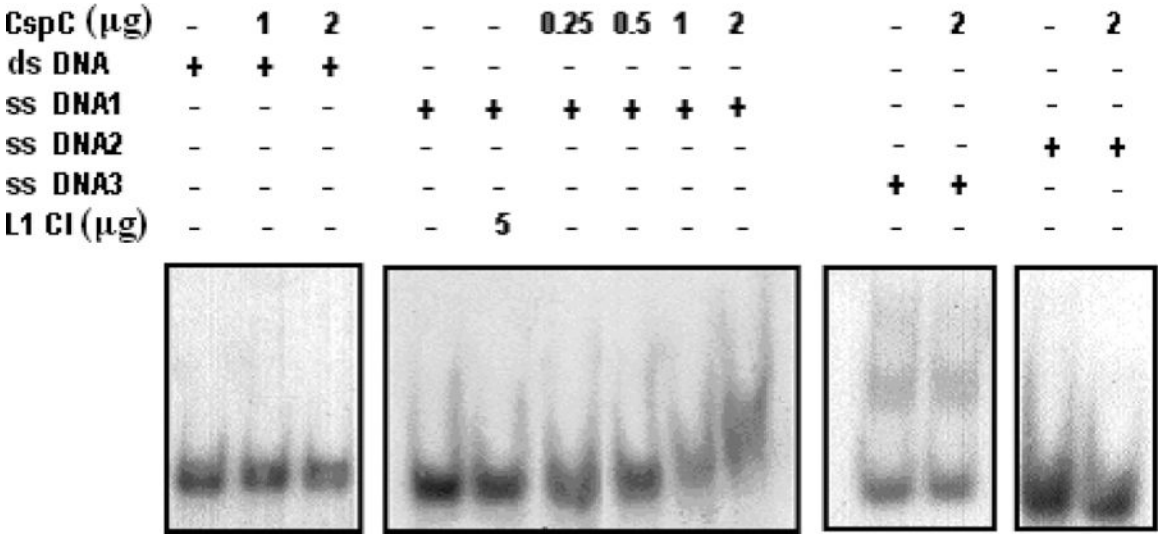

Figure 3.

DNA-CspC interaction. Interaction of indicated amounts of CspC and L1 repressor [19] with ^{32}P labeled DNAs were investigated by the standard gel shift assays. Autoradiogram of the gel shift assays show the migration of different (ss/ds) DNAs. Abbreviations: ds DNA-PCR made DNA using Newman DNA, ssDNA2 and ssDNA3.

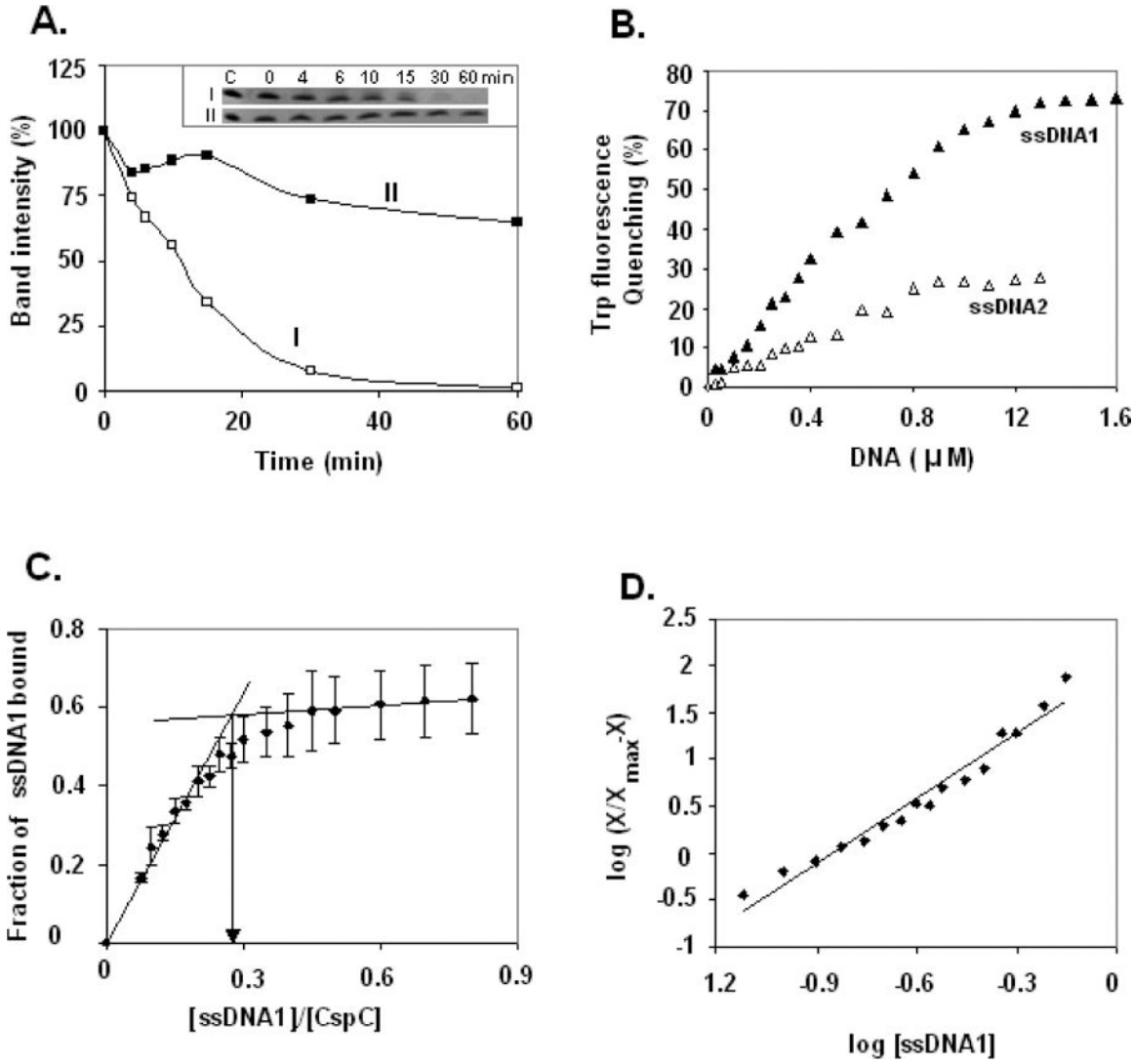

Figure 4.

Effect of ssDNA on CspC. (A) Trypsinolysis of CspC. About 1 μg CspC was incubated with 0.05 μg trypsin at 25 °C in 20 μl Buffer D and aliquots, withdrawn at indicated time intervals, were analyzed by Tris-Tricine SDS –15% PAGE (inset pictures). Tryptic digestion was performed both in the presence (II) /absence (I) of 6 μM ssDNA1. C, trypsin untreated CspC. Band intensities determined by scanning the CspC bands in the above polyacrylamide gel are plotted against time of digestion. (B) Tryptophan fluorescence quenching of 0.3 μM CspC in the presence of 0 – 1.6 μM ssDNAs. Filled and empty triangles denote percent quenching of tryptophan fluorescence intensities of CspC in the presence of ssDNA1 and ssDNA2, respectively. (C) Binding stoichiometry. From the Trp fluorescence quenching of 3 μM CspC in the presence of 0 – 2.4 μM ssDNA1, fractions of ssDNA2 bound by CspC were determined according to Lopez and Makhatadze [15] and plotted against the ratio of [ssDNA1]/[CspC]. The break point (denoted by arrow) indicates that all input ssDNA1 molecules are bound by CspC. (D) Hill plot as developed from the data in Fig. 4C. X and

X_{max} denote the mean fraction of ssDNA1 and maximum amount of ssDNA1 bound by CspC, respectively.