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Review

Bacterial cold-shock proteins

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Abstract. Members of a family of small cold-shock proteins (CSPs) are induced during bacterial cell response to a temperature decrease. Here we review available data about the structure, molecular properties, mechanism of induction and possible functions of CSPs. CSPs preferentially bind single-stranded RNA and DNA and appear to play an important role in cell physiology under both normal and cold-shock conditions. Although the function of CSPs in cold-shock adaptation has not yet been elucidated in detail, a number of experimental evidences suggests that CSPs bind messenger RNA (mRNA) and regulate ribosomal translation, rate of mRNA degradation and termination of transcription.

Key words. ssDNA/RNA binding protein; RNA chaperone; regulation of transcription; regulation of translation; bacterial cold shock; cold adaptation.

Introduction

Bacteria respond to a decrease in temperature in a specific manner. A temperature downshift results in inhibition of cell growth and proliferation, and changes in protein expression patterns [1]. The synthesis of most cellular proteins is inhibited after a decrease in temperature [1]. However, a number of proteins are found to be induced under cold-shock conditions (for review see [2–5]). Expression of the cold-shock proteins reaches a maximum level during the phase of the cold-shock adaptation, the so-called acclimation. After this, synthesis of cold-shock proteins declines and a new steady-state level of protein expression is established which is lower than before the cold shock.
Among cold-shock proteins, small homologous proteins [65–70 amino acid residues long) were found to be dominant under cold-shock conditions in different species of bacteria [6]. They are the most common cold-inducible proteins identified to date. The first protein described as a major protein induced upon decrease in temperature was CspA of *Escherichia coli* [7]. CspA production reached concentrations of 100 μM within 1–1.5 h after the temperature downshift [7, 8]. Eight more proteins named in alphabetical order from CspB to CspI that are homologous to CspA are identified in *E. coli* [6]. CspB, CspG and CspI were found to be inducible by a temperature downshift similarly to CspA [9–11]. CspC and CspE are expressed at both high and low temperature [12], while CspD is induced during stationary phase and upon nutrition starvation [13]. In *Bacillus subtilis* three proteins homologous to *E. coli* CspA (CspB, CspC and CspD) are induced upon temperature downshift [14, 15]. CSP-like proteins were found in more than 50 other bacterial species [2, 16]. The first eukaryotic true homologue of CSPs, protein Cla h 8, was recently identified in the mould *Cladosporium herbarum* [17]. It appears that proteins of the CspA family not only play a major role during cold-shock adaptation but also are important under normal growth conditions. Despite the fact that CSPs were extensively studied during the last decade, their exact function both at normal and cold-shock conditions is not elucidated yet. Below we will focus on the known molecular properties, mechanisms of

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induction of the CspA protein family members from *E. coli* and *B. subtilis,* and their possible connections to cold-shock adaptation.

Structure and molecular properties of CSPs

While information about cellular functions of CSPs is limited, the structure and molecular properties of CSPs are relatively well studied.

Three-dimensional (3-D) structures of CspA from *E. coli* [18, 19], CspB from *B. subtilis* [20, 21], *B. caldolyticus* [22] and *Thermotoga maritima* [23] were solved. All of the structures contain five antiparallel β strands that form a β barrel (fig 1). $CspA_{EC}$ exists as a monomer in both solution and the crystal [18, 19]. $CspB_{BC}$ exists as dimer in the crystal [20] and in solution in the absence of phosphate ions [24]; in the presence of 30–50 mM of phosphate, $CspB_{BC}$ exists predominately as monomer [24]. $CspD_{EC}$ forms dimers in solution [25].

The cold-shock proteins form a cold-shock domain fold that belongs to the family of five-strand β-barrel proteins that bind oligonucleotides and/or oligosaccharides (OB fold) [26]. In particular, a domain fold similar to a cold-shock domain fold was found in ribosomal protein S1 and several different cold-inducible proteins, NusA, PNPase and IF1 [27, 28] (fig. 1). Based on the Structural Classification of Proteins (SCOP) database [29] cold-shock domains are also found in archaeal initiation factor-1 a, aIF1 a from *Methanococcus jannaschii*, translation initiation factor-1 a, eIF1 a (*Homo sapiens*), the N-terminal RNA-binding domain of Rho termination factor (*E. coli*), the C-terminal domain of eukaryotic initiation translation factor 5 a (*M. jannaschii and Pyrobaculum aerophilum*), the N-terminal domain of ribosomal protein L2 (*Bacillus stearothermophilus* and *Haloarcula marismortui*), ribosomal protein S12 (*Thermus thermophilus*) and ribosomal protein S17 (*T. thermophilus* and *B. stearothermophilus*). Interestingly, significant sequence conservation of the cold-shock domain was demonstrated not only in bacteria but also among eukaryotes. A sequence alignment of several cold-shock domain-containing proteins is presented on figure 2. Significant sequence homology is found [30] between CSPs and the nucleic acid binding domains of the eukaryotic gene-regulatory Y-box factors (fig. 2). Y-box proteins were identified as proteins recognizing the ATTGG motif known as the 'Y-box', a cis-acting DNA regulatory element [31]. Y-box proteins are involved in regulation of transcription and translation (for reviews see references [31, 32]. CSPs and Y-box proteins share the canonical nucleic acid-binding sequence motifs RNP1 (K/R-G-F/Y-G/A-F-V/I-X-F/Y) and RNP2 (L/I-F/Y-V/I-G/K-N/G-L), see figure 2 [20, 33, 34]. The isolated cold-shock domain of the Y-box protein YB-1 has a structure very similar to that of the CSPs (fig. 1) – five-stranded antiparallel β barrel [35]. Cold-shock domains were also found in several other eukaryotic proteins that do not belong to the Y-box protein family (fig. 2). The human RNA-binding protein Unr is required for internal ribosomal entry site (IRES)-dependent translation [36], and the calcium-regulated protein CRHSP-24 [37] carrying the cold-shock domain (see fig. 2) does not show any significant similarity with YB-1 except in the part of the sequence corresponding to the cold-shock domain. The first true homologue of CSPs among eukaryotes – protein Clah 8, which consists only of a cold-shock domain – was recently found in *Cladosporium herbarum* [17].

It is clear that CSPs belong to a large family of structurally related nucleic acid-binding proteins, suggesting similar functions for CSPs. It appears that CSPs bind single-stranded RNA and DNA (ssRNA/ssDNA), but not double-stranded DNA (dsDNA) [38–40]. Although binding of $CspA_{EC}$ to a double-stranded 110 bp fragment of the promoter region of the H-NS protein operon was shown in a crude cell extract [41], purified $CspA_{EC}$ bound the same DNA fragment with significantly lower affinity [42]. Thus, dsDNA binding by $CspA_{EC}$ is probably mediated by the presence of other proteins. There is only one report that contradicts the data about CSP binding properties: the eukaryotic CSP homologue, Cla h 8, binds both ssDNA and dsDNA [43].

The affinity of CSPs binding to ssRNA/ssDNA is in the micromolar range. The only exception is Cla h 8, which binds both ssDNA and dsDNA with nanomolar affinity (K_D ~ 5–10 nM) [17]. The minimal concentration of $CspA_{EC}$ required to retard ssRNA in gel-shift assay is only 27 µM [38]. Although an in vitro selection approach

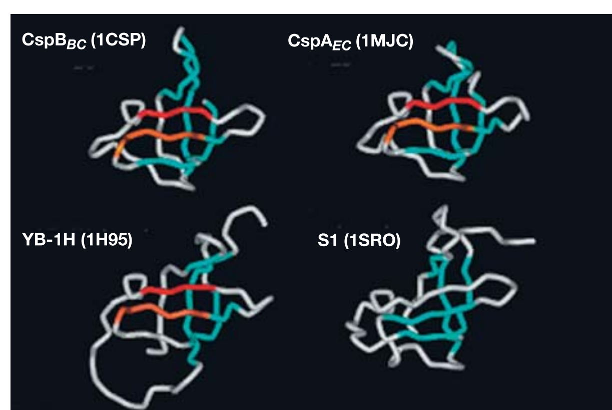

Figure 1. Comparison of the cold-shock domain structures of CspA from *E. coli* (1MJC) [19], CspB from *B. subtilis* (1 CSQ) (20], the S1 RNA binding domain of PNPase (1SRO) [27] and the cold-shock domain of human Y-box protein 1 (1H95) [35], revealing similarity in fold. RNP1 and RNP2 nucleic acid binding motifs are colored orange and red, respectively. RNP1 and RNP2 are not colored in the S1 RNA binding domain of PNPase, because sequence of this protein does not show any significant similarity with CspA from *E. coli* or CspB from *B. subtilis*.

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                                                    RNP1          RNP2
CSPA E.coli                          /4-70     KMTGIVKWFNADKGFGFITPDDGSKDVFVHFSAIQNDG----YKSLDEGQKVSF---TIES--GAKGPAAGNVTSL
CSPB E.coli                          /4-70     KMTGLVKWFNADKGFGFISPVDGSKDVFVHFSAIQNDN----YRTLFEGQKVTF---SIES--GAKGPAAANVIIT
CSPC E.coli                          /2-68     KIKGQVKWFNESKGFGFITPADGSKDVFVHFSAIQGNG----FKTLAEGQNVEF---EIQD--GQKGPAAVNVTAI
CSPD E.coli                          /1-67     MEKGTVKWFNNAKGFGFICPEGGGEDIFAHYSTIQMDG----YRTLKAGQSVQF---DVHQ--GPKGNHASVIVPV
CSPE E.coli                          /2-68     KIKGNVKWFNESKGFGFITPEDGSKDVFVHFSAIQTNG----FKTLAEGQRVEF---EITN--GAKGPSAANVIAL
CSPF E.coli                          /3-70     KMTGIVKTFDGKSGKGLITPSDGRIDVQLHVSALNLRD----AEEITTGLRVEF---CRIN--GLRGPSAANVYLS
CSPG E.coli                          /4-70     KMTGLVKWFNADKGFGFITPDDGSKDVFVHFTAIQSNE----FRTLNENQKVEF---SIEQ--GQRGPAAANVVTL
CSPH E.coli                          /3-70     KMTGIVKTFDRKSGKGFIIPSDGRKEVQVHISAFTPRD----AEVLIPGLRVEF---CRVN--GLRGPTAANVYLS
CSPI E.coli                          /4-70     KMTGLVKWFNPEKGFGFITPKDGSKDVFVHFSAIQSND----FKTLTENQEVEF---GIEN--GPKGPAAVHVVAL
CSPB B.subtilis                      /1-66     MLEGKVKWFNSEKGFGFIEVEGQ-DDVFVHFSAIQGEG----FKTLEEGQAVSF---EIVE--GNRGPQAANVTKE
CSPC B.subtilis                      /1-66     MEQGTVKWFNAEKGFGFIERENG-DDVFVHFSAIQSDG----FKSLDEGQKVSF---DVEQ--GARGAQAANVQKA
CSPD B.subtilis                      /1-66     MEKGTVKWFNNAKGFGFICPEGGGEDIFAHYSTIQMDG----YRTLKAGQSVQF---DVHQ—-GPKGNHASVIVPV
CSPA Salmonella typhimurium          /4-70     KMTGIVKWFNADKGFGFITPDDGSKDVFVHFSAIQNDG----YKSLDEGQKVSF---TIES--GAKGPAAGNVTSL
CSPA Streptococcus pyogenes          /1-66     MAQGTVKWFNAEKGFGFISTENGQ-DVFAHFSAIQTNG----FKTLEEGQKVAF---DVEE--GQRGPQAVNITKL
CSPA Mycobacterium tuberculosis      /1-67     MPQGTVKWFNAEKGFGFIAPEDGSADVFVHYTEIQGTG----FRTLEENQKVEF---EIGH--SPKGPQATGVRSL
CSPA Micrococcus luteus              /1-67     MAVGTVKWFNAEKGYGFIAPEDNSADVFVHFSAIQGNG----FKELQENDRVEF---ETQD--GPKGLQAANVTKL
CSPA Pseudomonas aeruginosa          /4-69     RQNGTVKWFNDAKGFGFITPESGN-DLFVHFRSIQGTG----FKSLQEGQKVSF---VVVN--GQKGLQADEVQVV
CSPA Listeria monocytogenes          /1-66     MEQGTVKWFNAEKGFGFIERENGD-DVFVHFSAIQGDG----FKSLDEGQAVTF---DVEE--GQRGPQAANVQKA
CSPB Yersinia pestis                 /4-70     KMTGLVKWFDAGKGFGFISPADGSKDVFVHFSAIQGND----YKTLDEGQNVEF---SIEQ--GQKGPSAVNVVAL
CSPD Haemophilus influenzae          /1-67     MEIGIVKWFNNAKGFGFISAEGVDADIFAHYSVIEMDG----YRSLKAGQKVQF---EVLH--SDKGSHATKIIPI
YB-1 human                           /58-128   KVLGTVKWFNVRNGYGFINRNDTKEDVFVHQTAIKKNNPRKYLRSVGDGETVEF---DVVE--GEKGAEAANVTGP
DBPA human                           /90-160   KVLGTVKWFNVRNGYGFINRNDTKEDVFVHQTAIKKNNPRKYLRSVGDGETVEF---DVVE—-GEKGAEAANVTGP
Cla h 8 Caldosporium herbarum         /7-73     RQNGTVKWFNDEKGYGFITPENGSADLFVHFRAIEKDG----FKSLKEGEAVTF---VAEQ--GQKGMQASSVRNN
LIN-28 C.elegans                     /52-120   RYFGSCKWFNVSKGYGFVIDDITGEDLFVHQSNLNMQG----FRSLDEGERVSY---YIQERSNGKGREAYAVSGE
CRHSP-24 human                       /62-129   VYKGVCKCFCRSKGHGFITPADGGPDIFLHISDVEGEY------VPVEGDEVTYKMCSIPP—-KNEKLQAVEVVIT
UNR human                            /185-248  RCQGVVCAMKE--AFGFIERGDVVKEIFFHYSEFKGDL-----ETLQPGDDVEF---TIKD--RNGKEVATDVRLL
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Figure 2. A sequence alignment of the cold-shock domains from different proteins. The numbers correspond to the position of the cold-shock domain in the amino acid sequence of the entire proteins. Residues conserved in more than 80% of these sequences are colored red, and more than 60% in blue. Canonical nucleic acid binding sequence motifs RNP1 and RNP2 are bold and underlined. Swiss-Prot access numbers for these sequences are $CspA_{EC}$-P15277; $CspB_{EC}$-P36995; $CspC_{EC}$-P36996; $CspD_{EC}$-P24245; $CspE_{EC}$-P36997; $CspF_{EC}$-P39819; $CspG_{EC}$-Q47130; $CspH_{EC}$-P56253; $CspI_{EC}$-P77605; $CspB_{BC}$-P32081; $CspC_{BC}$-P39158; $CspD_{BC}$-P51777; CspA *Salmonella typhimurium*-P15277; CspA *Streptococcus pyogenes*-Q54974, CspA *Mycobacterium tuberculosis*-O06360; CspA *Micrococcus luteus*-O30875; CspA *Pseudomonas aeruginosa*-P95459; CspA *Listeria monocytogenes*-Q48770; CspB *Yersinia pestis*-O85593; CspD *Haemophilus influenzae*-P46449; YB-1 human-P16991; DBPA human-P16989; LIN-28 *C. elegans*-P92186; CRHSP-24 human-Q9Y2V2; UNR human-O75534. Protein sequence of Cla h 8 was taken from [17, 43].

(SELEX) was insufficient to find the preferable sequence for $CspA_{EC}$ RNA binding [38], it was shown that $CspA_{EC}$ binds with higher affinity to poly-pyrimidines ssDNA oligonucleotides than to polypurine ssDNA templates [39]. In the cases of $CspB_{EC}$, $CspC_{EC}$ and $CspE_{EC}$, SELEX revealed the specific consensus sequences UUUUU, AGGGAGGGA and AAAUUU, respectively [44]. However, the K_D values of specific binding differed marginally from the K_D of randomized sequence binding. K_D values were in the range of 1–10 µM, which is similar to the $CspA_{EC}$ affinity to ssRNA [44]. It was shown that $CspB_{BS}$ binds the Y-box recognition sequence ATTGG, as well as the complementary sequence CCAAT [45]. $CspB_{BS}$ has higher affinity to the ATTGG sequence than to CCAAT. However, binding to CCAAT is preferential over other sequences [45]. More detailed studies showed that CspB binding is not limited to binding these sequences [40, 46]. $CspB_{BS}$ preferentially binds polypyrimidine but not polypurine ssDNA templates. Moreover, binding of T-based oligos occurs with an affinity on the order of 0.1 µM or higher and is salt independent, while binding of poly-C is one order of magnitude lower and is strongly salt dependent [40]. The binding properties of CspB to continuous and noncontinuous stretches of T bases are similar [47]. The capacity of $CspA_{EC}$ to bind ssRNA [38] and $CspA_{EC}$ and $CspB_{BS}$ to bind ssDNA is moderately cooperative [39]. $CspD_{EC}$ exists exclusively as a dimer in solution and binds noncooperatively ssDNA and RNA but not dsDNA [25].

Despite these differences in the binding properties of CSPs, all cold-shock proteins preferentially bind pyrimidine-rich regions of ssDNA and ssRNA but not dsDNA. Similar binding properties were observed for other cold-shock-domain-containing proteins, such as ribosomal protein S1 [48], and the isolated cold-shock domain of YB-1 [35], which preferentially binds pyrimidine-rich ssDNA and RNA. The binding of dsDNA by Y-box proteins, particularly Y-box sequence binding, is probably mediated by other domains of YB-1 [35, 49].

Several basic and aromatic residues (K_7, K_{13}, H_{29}, R_{56}, W_8, F_{15}, F_{17}, F_{27}, F_{30} in $CspB_{BS}$) conserved in cold-shock domain proteins are arranged on one side of the protein and proposed to form a nucleic acid-binding surface [20, 50]. This hypothesis was supported by chemical shift perturbation analysis of complexes between $CspA_{EC}$ and ssDNA [18]. Single substitutions of F_{15}, F_{17}, F_{27} with alanine and H_{29} with glutamine abolished ssDNA retardation in gel-shift assay using $CspB_{BS}$ [50]. The mutation of F_{18}, F_{20} and F_{31} in $CspA_{EC}$ to serine affects ssDNA binding [51]. Three molecules of $CspA_{EC}$ and $CspB_{BS}$ bind the 23-base-long poly T oligonucleotide [39, 40]. Thus, the size of the site at least on the poly T template for $CspA_{EC}$ and $CspB_{BS}$ is six to seven bases per molecule of protein.

Surprisingly, aromatic residues involved in nucleic acid binding also contribute to protein stability and participate in the rate-limiting step of the protein folding [51–53]. CSPs from mesophilic bacteria fold extremely rapidly (time constant of folding is approximately 1 ms) in vitro

and have very low thermodynamic stabilities [22, 53, 54]. However, CSPs are stable in vivo [55]. In the presence of nucleic acid ligands CSPs become less sensitive to proteolytic degradation in vitro, suggesting that the high stability of CSPs in vivo is mediated by nucleic acid binding [55].

The preferential binding of ssRNA versus dsRNA by CSPs led to the hypothesis that CSPs can destabilize the secondary structure of RNA and act as an RNA chaperone in the cell [38]. It was shown that $CspA_{EC}$ stimulates the hydrolysis of RNA by ribonucleases, presumably by preventing formation of RNase-resistant secondary structures [38]. RNA chaperones were defined as 'proteins that aid in the process of RNA folding by preventing misfolding or by resolving misfolded species' [56]. The popular hypothesis is that the major role of CSPs is an RNA chaperon function [2, 4]. However, it should be pointed out that there is no direct evidence of CSP involvement in biologically relevant RNA folding. It remains to be shown that CSPs are involved in the folding of transfer RNAs (tRNAs), ribosomal RNAs and ribozymes. Moreover, despite the fact that CSPs bind ssRNA/DNA, the direct relation between the ability of CSPs to destabilize the secondary structure of RNA and cold-shock adaptation has not yet been demonstrated.

Cellular functions of the CSPs

There is only limited information available regarding the role of CSPs in cell physiology. CSPs are thought to be involved in the regulation of a number of cellular processes: translation, transcription, nucleoid condensation, coupling of translation and transcription [2, 5, 57–59]. Only four of nine members of the Csp family, $CspA_{EC}$, $CspB_{EC}$, $CspG_{EC}$ and $CspI_{EC}$, are cold inducible in *E. coli*. $CspE_{EC}$ and $CspC_{EC}$ are constitutively expressed at 37 °C. However, CspE becomes highly expressed in a triple-deletion strain lacking three cold-inducible CSPs (*ΔcspAΔcspB-ΔcspG*), indicating that these proteins can perform overlapping functions [60]. Constitutively expressed CSPs were found to be functionally important. $CspC_{EC}$ and $CspE_{EC}$ are thought to be regulators of the expression of two proteins involved in the stress response network of *E. coli*, RpoS and UspA [61]. $CspE_{EC}$ and $CspC_{EC}$ suppress mutations in the chromosomal partition gene *mukB* [12]. Overproduction of $CspE_{EC}$ also leads to resistance to camphor treatment [62], by preventing the unfolding of the nucleoid caused by camphor [63]. These results indicate that $CspE_{EC}$ and $CspC_{EC}$ are probably involved in nucleoid condensation.

The expression of $CspD_{EC}$ is upregulated in the stationary phase of cell growth and upon glucose starvation [13]. $CspD_{EC}$ is associated with the nucleoid in the late exponential phase of growth [64]. It was suggested that $CspD_{EC}$ binds to single-stranded regions of the replication fork and blocks DNA replication, thus leading to the observed lethal phenotype [25]. No information regarding the cellular functions is available for other cold-uninducible CSPs, $CspF_{EC}$ and $CspH_{EC}$. The quadruple-deletion strain lacking *cspA*, *cspB*, *cspE* and *cspG* acquired cold sensitivity and formed filamentous cells at 15 °C. However, overexpresion of any member of the CspA family, except $CspD_{EC}$, suppressed the cold sensitivity of the quadruple-deletion strain of *E. coli* [60], indicating that the cold-uninducible CSPs have at least some overlapping functions with the cold-inducible proteins.

Cold-inducible CSPs are also produced under normal conditions. It was shown that the intracellular concentration of $CspA_{EC}$ in *E. coli* is about 50 µM during early exponential growth at 37 °C [8]. The presence of at least one of $CspB_{BS}$, $CspC_{BS}$ and $CspD_{BS}$ is essential for viability in *B. subtilis* at 37 °C [46]. $CspB_{BS}$ and $CspC_{BS}$ are major stationary-phase-induced proteins in *B. subtilis*, and their deletion leads to cell lysis during the stationary phase [65]. These findings lead to the suggestion that cold-inducible CSPs play some important functional roles even under normal growth conditions.

CSPs may be messenger RNA (mRNA) binding proteins. CSPs bind a broad range of ssRNA sequences with approximately micromolar affinity [39, 40, 47]. There are 10^6 copies of $CspA_{EC}$ per cell [8] and significant amounts of other CSPs under cold-shock conditions in *E. coli*. The average amount of the total mRNA in *E. coli* is 10^{-9} µg per cell [66, 67], which corresponds to approximately 10^3 copies of total mRNA [67]. Therefore, one can expect that at least under cold-shock conditions, CSPs are bound to cellular mRNAs in several copies per molecule of mRNA. CSPs in *B. subtilis* are localized around nucleoids in growing cells, when transcription is intensive and is coupled to translation. When transcription and translation are not intensive and are not coupled, CSPs are distributed throughout the cells [58, 59]. A simple explanation of these results is that CSPs are indeed associated with mRNA in cells. Moreover, the absence of cold-inducible CSPs in mutant strains of *E. coli* and *B. subtilis* can be compensated by the overexpression of other proteins with similar structures and probably similar nucleic acid binding properties, such as IF1 [68] and the S1-domain of polynucleotide phosphorylase (PNPase) [60]. The CSPs binding to mRNA might regulate translation and the rate of mRNA degradation.

The association of CSPs with isolated ribosomes was documented in *Streptomyces aureofaciens* [69]. The structural similarity of CSPs, ribosomal protein S1 [27] and initiation factor IF1 [28] leads to the hypothesis that CSPs are also involved in translation initiation. It was shown that $CspA_{EC}$ enhances its own mRNA translation in vitro [70]. Recently, it was demonstrated that the expression of IF1 from *E. coli* in *B. subtilis* suppresses the

growth-defective phenotype of the *cspB*$_{BS}$ and *cspC*$_{BS}$ double deletion. Based on this observation it was suggested that IF1 and CSPs have at least some overlapping cellular function(s) [68].

The major demonstrated property of all cold-shock proteins is binding of poly-pyrimidine tracts of ssRNA and ssDNA [38–40]. Thus, they can destabilize dsRNA, and this function may contribute to many molecular processes in the cell [38]. CSP's abilities to destabilize the secondary structure of RNA were implicated in transcription antitermination [57]. It was shown that CspA$_{EC}$, CspE$_{EC}$ and CspC$_{EC}$ have decreased transcription termination at several terminator in vitro. The overexpresion of CspA$_{EC}$ and CspE$_{EC}$ at 37 °C induced transcription of the metY-rpsO operon genes *nusA, infB, rbfA* and *pnp* located downstream from the multiple transcriptional terminator [57]. Products of these genes – NusA, IF2, RbfA and PNP – are known as cold-shock-inducible proteins [4]. It was suggested that the transcription antiterminator function of CspA$_{EC}$, CspE$_{EC}$ and CspC$_{EC}$ at ρ-independent terminators is carried out by preventing formation of the secondary structure in the nascent mRNA [57]. The interaction between CspE$_{EC}$ and nascent RNA molecules was also observed [71]. On the other hand, CspE$_{EC}$ is capable of inhibiting Q-mediated transcriptional antitermination from $P_{R'}$ promoter of λ phage in vitro [71].

It was also observed that CSPs can act as transcriptional activators. Binding of CspA$_{EC}$ to the promoter regions results in an increase in transcription of two cold-inducible proteins, GyrA (subunit of DNA gyrase) [72] and H-NS (nucleoid-associated protein, which is involved in environmental regulation of genes expression) [41, 42]. CspB$_{BS}$ expressed in *E. coli* also increased the transcription of H-NS [73]. Furthermore, induction of H-NS was found to be essential for cold-shock adaptation [74, 75]. Experimental data accumulated for CSPs implicates them in a number of cellular processes; however, it is not clear which of these reported functions are related to cold-shock adaptation.

Mechanism of the temperature downshift-dependent induction

CSPs were originally identified as proteins induced upon a temperature downshift. The cold-shock response is not induced by a temperature decrease to a certain temperature but by any downshift of 10 °C or more. The greater the magnitude of the temperature shift and the lower new absolute temperature, the higher the induction [76].

The mechanisms of induction and control of CspA$_{EC}$ expression were studied extensively among other CSPs. CspA$_{EC}$ was identified as a major protein induced in *E. coli* during the acclimation phase of the cold shock [7]. However, it was recently demonstrated that a significant level (50 µM concentration) of CspA$_{EC}$ is present in the cell during the early exponential growth under nonstress conditions. The CspA$_{EC}$ level declines and becomes almost undetectable in the late phase of exponential growth. The expression of CspA$_{EC}$ was increased approximately 30-fold during the late phase of exponential growth after cold shock, but the total amount of CspA$_{EC}$ (100 µM concentration) was not more than 2.5-fold above its maximum level detected during early exponential growth at 37 °C [8]. CspA$_{EC}$ expression is triggered not only by a temperature downshift; it was found that CspA$_{EC}$ can also be induced by culture dilution at 37 °C [8] and by addition of nutrients [77]. Antibiotics blocking ribosomal translation at the stage of aminoacyl-tRNA binding (tetracycline), peptidyl transferase reaction (chloramphenicol, erythromycin, spiramycin) and translocation (fusidic acid) during ribosomal elongation were shown to be able to induce the cold-shock response [78, 79]. Furthermore CspA$_{EC}$, CspB$_{EC}$ and CspG$_{EC}$ are induced at a low temperature at concentrations of kanamycin and chloramphenicol that completely block protein synthesis [80]. Although factors, such as temperature downshift and addition of antibiotics and nutrients, which induce CSPs expression were identified, the general mechanism triggering an increase in CSPs level is not clear.

CspA$_{EC}$ expression is regulated at a transcriptional level during cold shock [81, 82]. The expression of β-galactosidase under the control of the CspA$_{EC}$ promoter was increased 3- to 5-fold upon an incremental temperature decrease from 37 to 15 °C. Maximum induction was observed at 20 °C [83]. It was shown that transcription from the CspA$_{EC}$ promoter is stimulated by Fis protein and inhibited by H-NS, the transcription of which is positively regulated by CspA$_{EC}$ [8]. A direct negative regulation of CspA$_{EC}$ transcription by CspA$_{EC}$ was also suggested [8, 84]. Thus, CspA negatively regulates its own expression at a transcriptional level through the negative feedback loop [8, 84]. CspE also inhibits transcription of CspA [85]. However, transcriptional activation of CspA under cold-shock conditions was found to be quantitatively modest [82].

The effects of a temperature downshift on posttranscriptional regulation of CspA expression are more dramatic. The half-life of CspA mRNA is very low at 37 °C and significantly increases under cold-shock conditions [70, 86]. Similar stabilizations were observed for the mRNA of CspB$_{BS}$ and CspC$_{BS}$, polynucleotide phosphorylase of *E. coli*, CrhC DEAD-box RNA helicase from cyanobacterium *Anabaena* sp., all known as cold-shock inducible proteins [87–89].

Although an increase of half-life of mRNA under cold-shock conditions is dramatic, the mechanism of decay regulation remains unclear. The level of mRNA is higher in the presence of an extra copy of the csdA gene [8]. The product of this *E. coli* gene is a cold-shock inducible

DEAD-box RNA helicase CsdA [90]. Induction of DEAD-box helicases under cold-shock conditions was also found in cyanobacterium *Anabaena sp.* [91] and archea *Methanococcoides burtonii* [92]. The stabilization of cellular mRNAs by DEAD-box helicases has been observed before [93]. It was proposed that CsdA helicase activity interferes with the formation of extended stem-loop structures, predicted for the 5′-untranslated region (UTR) of $CspA_{EC}$, which could be the target of nucleases [8]. Indeed, inactivation of RNaseE in vivo by temperature-sensitive mutations lead to an increase of $CspA_{EC}$ mRNA half-life [94]. However, RNaseE appears to cleave single stranded RNA much more efficiently than dsRNA [95]. The other key endonuclease of mRNA decay in bacteria, RNaseIII, cleaves dsRNA [95] and might be responsible for rapid degradation of CSPs mRNA. Thus CsdA, induced under cold-shock conditions, could unwind the secondary structure of CSPs mRNA, protecting it from cleavage by RNaseIII, and this could be the triggering factor that increases levels of CspA.

Unusually long (159 bs in the case of $CspA_{EC}$) 5′ UTRs are found in CSPs and CsdA mRNA and are thought to be crucial in regulating $CspA_{EC}$ expression [4]. However, a detailed role of the 5′ UTR remains controversial. On the one hand, it was shown that the deletion of the $CspA_{EC}$ 5′ UTR leads to constitutive expression of $CspA_{EC}$ at 37 °C [96], indicating that 5′ UTR negatively regulates synthesis of $CspA_{EC}$ at 37 °C. This deletion, however, did not have an effect on half-life of mRNA [96]. It was also shown that overexpression of the 5′ UTR of $CspA_{EC}$ results in continuation of $CspA_{EC}$ transient induction under cold-shock conditions, probably due to titration of a hypothetic repressor of $CspA_{EC}$ expression [97, 98]. However, another group was not able to reproduce this effect [92]. On the other hand, the presence of the 5′ UTR increases the half-life of $CspA_{EC}$ mRNA at a low temperature [99]. The sequence at the 5′-end of 5′ UTR, the so called ‘cold box’, is highly conserved in CSPs and bacterial DEAD-helicases [92, 97]. Two opposing observations have been reported: (i) deletion of the ‘cold box’ has a small effect on $CspA_{EC}$ induction under cold-shock conditions [100] and (ii) deletion of the cold box leads to significant decrease in half-life of $CspA_{EC}$ mRNA [101]. Therefore, the involvement of CSP’s 5′ UTR in cold-shock induction is not yet clear.

The explanation for the positive effect on CSP induction of 5′ UTR under cold conditions was proposed for $CspB_{BC}$ [47]. It was shown that $CspB_{BC}$ preferentially binds polyT as well as polyU stretches in ssDNA [40]. PolyU-rich regions were found in the 5′ UTR of Csp_{BC}. It was suggested that $CspB_{BC}$ specifically binds these U-rich regions and destabilizes the secondary structure of the 5′ UTR [47]. The secondary structure of the 5′ UTR can limit accessibility of the Shine-Dalgarno (SD) sequence, required for initiation of translation, for ribosomes. This secondary structure can also be a target for RNaseIII. Therefore, destabilization of the 5′ UTR Csp_{BC} mRNA secondary structure by $CspB_{BC}$ might increase the efficiency of translation and inhibit mRNA decay. In a similar way, $CspB_{BC}$ might induce other CSPs under cold-shock conditions.

It was suggested that $CspA_{EC}$ mRNA not only becomes more stable under cold-shock conditions but also is more efficiently translated by the ribosomes. Indeed, an S30 extract, as well as 70S ribosomes prepared from cold-shocked cells, translates $CspA_{EC}$ mRNA (but not phage MS2 mRNA) two to three times more efficiently at 37 °C than an extract and ribosomes obtained from cells grown at 37 °C [70]. Since ribosomes generally appear to protect mRNA against degradation [95], the more efficient translation of CSP mRNA may contribute to the observed increase of CSP mRNA half-life under cold conditions.

It was proposed that translational enhancement during cold-shock induction of CSPs requires a sequence, called the downstream box (DB), located 12 bp downstream from the initiation codon [99]. The DB can potentially base-pair with nucleotides 1469–1483 of 16S RNA in addition to the SD – anti-SD mRNA sequence interaction [99, 102, 103]. Similar sequences were also found in several phage and bacterial mRNAs [104]. However, the existence of a DB-anti-DB interaction and the role of DB in translation enhancement is still under debate [105–108]. Based on chemical foot-printing of ribosomes of *E. coli* [109] and the crystal structure of the 30S subunit of *T. thermophilus* ribosome [110], the anti-DB sequence is positioned within a stable helix region (helix 44) of 16S RNA and is not available for base pairing with the template. The strain of *E. coli* with ribosomes carrying an inversion of the anti-DB sequence of 16S RNA was capable of cold acclimation, and ribosomes translated $CspA_{EC}$ mRNA with the same efficiency as the wild-type ribosomes [109]. Another sequence in the 5′ UTR of $CpsA_{EC}$, $CspB_{EC}$, $CspG_{EC}$ and $CspI_{EC}$, 11 bases upstream of the SD and named the ‘upstream box’, is complementary to the region 1035–1023 of 16S RNA of *E. coli.* Deletion of bases 118–143 of 5′ UTR decreases the level of $CspA_{EC}$ expression under cold-shock conditions [100]. Based on these observations, it was suggested that the ‘upstream box’ might be a translational enhancer [100], but there is no direct evidence for this.

Another interesting effect related to cold-shock induction was observed recently. Nonsense mutations in the cspA gene cause growth inhibition and cell death of *E. coli* at low temperatures [111, 112]. This phenomenon was called the low temperature-dependent antibiotics effect (LACE) of truncated cspA expression. LACE can be observed even when the nonsense mutation is made in the second codon of the $CspA_{EC}$ ORF. The 5′ UTR is suggested to be

such a strong enhancer of translation that all ribosomes become trapped by the truncated $CspA_{EC}$ mRNA and are no longer able to synthesize cellular proteins. Indeed, loss of SD or the initiation codon in truncated $CspA_{EC}$ rescued cells from LACE [112]. However, termination of the translation at an introduced stop-codon should release ribosomes, and it is not clear why they remain trapped by $CspA_{EC}$ mRNA. Thus, the mechanism of LACE is still unclear.

Recently, new mechanisms of temperature-dependent regulation of gene expression by small RNAs was uncovered [113, 114]. It was shown that at low temperature 87-nucleotide-long DsrA RNA accumulates and regulates translation of two global transcriptional regulators, H-NS and RpoS [113]. DsrA inhibits translation of H-NS and decreases H-NS mRNA half-life and stimulates translation of RpoS [113, 114]. It is possible that small RNAs are involved in regulation of other cold-inducible proteins such as CSPs.

Figure 3 presents a possible scheme for the regulation of $CspA_{EC}$ expression. CSPs (at least the best-studied $CspA_{EC}$ in *E. coli*) are expressed during the exponential phase of growth and then suppressed in the stationary phase at the transcriptional level by the H-NS protein. At 37 °C $CspA_{EC}$ negatively regulates its own expression by stimulating synthesis of H-NS. The main mechanism of CSPs induction under cold-shock conditions is stabilization of their own mRNA. The half-life of CSP mRNA is low at 37 °C but greatly increases at low temperature. A key factor here could be the CsdA helicase that was found to decrease the rate of $CspA_{EC}$ mRNA degradation [8]. CSPs themselves may stimulate their own translation and stability of mRNA by melting the 5′ UTR secondary structure. Induction of CSPs at the level of transcription is modest. Details of the CSPs induction mechanism remain to be uncovered.

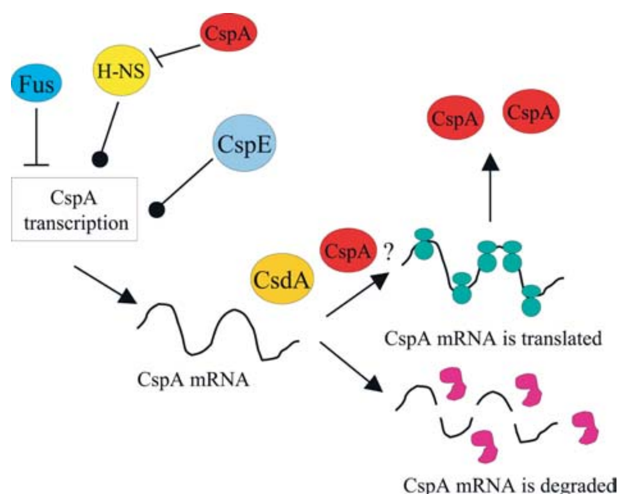

Figure 3. Schematic representation of CspA expression regulation in *E. coli*. See details in text.

Mechanism of cold-shock adaptation in the bacterial cell

Why does a decrease in temperature pose a significant problem for the cell and induce cold-shock response? A number of hypotheses are discussed in the literature, for example the membrane fluidity is low; ribosomal translation is inhibited; superhelical density of the DNA is too high for opening of the double helix; enzyme activities decrease to a different extent, so that protein levels must be adjusted; protein folding is too slow, especially formation of disufide bridges [2, 3]. Obviously, a shift in temperature affects the rates of all of the biochemical reactions in the cell and can influence many other cellular processes. However, the two best-documented effects of cold shock on cellular processes are a decrease in membrane fluidity and inhibition of ribosomal translation.

It was shown that *B. subtilis* responds to decrease in temperature by introducing double bonds into preexisting fatty acid tails of phospholipids within the cellular membrane. The reaction is catalyzed by the desaturase enzyme [115, 116]. Induction of Δ5-desaturase (product of *des* genes) in *B. subtilis* under cold-shock conditions has been reported [116–118], and the signal transduction pathway of *des* cold induction has been recently identified [119]. The trans-membrane protein DesK is suggested to respond to the physical state of the membrane bilayer, and on decrease in temperature it phosphorylates the transcriptional activator DesR. The phosphorylated form of DesR binds to the promoter of the *des* gene and activates transcription of the Δ5-desaturase [119]. This is perhaps the only example when the mechanism of the cold-shock induction of the entire pathway is well established and a functional connection between induced protein and cold adaptation is demonstrated. Interestingly, the cold-induced expression of Δ9-desaturase that also restores membrane fluidity was shown in carp upon a decrease in temperature from 30 to 10 °C [120].

The second best-studied effect of decrease in temperature on cellular physiology is inhibition of ribosomal translation. During incubation at low temperature the polysomal fraction of the ribosome disappears, and monosomes and individual small and large ribosomal subunits accumulate [121–124]. It appears that at low temperature initiation of translation is completely blocked [122, 125] and ribosomal translocation during elongation is inhibited [126, 127], but elongation of translation still occurs [125]. What is the molecular basis of the translation inhibition under cold-shock conditions, and what is the role of CSPs in overcoming the translation block during cold-shock adaptation? Two possible explanations can be proposed. One, inhibition is passive and is a direct consequence of the temperature decrease. Two, inhibition of translation is a cell-regulated process carried out in response to temperature stress.

Several lines of evidence support the direct inhibition of translation by temperature. It is well known that the translational apparatus is very sensitive to the strength of RNA-RNA interactions (modulated, for example, by the changes in concentration of Mg^{2+} ions). The effect of changes in concentration of Mg^{2+} ions on different stages of translation is well documented (for review see [128]). One can expect that the decrease in temperature should enhance RNA-RNA interactions as it does upon the increase of magnesium ions concentration. Thus, the effects of a temperature downshift on translation should be similar to all well-known effects of an increase in magnesium ion concentration. Following these arguments, a temperature downshift should increase the level of miscoding and inhibit translocation. All these have been demonstrated experimentally [126, 127, 129, 130]. Dissociation of ribosomal subunits after translation termination, which is important for the initiation of the next round of protein synthesis, might be also blocked by the low temperature. Nevertheless, it is not clear how the induction of CSPs can help cells to overcome these effects of a temperature downshift.

Another direct consequence of a temperature downshift can be stabilization of the secondary structure of mRNA, particularly the 5′ UTR, which can make the SD sequence unavailable for ribosomes and prevent synthesis of most polypeptides in the cell [2, 5, 47]. Members of the $CspA_{EC}$ family exhibit properties of mRNA chaperones [38]. It was proposed that the induction of CSPs helps to unfold elements of the secondary structure of mRNA and restore translation initiation efficiency [2]. There is evidence (see above) that $CspA_{EC}$ can indeed facilitate translation, although, this has only been demonstrated in vitro for translation of CspA itself [70]. Another fact supporting the hypothesis that on decrease in temperature the secondary structure formation of mRNA becomes a problem for the cell is that dsRNA helicase CsdA was found to be cold inducible and bound to the ribosome under cold-shock conditions [90]. However, the other possible role of CsdA is CSP induction through a decreased rate of degradation of CSP mRNA (see above).

If a temperature downshift inhibits translation by stabilizing of mRNA secondary structure, then a gradual decrease in temperature should inhibit the expression of different proteins to various degrees due to differences in both length and propensities of 5′UTR to form secondary structures. Some of the proteins would be expressed, while others would not. Yet, synthesis of most of the cellular proteins is inhibited to a similar extent. The cold-shock response seems to be an 'all or nothing' rather than gradual [76]. Thus it appears that stabilization of mRNA secondary structure under cold-shock conditions is an unlikely explanation for inhibition of ribosomal translation.

In contrast to the hypotheses discussed above, an alternative could be that under cold-shock conditions, ribosomes are inhibited by cellular factors in response to the temperature downshift, which can help to find a functional relationship between CSP induction and inhibition of ribosomal translation. It has been shown that ribosomes isolated from cells incubated at low temperature for several hours synthesize polypeptides at 0 °C with lower efficiency than ribosomes isolated from cells incubated at 37 °C [121]. Translation efficiency of ribosomes from 'cold' cells can quickly be restored at 37 °C [121]. This suggests that indeed ribosomes are inhibited under cold-shock conditions by some cellular factors.

Why might ribosomal translation be inhibited under cold-shock conditions by cellular factors? A temperature downshift leads to an increase in the level of miscoding [129, 130]. Suppression of translation termination at a UAG codon at low temperatures was also demonstrated [131]. Accumulation of translation errors might be the factor triggering the cold-shock response and CSP induction. Antibiotics that affect ribosomal translation were found to induce cold-shock proteins [78], supporting the hypothesis that CSPs appear to be induced in response to low fidelity of ribosomal translation in general. A decrease in translation fidelity upon amino acid starvation leads to synthesis of ppGpp, inhibiting translation [132]. It has been suggested that synthesis of ppGpp could trigger CSP induction [78], although experimental data do not support this hypothesis [76].

One might therefore expect to find two groups of factors: one that blocks translation and another that adapts the translational apparatus to cold-shock conditions. Indeed, several proteins were found to be associated with the ribosome only at low temperature. CsdA RNA-helicase is associated with ribosomes under cold-shock conditions [90]. Cold-inducible protein RbfA binds the small ribosomal subunit and suppresses the cold-sensitive mutation of 16S rRNA [124, 133]. Recently, a new protein named Y was found to be associated with ribosomes under cold-shock conditions [134]. Protein Y inhibits translation elongation by preventing binding of aminoacyl-tRNA to the A-site of the ribosome [134].

CSPs are possible candidates for regulation of translation initiation at low temperature. Despite significant differences in the mechanism of translation initiation in prokaryotes and eukaryotes, proteins carrying cold-shock domains and having nucleic acid binding properties similar to CSPs (protein S1 of *E. coli* ribosome and Y-box protein p50) regulate translation initiation in a concentration-dependent manner, interacting with mRNA. Both S1 and p50 bind mRNA and stimulate translation initiation at low concentrations (S1 being associated with the ribosome in a one-to-one ratio) but inhibit it at high concentrations [135–137]. The Y-box protein YB-1 also inhibits translation at the stage of initiation [49]. It appears that p50 destabilizes elements of the secondary structure in mRNA

at low concentrations, but at higher concentration p50 makes mRNA inaccessible for the ribosomes and translational factors [138]. The regulation of translation initiation during cold shock could occur by a similar concentration-dependent mechanism when the high level of CSPs inhibits translation. Since it was shown that CspA does not influence MS2 mRNA translation but slightly stimulates translation of its own mRNA at 37 °C [70], effects of CSPs on translation of different mRNAs at lower temperature in a broad range of CSP concentrations need to be reexamined. Another possible function of CSPs that might be proposed is to store mRNAs in an RNA-protein complex while the cell adapts its translational apparatus to the cold-shock conditions.

Conclusions and future prospects

Even without favoring a particular hypothesis about CSP functions and mechanisms of cold-shock adaptation, it is still possible to make several conclusions. The most common documented property of CSPs is to bind a broad range of sequences (preferentially poly-pyrimidine regions) of ssRNA/DNA with micromolar affinity but not dsRNA/DNA. Therefore, CSPs might destabilize RNA secondary structure and play an RNA chaperone role. Several lines of evidence suggest that CSPs bind mRNA and regulate ribosomal translation, mRNA decay and termination of transcription. These functions appear to be important not only during cold shock but under normal growth conditions as well.

Several questions remain open and need to be clarified. Some information about the regulation of CSP expression is available, but it is not clear what thermo-sensitive factor is triggering the cold-shock response. It appears that initiation of translation is blocked under cold-shock conditions, but it is not known whether this is a direct consequence of the temperature decrease or a cell-regulated response to the cold shock. There are a number of indications that CSPs bind mRNA and are involved in regulation of translation, but effects of CSPs on translation of different mRNAs at different temperatures using in vitro translation systems have not been studied in detail. Answers to these questions will significantly contribute to our understanding of CSP biology.

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