Three Amino Acids in *Escherichia coli* CspE Surface-exposed Aromatic Patch Are Critical for Nucleic Acid Melting Activity Leading to Transcription Antitermination and Cold Acclimation of Cells*

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Cold-shock proteins of the CspA family of *Escherichia coli* help the cells to acclimatize to low temperature conditions through an unknown mechanism. *In vitro*, these proteins bind to single-stranded nucleic acids and destabilize nucleic acid secondary structures. An unusual surface-exposed patch of 6 evolutionarily conserved aromatic amino acids is thought to be involved in RNA binding by the cold-shock proteins. Here we investigated the functional role of the aromatic patch in *E. coli* CspE by substituting individual aromatic residues with positively charged Arg residues. These substitutions do not affect the RNA binding activity of the CspE mutants. We show that substitutions of three centrally located aromatic patch amino acid residues, Phe\(^{17}\), Phe\(^{30}\), and His\(^{32}\), abolish the ability of the mutant CspE to acclimatize cells to cold, antiterminate transcription and melt nucleic acids but have no effect on RNA binding. On the other hand, peripherally located Trp\(^{10}\), Phe\(^{19}\), and Phe\(^{33}\) can be substituted with Arg without loss of any of the *in vivo* and *in vitro* CspE functions tested. The results thus indicate that these aromatic patch residues have clearly distinct functional roles and further extend the correlation between the essential function of CspA homologues in cold acclimation and their ability to antiterminate transcription.

The cold-shock response is a physiological response of living cells to temperature downshift (1). When an exponentially growing culture of *Escherichia coli* is shifted from 37 to 15 °C, there is a transient arrest of cell growth characterized by severe inhibition of general protein synthesis. However, synthesis of a number of proteins, the so-called cold-shock proteins, is induced under these conditions. Synthesis of CspA protein is dramatically induced immediately following the temperature downshift, and this protein becomes one of the most abundant proteins in the cell (for a review, see Refs. 2 and 3). Eventually, the synthesis of CspA decreases, cells become acclimated to low temperature, and growth resumes.

CspA homologues are widely distributed in prokaryotes, and the cold-shock domain of human Y-box protein YB-1 and of other eukaryotic Y-box proteins is also homologous to CspA (for a review, see Refs. 4 and 5). In *E. coli*, the CspA protein family is represented by nine homologous proteins, from CspA to CspI, of which CspA, CspB, CspG, and CspI are cold-shock-inducible. However, cells harboring double or triple deletions of genes coding for cold-shock-inducible CspA homologues (ΔcspAΔcspB, ΔcspAΔcspG, ΔcspBΔcspG, ΔcspAΔcspI, or ΔcspAΔcspBΔcspG) are not cold-sensitive (6). This and the fact that in a triple deletion strain ΔcspAΔcspBΔcspG, the CspE protein, which is normally constitutively produced at 37 °C, is overproduced at low temperatures, suggest that the functions of the CspA family members overlap and that they are able to functionally substitute for each other during cold acclimation.

The exact mechanism(s) by which CspA family proteins allow cells to acclimate to cold is not known. *In vitro*, CspA, CspB, CspC, and CspE are able to bind RNA and single-stranded DNA with low sequence specificity and low binding affinity (7, 8). Binding of CspA and CspE to RNA destabilizes RNA secondary structures (7, 9). Known biochemical and physiological properties of CspA homologues appear to stem from either nucleic acid binding or melting activities. For example, purified CspE impedes poly(A)-mediated 3′ to 5′ exonucleolytic decay by PNPase and inhibits both internal cleavage and poly(A) tail removal by RNase E (10). Hanna and Liu (11) demonstrated that CspE interacted with the nascent RNA in transcription elongation complexes and interfered with Q-mediated transcription antitermination. The link between Csp protein function and transcription regulation was further strengthened by the demonstration that several Csp proteins, including CspA and CspE, can act as transcription antiterminators in *vivo* and *in vitro*. During cold shock, the expression of several promoter-distal genes of the metY-*rpsO* operon whose products themselves are involved in cold acclimation is increased at the level of transcription antitermination. Overproduction of either CspA, CspE, or CspC at high temperature leads to similar increase in promoter-distal metY-*rpsO* operon gene expression (9). Thus, it appears that CspA homologue function at cold shock is at least partially due to its ability to antiterminate transcription. Nucleic acid melting activity is required for both cold acclimation and transcription antitermination functions of CspE (12). Additional consequences of overproduction of CspA homologues include (i) up-regulation of *rpoS* expression, which appears to depend...
only on Csp RNA binding (13) and (ii) induction of cell resistance to camphor, through undefined mechanisms (14).

The three-dimensional structure of CspA from E. coli has been solved by both x-ray crystallography and NMR (15–17). The structure consists of five antiparallel β-strands, β1 to β5, forming a β-barrel structure with two β-sheets. Two evolutionarily conserved RNA-binding motifs, RNP1 and RNP2, are located on the β2 and β3 strands. The RNP1 Trp13, Phe18, and Phe30 and the RNP2 Phe31, His33, and Phe34 (the corresponding residues in CspE are Trp10, Phe17, Phe19, Phe30, His32, and Phe33, respectively) form a compact surface-exposed aromatic patch on the CspA three-dimensional structure (Fig. 1) (15–17). The three central aromatic patch residues are shown in magenta. The three peripheral residues are shown in yellow. Lys residues surrounding the aromatic patch are shown in green.

Fig. 1. Structural context of CspA aromatic patch. A surface representation of the CspA structural model determined by x-ray crystallography (17) is shown. The three central aromatic patch residues are shown in magenta. The three peripheral residues are shown in yellow. Lys residues surrounding the aromatic patch are shown in green.

**Experimental Procedures**

**Bacterial Strains—**E. coli strain JM109 (20), its quadruple deletion strain of ΔcspaΔcspBΔcspEΔcspG (6), and the strain RL211 (21) were used in this study. The bacterial cultures were grown in Luria broth (LB). Antibiotics such as ampicillin (50 μg ml⁻¹) or chloramphenicol (30 μg ml⁻¹) were supplemented as required.

**Site-directed Mutagenesis—**The single amino acid point mutations within the cspE coding region were introduced through site-directed mutagenesis using PCR reaction. The resultant DNA fragments were cloned in pINIII and pET11a vectors. The plasmids (pINIII-cspE-W10R, pINIII-cspE-F17R, pINIII-cspE-F30R, pINIII-cspE-F33R, pET11a-cspE-F17R, pET11a-cspE-F30R, and pET11a-cspE-F33R) were sequenced to confirm the mutant sequences.

**Expression and Purification of the Proteins—**In vivo expression of CspE and its mutants was examined using corresponding isopropyl-β-D-thiogalactopyranoside (IPTG) inducible pRPI1 expression vectors as described previously (9, 13). The cells were grown at 37 °C to an OD₆₀₀ of 0.5, and the CspE expression was induced with 1 mM IPTG for 60 min at 37 °C followed by analysis with SDS-PAGE. CspE and its mutant proteins were purified by Q-Sepharose and hydroxyapatite column chromatography as described previously (9).

**Circular Dichroism Studies—**CD measurements were performed on an automated AVIV 60DS spectrophotometer fitted with a thermostated cell holder that is controlled by an on-line temperature control unit. Quartz rectangular cells (Precision cells, Hicksville, NY) with a path length of 1 mm were used. These cells were maintained at 4 °C during the experiment. For CD studies, CspE and its mutant proteins in 20 mM potassium phosphate buffer, pH 7.0, were used. Solutions were filtered through 0.22-μm filters before use. In the thermal unfolding experiments, temperatures were increased from 10 to 90 °C in 1 °C intervals with a 30-s equilibration at each temperature.

**In Vitro Transcription—**The in vitro transcription was carried out as described previously (9). A DNA template containing the T7 A1 promoter fused to the T3 terminator was used. The elongation complexes stalled at position +20 (EC20) were prepared in transcription buffer (40 mM KCl, 40 mM Tris-HCl, pH 7.9, and 10 mM MgCl₂) containing NTPs (250 μM) and T7 RNA polymerase, and 0.5 mM, adenylyl (3’ → 5’) uridine ammonium salt (ApU). Reaction mixtures were incubated at 37 °C for 15 min to form the open complexes and then were transferred to room temperature. 50 μM ATP, GTP, and 2.5 μM [α-³²P]CTP (300 Ci/mmol) were added. After a 10-min incubation, the agarose beads were thoroughly washed with transcription buffer. Equal aliquots of purified EC20 were then supplemented with Csp proteins (1.5 μg) and NTPs (250 μM), and the reactions were incubated at room temperature for 10 min. After the transcription reactions, 20 μl EDTA and 10 μl of the reaction mixture was added.

1 The abbreviation used is: IPTG, isopropyl-β-D-thiogalactopyranoside.
mg/ml heparin were added to the reaction mixtures to avoid nonspecific retardation of RNA in the gel. The reactions were terminated by formamide-containing loading buffer. The products were analyzed by urea-PAGE electrophoresis (7 M urea/10% polyacrylamide) followed by autoradiography and phosphorimaging device analysis.

RNA Binding Assay—The 82-nucleotide RNA fragment (5'-TTGGG-ACATATTATATACACATCTCAAGGCAGUAUAGCAAAUU-UACCCCAAAGTGGTGCAAATCTGCGTTGCTGGCC-3') to which CspE binds preferentially as shown previously by SELEX (systematic evolution of ligands by exponential enrichment) was used, and the filter binding assays were carried out as described before (8). The T7 RNA polymerase reaction was carried out in the presence of [α-32P]UTP to prepare the RNA probe. The RNA was then purified by phenol/chloroform and ethanol precipitation, and its integrity was checked by urea-acrylamide gel electrophoresis. RNA concentration was estimated by quantitating [α-32P]UTP incorporation.

Binding assay was carried out in a 15-μl reaction mixture containing binding buffer (10 mM Tris-HCl buffer, pH 8.0, containing 1 mM EDTA, 10 mM KCl, and 7.4% glycerol) and 50 fmol of RNA. The reaction mixtures were passed through nitrocellulose filters, which were washed thoroughly to remove unbound RNA. Radioactivity retained on the filter was measured by liquid scintillation counter.

Molecular Beacons—The molecular beacon used was described previously (12). It is an 82-nucleotide-long hairpin-shaped molecule labeled with a fluorophore-tetramethyl rhodamine and quencher-DABCYL (4,4'-dimethylaminophenylazo)benzoic acid.

Fluorescence measurements were performed on an LS-5B spectrophotometer (PerkinElmer Life Sciences) using 1-cm path length QS cuvettes (Hellma). The temperature of the cuvette was controlled by a circulating bath. The excitation and emission wavelengths used were 555 and 575 nm, respectively. The fluorescence of a 100-μM solution of a 32-nM molecular beacon dissolved in 20 mM Tris–HCl, pH 7.5, containing 1 mM MgCl2 was monitored as CspE or its mutant proteins (1.5 μg) were added. The reactions were carried out at room temperature.

RESULTS

Genetic and Structural Context of Csp Aromatic Patch—Structural analysis of E. coli CspA reveals an unusual continuous surface-exposed aromatic patch composed of 6 amino acid residues, Trp11, Phe18, Phe20, Phe31, His33, and Phe34 (15–17). Our goal was to determine the contributions of individual aromatic patch residues of E. coli CspE to cold acclimation and to in vitro nucleic acid melting activity. The aromatic patch residues have been strictly conserved among the CspA family members except for CspD (6). We introduced pINIII-based plasmids overexpressing wild-type CspE, CspE mutants, or the pINIII vector alone in the quadruple deletion strain and examined growth at 15 and 37 °C on LB plates with and without IPTG. All clones could grow at 37 °C with or without IPTG (Fig. 3). The plasmid overproducing wild-type cspE complemented the low temperature growth defect of the quadruple deletion strain only in the presence of IPTG, whereas cells with pINIII vector alone did not from colonies at 15 °C either in the presence or in the absence of IPTG, as expected. Plasmids overexpressing the F17R or the F30R mutant did not complement the growth defect of the quadruple deletion strain. In contrast, overproduction of W10R, F19R, and F33R proteins allowed growth at low temperature. However, the F19R mutant appeared to be somewhat less effective than the wild-type, W10R, and F33R CspE in supporting growth at low temperature.

Transcription Antitermination by Mutant CspE Proteins in Vivo and in Vitro—The exact biochemical property of CspA homologues that allows the cell to survive at low temperature is not known. Previously, we suggested that transcription antitermination function of these proteins may be essential for low temperature survival (9, 12). We therefore determined the effects of substitutions in CspE surface-exposed aromatic patch on transcription antitermination in vivo. The E. coli strain RL211 designed by Landick et al. (21) contains the cat gene preceded by a strong p-independent trpL terminator and is therefore sensitive to chloramphenicol. When transcription termination at trpL is reduced, the cat gene is expressed, and the cells become resistant to chloramphenicol. The RL211 cells transformed with plasmids expressing cspE mutants were spotted on LB plates containing ampicillin and IPTG, in the presence or in the absence of chloramphenicol. Cells carrying the wild-type cspE expression plasmid or pINIII vector alone were used as controls. As expected, cells carrying all the constructs showed growth in the absence of chloramphenicol, and cells carrying the wild-type cspE expression plasmid but not the pINIII vector grew in the presence of chloramphenicol (Fig. 4A). In agreement with low temperature growth complementation data, overproduction of W10R, F19R, and F33R CspE mutants, but not of F17R and F30R, allowed RL211 cells to grow in the presence of chloramphenicol (Fig. 4A). We conclude that the lack of growth in the presence of chloramphenicol by cells overproducing F17R and F30R mutants is likely due to a defect in transcription antitermination by mutant proteins.
We confirmed the in vivo result in vitro using purified proteins. Two non-functional CspE mutants, F17R and F30R, were overproduced and purified to homogeneity. The wild-type CspE, as well as one of the functional mutants, F19R, which showed a slight defect in cold acclimation test, were also purified as controls. Stalled E. coli RNA polymerase elongation complexes were prepared on DNA template containing the T7 A1 promoter followed by a /H9267-independent terminator tR2 (22). Transcription was resumed by the addition of NTPs in the presence or absence of CspE proteins. As seen from Fig. 4B, the wild-type and F19R CspE decreased transcription antitermination at tR2. The mean read-through efficiency values, defined as the fraction of the run-off transcripts of the total transcripts produced, were calculated for three independent experiments and are presented in Fig. 4B. As can be seen, the read-through efficiency value was less than 30% in the control reaction in the absence of CspE or in the presence of the F17R and F30R mutants. Addition of the wild-type CspE or the F19R mutant protein resulted in increase in the read-through efficiency values to 57 and 58%, respectively. These results are consistent with the in vivo data shown above and suggest that only the single amino acid substitutions of Phe residues with Arg that result in the loss of the nucleic acid melting activity of CspE lead to loss of its ability to cause transcription antitermination.

Loss of Cold Acclimation and Transcription Antitermination Activities Is Not Due to the Destabilization of CspE by Mutations—Structural integrity of CspE mutants was examined by CD spectroscopy. Similar to the CD spectrum of CspA (23), the far UV spectrum of wild-type CspE is unusual (12), with positive ellipticity around 225–228 nm, which is due to aromatic chromophores. In agreement with the results of CD spectra analysis of CspA aromatic patch mutants performed by the Gregoret group (18), the CD spectra of some of our mutants (i.e.
F19R and F30R) do not closely resemble the wild-type spectrum, but this difference does not necessarily mean dramatic change in the overall structure of CspE since the mutations affect the number of aromatic residues in the protein. The presence of characteristic minima at around 210–220 nm indicative of the β-sheet structure is seen for all proteins. Further, the 260–320-nm CD spectrum of the F30R mutant, whose far UV spectrum differed the most from the wild-type CspE spectrum, was similar to the wild-type and F17R spectra, suggesting that the tertiary packing of the mutant protein is similar to the wild type (Fig. 5B). This, and the fact that none of the mutants was affected in RNA binding (see below), allows us to conclude that none of the aromatic patch mutations significantly perturb CspE structure.

Analysis of the melting patterns (Fig. 5C) showed that all mutants were less stable (T_m values of 42, 50, and 49 °C, for F17R, F19R, and F30R, respectively) than the wild-type CspE (T_m value of 62 °C). However, since the biochemical and microbiological assays that are presented here were performed at 25 °C, at which temperature the stability of mutant proteins was not significantly different from that of the wild-type CspE, and since the F19R mutant with reduced T_m (50 °C) showed unaffected nucleic acid melting activity (see below), we conclude that the effect of mutations is not a manifestation of the differences in the stability of these proteins.

**Mutations Substituting Aromatic Patch Residues with Arg Do Not Affect the RNA Binding Activity of CspE**—The RNA binding activity of CspE mutants was examined in filter binding assays using an 82-nucleotide-long, radioactively labeled AU-rich RNA substrate as described previously (8). The apparent K_d value of the binding reaction was defined as the concentration of protein at which half of the maximum binding was obtained (24). As can be seen from the data presented in Fig. 6 and consistent with the previous report (8), the calculated K_d value for CspE was 0.3 × 10^{-6} M. The calculated K_d values for the F17R, F19R, and F30R mutants were 0.09, 0.05, and 0.06 × 10^{-6} M, respectively. Thus, the substitutions of residues of the surface-exposed aromatic patch with positively charged Arg residues increased the RNA binding ability of CspE, as expected. We note that the previously described substitution of the aromatic patch His^{32} to Arg also enhanced the RNA binding of CspE and left the protein structurally intact (12).
structure. A fluorescent moiety and a nonfluorescent quenching moiety are attached to the ends of two arms. The two moieties are in close proximity to each other because of the stem, and this results in efficient quenching of the fluorophore fluorescence (25). When a protein "opens up" the hairpin loop structure, the arms of the beacon move apart, causing the fluorophore and the quencher to move away from each other as well. Since the fluorophore is no longer in close proximity to the quencher, its fluorescence will increase. Addition of purified CspE resulted in the stably increased beacon fluorescence (Fig. 7) (12). The molecular beacon experiment was next repeated with CspE mutants. The maximum fluorescence obtained with the wild-type CspE was assumed as 100%, and the relative fluorescence obtained for each protein is expressed as relative percent (Fig. 7). As can be seen, the F19R mutant fully retained the nucleic acid melting activity (102% fluorescence), whereas the F30R mutant showed only 7.9% fluorescence. The F17R mutant showed 30% of the wild-type activity. Increasing the amount of any of the proteins did not lead to increased fluorescence, and none of the mutant proteins showed fluorescence by themselves (data not shown). We conclude that the Phe17 and Phe30 contribute to the nucleic acid melting activity of CspE with the later residue making the most important contribution. On the other hand, Phe19 does not contribute to the melting activity.

**Discussion**

The research into the physiological functions of CspA homologues has been mostly focused on protein stability and folding and RNA binding (18, 19). Ever since the structures of CspA homologues became available, it has been assumed that an unusual surface-exposed patch of 6 evolutionarily conserved amino acid residues participates in nucleic acid binding. Several groups showed that substitutions of aromatic residues in these proteins lead to a loss of the nucleic acid binding ability and also destabilize the structure. In particular, Phe18, Phe20, and Phe31 of E. coli CspA or corresponding residues in *Bacillus* CspB are critical in this aspect (18, 19, 26). General considerations as well as some recent data suggest that it is the ability of CspA homologues to destabilize nucleic acid secondary structures that are critical for their ability to function in cold acclimation. At low temperatures, the secondary structures of RNA are stabilized, slowing down transcription elongation and ribosomal movement on RNA. CspA, acting like "RNA chaperones," could destabilize the secondary structures in RNA and thus facilitate transcription and translation (5, 7).

The role of aromatic patch residues in nucleic acid melting was not looked at previously. Mutational analysis of nucleic acid melting by Csp is complicated by the fact that mutant proteins under study should retain their nucleic acid binding ability. Recently, we isolated a mutation in CspE, changing aromatic patch His32 to Arg in an *in vivo* genetic screen (12). This mutant did not affect nucleic acid binding but abolished the nucleic acid melting function of the protein. The mutant was defective in cold acclimation and transcription anticodon termination, supporting the idea that nucleic acid melting is critical for these functions of CspE. Here, we extended the results of our original work by performing systematic substitutions of each of the aromatic patch residues with Arg and studying the effects on *in vivo* and *in vitro* CspE functions. The results clearly demonstrate functional specialization within the aromatic patch residues. The 3 central amino acid residues, Phe17, Phe30, and His32, are each required for nucleic acid melting by CspE. On the other hand, the 3 peripheral amino acid residues (Trp10, Phe19, and Phe33) (Fig. 1, indicated in yellow) can be substituted with Arg without loss of nucleic acid melting. The results thus support the following hypothetical scenario of the interaction of CspA homologues with nucleic acids. The primary interaction is probably due to a set of positively charged Lys residues (Lys4, Lys10, Lys16, Lys28, Lys43, and Lys60) that surround the aromatic patch (Fig. 1, indicated in green). This primary electrostatic interaction must position CspE such that Phe17, Phe30, and His32 residues can intercalate between the bases and thus initiate separation of the nucleic acid strands. Substitution of any one of these residues by Arg actually increases RNA binding presumably by electrostatic interactions but completely prevents melting since its side chain containing the guanidino group is unable to intercalate between the bases. Once initiated at the center of the aromatic patch, melting could propagate further outside in both directions, and Trp10, Phe19, Phe33 may help at this stage, although these additional interactions may not be essential in the context of the Arg substitutions studied here. Our data do not allow us to determine whether only one of the central aromatic patch residues, which are all in Van der Waals contact with each other, is particularly important for the initiation of melting, or whether all three residues act in concert. Future analysis of melting intermediates using model substrates and the CspE mutants described here should clarify this issue.

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