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Review

Structure and function of bacterial cold shock proteins

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Abstract. Cold shock proteins (Csps) comprise a family of small proteins that are structurally highly conserved and bind to single-stranded nucleic acids via their nucleic acid binding motifs RNP1 and RNP2. Bacterial Csps are mainly induced after a rapid temperature downshift to regulate the adaptation to cold stress, but are also present under normal conditions to regulate other biological functions. The structural unit characteristic for Csps occurs also as a cold shock domain (CSD) in other proteins and can be found in wide variety of organisms from bacteria to vertebrates. Important examples are the Y-box proteins that are known to be involved in regulation of several transcription and translation processes. This review describes the role of Csps in protein expression during cold shock with special emphasis on structural aspects of Csps.

Keywords. Cold shock protein, cold shock domain, RNA chaperone, nucleic acid binding, cold adaptation, transcription.

Introduction

All organisms must possess the ability to adapt to environmental changes that include changes of the availability of different nutrients and of general physical parameters such as osmotic pressure and temperature. In higher organisms, there are many different strategies to minimize the effect of the changes on cellular level by global and local regulatory mechanisms, *e.g.*, temperature homeostasis in mammals. For single-cell organisms only direct cellular responses to external challenges are possible. When the external changes are outside the physiological range favored by the organism, specific stress responses can be observed [1].

With regard to the cold shock, several authors defined *ad hoc* practical conditions, *e.g.*, for mesophilic bacteria a cold shock was defined as a temperature downshift from 310 K (37°C) to 288 K (15°C) [2] in a few minutes, that is from an optimal growth temperature to a temperature T_f close to the lowest limit for growth. However, one can expect that the majority of the cellular processes observed after fast, large temperature shifts are also occurring at the acclimation to naturally occurring small, slow temperature downshifts (known as adaptation phase) as long as the temperature T_f is inside the physiological range. Although the speed of the change does not influence the final response in the new equilibrium state, nonlinear response theory most probably applies to this problem and requires that the initial response (that is mainly the time-dependent induced change of protein concentrations and activities) are strongly dependent on the speed and the size of the perturbation. This feature probably also explains some of the discrepancies in the literature concerning the quantitative changes in protein expression.

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The adjustments of cell metabolism and structural elements of the cell, such as membrane fluidity, are closely connected to the adjustment of the whole protein expression machinery to the new requirements. In general, upon a cold shock, the following scenario can be observed (Fig. 1): The downshift of temperature is sensed by the cells and signaled to the responsible elements, mainly to the transcriptional and translational apparatus and possibly to structural elements. Recent studies report a specific thermosensing system responsible for the adaptation of the membrane fluidity in *Bacillus subtilis* [3–5].

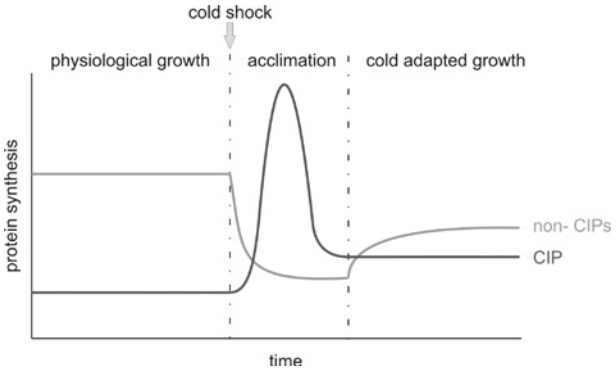

Figure 1. Schematic view of the pattern of protein expression after cold shock. The acclimation phase directly after a cold shock is characterized by a transient inhibition of non-cold shock proteins, a boost expression of cold-induced proteins (CIPs) including the cold shock proteins (Csps), and a decay of the corresponding mRNA. After that, the CIP expression declines and the bulk protein synthesis is restored with a new pattern adapted to the cold.

Additionally, a specific set of genes is induced immediately after the temperature shift and, nearly simultaneously, the protein production machinery of "bulk proteins" or non-cold shock proteins in the cell becomes transiently inhibited. *Escherichia coli* cells stop growing for about 4 h [6]. This phenomenon, also called 'translational bias', is not fully understood yet. The initial phase of the cold shock response, the acclimation phase, is characterized by high levels of these newly synthesized cold-induced proteins (CIPs) that are sometimes called cold shock proteins (Csps). However, the term cold shock protein is generally used for a class of small closely related proteins with high homology in sequence and structure. In this review we will reserve the expression Csps for these proteins. Not all members of this structural class are necessarily involved in the cold shock response, but have also more general functions in the cellular regulation. At the end of the acclimation phase, the synthesis of Csps decreases to a constant level and the cells restore their protein production machinery (Fig. 1). The analyses of genome-wide transcription patterns in cold-shocked *B. subtilis* cells identified a fundamental set of gene products involved in the metabolic processes to adapt to cold [7–9].

The full description of cold shock response and cold adaptation inside the cell is a topic of systems biology that is not yet solved. Here we give mainly only an overview of the role of those cold shock domain-containing proteins traditionally termed as Csps. As a number of detailed reviews on different aspects of the role of the Csps already exist [10–13], our intention is to focus on the structural aspects of Csps.

Cold shock response in bacteria

Cold shock-induced proteins in bacteria

After a rapid shift of temperature from 310 K (37°C) to 283 K (10°C) up to 26 different genes [14] are transiently induced in *E. coli.* A selection of important proteins with their presumed function has been summarized in a review by Thieringer et al. [11]. Most of the proteins are directly or indirectly involved in protein transcription or translation such as the classical Csps, the RNA helicase DeaD, the DNA gyrase GyrA, the transcription factor Nus A and translation factor Inf B. During the initial phase the expression of most other genes is suppressed followed by new steady-state level expression adapted to the modified growth conditions (Fig. 1).

Bacterial Csps

The Csps form a highly conserved family of structurally related nucleic acid-binding proteins. These small proteins with a molecular mass of approximately 7.4 kDa [15, 16] consist of the typical cold shock domain (CSD). They all bind single-stranded RNA and DNA (ssRNA/ssDNA) but no double-stranded DNA (dsDNA) [17–23]. Five antiparallel β-strands form a β-barrel adopting an OB (oligonucleotide and/or oligosaccharide binding) fold (see *Structural aspects of Csps*, below). The protein-nucleic acid interaction is performed by the moderately well-conserved canonical 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) motifs [13, 24–27] that are present with small variations in all Csps. In the Csps of *Escherichia coli*, *Bacillus subtilis*, *Thermotoga maritima*, *Salmonella typhimurium*, *Streptococcus pyogenes*, *Mycobacterium tuberculosis*, *Micrococcus luteus*, *Pseudomonas aeruginosa*, *Listeria monocytogenes*, *Yersinia pestis* and *Haemophilus influenzae* the canonical sequences are modified to (K/S-G-F/K/Y-G-F/L-I-X-X) and (L/I/V-F/Q-V/A/L-H-X-S/T/R). In the three-dimensional structures of Csps, the basic and

aromatic amino acid residues from these motifs form a nucleic acid-binding surface [27] that can recognize different nucleotide sequences (see *Structural aspects of Csps*). The best-studied Csps are from the mesophilic bacterium *E. coli.* Nine members of Csp family proteins have been identified so far, named in alphabetical order CspA to CspI. Two of them, CspE and CspC are constitutively expressed at physiological temperatures [28], CspD is induced under nutrient stress [29] and CspA, CspB, CspG and CspI are highly cold inducible [30–32]. Typically, the steady-state induction level of the latter proteins is increased two- to tenfold for *E. coli* cold shock genes after a cold shock [33].

CspA, originally identified as a 7.4-kDa cytoplasmic protein, CS7.4, is the most prominent Csp in *E. coli.* CspA is synthesized up to a level of 13 % of the total protein content after cells are subjected to a fast temperature shift from 310 K to 288 K [6, 34]. After a cold stimulus, its concentration reaches a level of about 100 μM [33]. Generally, many (almost all) cold inducible genes are also expressed under non-cold shock conditions, an observation that has been confirmed by a number of groups, indicating that most cold-induced proteins have additional functions in general cellular regulation and metabolism. This is also true for CspA, which is present under non-stress conditions in *E. coli*; during the early exponential growth phase its concentration is approximately 50 μM. CspA genes are also highly transcribed at 310 K, but their mRNA cannot be efficiently translated because of their instability and reduced lifetime at higher temperatures. However, under cold conditions the CspA mRNA achieves extreme stability, which favors its translation. In addition, CspA can regulate its own gene expression by binding to the 5′ untranslated region (UTR, 159 bp) of its own mRNA and facilitates the translation of Csps at low temperatures in this way [35, 36]. This sequence is unusually long compared with mRNAs from non-Csps and is considered to be responsible for the differential regulation of the expression pattern in the cold.

Since CspA is also constitutively expressed and abundant at all temperatures it should be considered as one of many factors regulating the cold stress response. The commonly used designation as the "major Csp" in *E. coli* therefore appears to be somewhat misleading.

According to Gualerzi et al. [14], a constitutive expression (although at lower levels) seems to exist also for the other Csps. This view is also confirmed by multiple deletion experiments of Csps in *E. coli.* CspE, which shares 69 % identity and is transiently induced at physiological temperatures, is able to rescue the cold adaptive phenotype cells with a triple deletion of inducible Csps (*ΔcspA ΔcspB ΔcspG*) [37, 38].

Jiang et al. [39] reported that in *E. coli* an inhibition of the general protein biosynthesis by chloramphenicol induces the transcription of CspA. Later on, Etchegaray and Inouye [40] examined the influence of chloramphenicol and kanamycin on the expression of Csps after cold stress using pulse-labeling experiments. Despite the presence of the antibiotics in concentrations sufficient to completely block the protein biosynthesis, Csps were expressed in reasonable amounts. They considered as a possible explanation that Csp mRNA might be too small for antibiotic inhibition. However, recently published *in vitro* translation data could not verify that effect since under inhibition with chloramphenicol or kanamycin the translation of cold shock mRNA and non-cold shock mRNA showed no significant differences and was equally inhibited [41].

Besides CspA, which influences transcriptional processes, the homologues CspE and CspC are also involved in transcription anti-termination since purified CspE inhibits the Q-mediated transcription antitermination [42, 43]. CspE binds to the polyA tail at the 3' end of mRNAs, and interferes with the degradation of both polynucleotide phoshorylase (PNPase) and RNase E at 310 K [44]. It has been suggested that the mechanism of transcription antitermination is based upon preventing the formation of secondary structures on the nascent mRNA [43, 45, 46]. More detailed studies about the Csp-induced melting process of nucleic acids with hydroxyl radical footprinting revealed that mechanistic details of the unwinding of double-stranded nucleic acids by Csp are dependent on its concentration [38]. Starting at low Csp concentration, a binding of Csp occurs only at the stem loop junction. When the concentration of Csp increases, the binding sites on the single-stranded loop regions become more and more occupied by Csps, completing the melting process. *In vitro* studies showed that the concentration necessary for the melting of a stem loop increases when the length of the unpaired region decreases [45]. It has been suggested that this effect is due to the limited length of the stretch of nucleotides (typically six bases), that is covered when a Csps binds. In line with this suggestion are results from mutational studies analyzing the mechanism of nucleic acid melting by Csps. Using double-stranded model substrates it was shown that CspE induces melting of the stem region [38, 47].

Csps are found not only in the mesophilic *E. coli* but in most other eubacteria, *e.g.*, in the psychrophilic *L. monocytogenes* [48], mesophilic *B. subtilis*, the thermophilic *B. caldolyticus* and the hyperthermophilic *T. maritima.* A genomic analysis has not yet revealed

the existence of Csps in archebacteria [49]. Three CspA-like proteins have been found *in* B. subtilis [50], named CspB, CspC, and CspD. CspB and CspC are induced upon cold shock response. The existence of at least one of them is essential for cell viability [51] and two of them (CspB and CspC) are also induced at stationary phase [52, 53]. CspB in *B. subtilis* plays the most important role under cold stress and has a similar function as CspA in *E. coli.* CspB deletion results in a cold-sensitive phenotype [54] and its function can be complemented by CspD at 310 K (37°C) and by CspC at lower temperatures [51, 55].

To evaluate the role of cold shock-induced proteins during cold shock response, recent results with microarray technologies should be considered. Microarrays allow a more complete gene expression profile than the traditionally used two-dimensional gel electrophoresis. Many genes that have been identified in cold-adapted cells of *B. subtilis* belong to the so-called "SigB-controlled general stress regulon", which is known to be induced in response of several stress stimuli including heat shock [56, 57]. Other genes repressed during the cold adaptation phase play a crucial role in metabolic pathways necessary for growth and surviving [7–9, 58].

One interesting cold adaptation that is clearly required from general biophysical considerations is the adaptation of the membrane fluidity to lower temperatures. At lower temperature, the membrane undergoes a phase transition from fluid to non-fluid that depends on the membrane fatty acid composition. High membrane fluidity is required for the unperturbed function of almost all membrane proteins and for the stability of the membrane itself. The melting points of the fatty acids of the membrane lipids strongly influence the membrane fluidity. The membrane fluidity can be preserved at lower temperatures when fatty acids with lower melting points are introduced. For *B. subtilis*, a mechanism of temperature sensing as a function of membrane adaptation to the cold has recently been found [4]. A two-component system for perception of temperature downshift and regulation of physical membrane condition was identified by Aguilar et al. [3], consisting of a sensor kinase DesK and a response regulator DesR [5]. *B. subtilis* contains a membrane-bound phospholipid desaturase Des that modifies already existing fatty acids to unsaturated fatty acids in the membrane. In addition, the membrane composition can be changed by *de novo* synthesis of branched chain fatty acids and unsaturated fatty acids. Thus, the membrane undergoes changes as adaptation to a period of low temperature environments.

The role of bacterial Csps in transcription

The first hint that Csps are able to regulate the cold shock response on transcriptional level was given by Dersch et al. [59] and Atlung et al. [60]. They demonstrated that in enterobacteria [61, 62] the small chromatin-associated cold-induced protein H-NS (a nucleoid-associated protein that contains a CSD domain) binds preferentially to dsDNA and is associated with cold shock regulation. The role of Csps on the transcriptional regulation was supported by the homology of Csps to Y-box proteins [63–66], which are known to play a role in transcription [67]. The H-NS:DNA ratio determined during cold shock increases three- to fourfold compared to the ratio at physiological conditions [68]. The boost of its expression is mediated by CspA that recognizes the 110-bp promoter region of the *h-ns* gene, and therefore acts as a transcriptional activator for H-NS expression. Interestingly, the conserved Y-box motif (CCAAT) necessary for Csp binding is not essential for this effect [69]. The transcriptional enhancement of the *h-ns* gene transcription from *E. coli* can also be initiated by heterologous expression of CspB from *B. subtilis* [51], an observation that once again demonstrates the close functional homology of these inducible Csps of different microorganisms.

Not only the h-ns transcription is sensible to activation by CspA but also the transcription of the *gyrA* gene (the A subunit of DNA gyrase) as the promoter region has specific binding sites [70]. It has been proposed that an increased level of gyrase is helpful for the adaptation of the cell to reduced temperatures.

In addition to the capability of Csps to bind to the promoter region of various genes and modify their transcription, Csps can function as transcriptional anti-terminators in *E. coli* (see above). The supposed mechanism is always the same: Csps as single-stranded nucleic acid-binding proteins favor the single-stranded state of RNA or DNA. Since the formation of stable secondary structures by base pairing is thought to inhibit transcriptional (and translational) processes Csps support these activities [46, 71]. This function of Csps is often termed RNA-chaperone function, using the common term for the protein folding aids. However, functionally, the chaperone effect for RNA differs from that observed in proteins since protein chaperones usually help to form the proper secondary and tertiary structure after destabilizing wrong secondary structures, whereas RNA chaperones only destroy unwanted secondary structures.

The effect of Csps on the transcription of non-cold shock-induced genes has not been fully elucidated in detail. First studies on *in vitro* transcription systems

consisting of a T7 promotor-based gene showed inhibitory effects in the presence of high levels of Csps [72]. This finding complements the working model describing the role of Csps in the transcription process. At the event of a cold shock, the constitutively present Csps bind to the promoter regions of those operons whose products are cold inducible and contain the correct binding site for Csps. When the level of Csp increases during the acclimation phase, one can assume that binding sites on the DNA with lower affinities are also occupied, so that promoter sites are masked and are inaccessible for the RNA polymerase (Fig. 2).

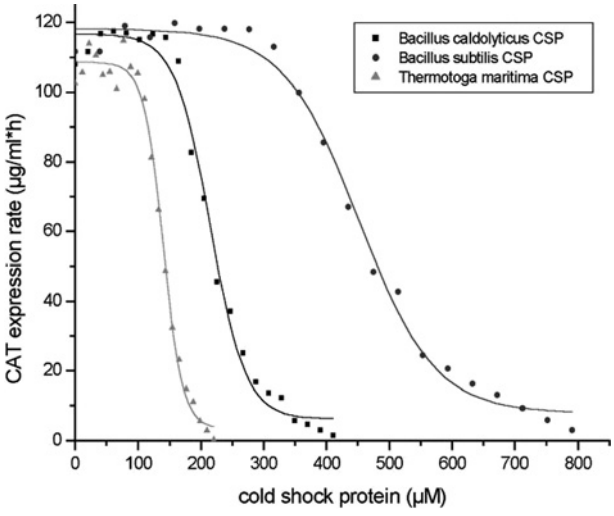

Figure 2. Influence of Csps from different organisms on coupled *in vitro* expression experiments. The synthesis rate of chloramphenicol-acetyltransferase in a coupled *in vitro* transcription-translation system from *E. coli* performed at 310 K is displayed as function of the concentration of Csp present in the assay: rectangles (hyperthermophilic *Tm*Csp), circles (thermophilic *Bc*Csp) or triangles (mesophilic *Bs*Csp) [72]. The corresponding apparent dissociation constants from RNA are listed in Table 1.

Post-transcriptional effects of bacterial Csps

Besides the limited effects on transcription itself, Csps have more extensive effects on the mRNA, by favoring states that are not base-paired [73]. The binding at the stem loop junction [45] enhances the RNA lifetime by increasing its stability against RNase degradation, since preferentially dsRNAs are recognized by the responsible RNases [13].

This activity of Csps is also important for the increased translation of their own mRNA at lower temperatures as studied extensively for the CspA gene from *E. coli.* As already discussed, the stability of CspA transcripts is reduced at 310 K but increases much at low temperatures [74–76]. In line with the general accepted hypothesis that the instability of the CspA mRNA at physiological temperatures is due to the presence of an extended secondary structure, the formation of the CspA mRNA leads to an increased decay via RNase III [6] that recognizes dsRNA.

CspA can also bind to the 159-nucleotide-long 5′ UTR of the *cspA* mRNA at low temperatures [35, 36], and might prevent the formation of secondary structures inducing the cleavage by RNases (reviewed in [13]). Csp is bound to the so-called "cold box" on the 5′ UTR, which is highly conserved in the genes of Csps and of the DEAD helicase CsdA [77, 78]. The interaction of CspA and its mRNA is due to the binding preference to polyU stretches of this cold box, as shown on CspB from *B. subtilis* [21]. During cold shock, the cold-inducible RNA helicase CsdA is proposed to unwind these secondary structures in cooperation with CspA, achieving a stabilization of the mRNA.

In principle, Csp could also bind to mRNA of other proteins and prevent secondary structure formation and thus increased enzymatic digestion of non-Csp mRNA at lower temperatures *in vivo.* However, this mechanism seems not to play a general role and is also difficult to separate from the increased translation by the chaperone-like function described in literature (see below).

The role of Csps in translation

Csps in bacterial cells are involved in the translational process in many different ways. One important aspect is the strong influence on the translation of their own mRNA. Unwinding double-stranded stretches of the mRNA not only reduces the cleavage by RNases and thus enhances the steady state mRNA concentration, but also facilitates the translation initiation where the formation of secondary structures is unfavorable [21] since these can mask the Shine-Dalgarno sequence.

Another mechanism to enhance its own translation has been found for CspB from *B. subtilis:* a downstream box on the mRNA is proposed to interact with rRNA [79–81] and thus can stabilize the binding of the ribosome to the Shine-Dalgarno sequence. However, besides the boost of its own expression (a primary regulatory event) important other cellular functions of Csps have to exist. The decrease of temperature influences the cell in almost all aspects since general biophysical, physicochemical and biochemical properties of its components are changed and, as such, the whole coupled system is strongly perturbed. According to Ermolenko et al. [13], the cell faces two major challenges after a decrease of temperature: On the one hand, the membrane fluidity is reduced, and on

Table 1. Apparent affinities of Csps to mRNA.[a]

Csp source	T_{opt} [K] [b]	K_D^{app} at 310 K [μM] [c]	K_D^{app} at T_{opt} [μM] [d]	K_D^{app} at T_{cs} [μM] [e]
T. maritima	353	0.59	3.39	1.46
B. caldolyticus	333	2.33	5.71	2.43
B. subtilis	308	4.51	4.16	1.60

[a] Apparent dissociation constants from mRNA K_D^{app} were determined in *in vitro* transcription-translation assays *(E. coli)* performed at 310 K [72]. The corresponding data are depicted in Fig. 2.
[b] T_{opt}, optimal growth temperature of the organism.
[c] Apparent K_D from the fit of the *in vitro* data according to ref [72]. The obtained apparent dissociation constants are largely independent of the other free-fit parameters as pointed out in ref [72].
[d] K_D^{app} was extrapolated using the van't Hoff relation neglecting the temperature dependence of ΔG.
[e] T_{cs}, cold shock temperature define as $T_{opt} - 22$ K.

the other hand the stability of nucleic acid base pairings increases and the protein translation is blocked. In particular, as the translation initiation and elongation are severely affected, a drop in temperature causes severe problems for the cell viability [82, 83]. As Csps have no described function in membrane metabolism, the most likely additional function of Csps could be the bulk protein translation. In agreement with this hypothesis, Weber et al. [84] show that the cold-sensitive phenotype of a Csp-double deletion mutant from *B. subtilis* can be complemented by the heterologous expression of the translation initiation factor 1 from *E. coli.* This observation suggests at least partly overlapping functions of these proteins.

However, with respect to cold shock, it is often forgotten that a temperature change also influences the protein system directly. All individual equilibrium and velocity constants are changed by temperature, thus the network of enzymatic activities and regulatory protein-protein interactions is strongly perturbed. Therefore, the main point of cold shock response is not the stabilization of the old equilibrium of the cellular components but the fast establishment of a new equilibrium with a new pattern of protein concentrations. If the drop in temperature was sufficiently large and fast (a real cold shock, not a slow cold adaptation), then a stop of the general protein expression would free the protein expression machinery (especially the ribosomes) for the creation of a new, temperature adapted protein pattern.

Indeed, such an effect is observed experimentally (Fig. 1). The observed dramatic decrease of the physiological protein expression during acclimation phase could be caused directly by the temperature (see [13]), leading to an increase in miscoding during elongation and in increased secondary structure formation on the mRNA [85]. However, cold-induced secondary structure formation is a very unlikely mechanism for the all-or-nothing inhibition of bulk protein expression, since a gradual decrease of temperature does not lead to a selective block of gene expression as would be expected due to the different properties of their 5′ UTRs [70]. Therefore, it is more likely that this is a direct transient regulatory effect of Csps on protein transcription and translation to help to establish the new protein expression pattern rapidly [72]. This translation stop could also avoid an increase in miscoding, resulting in an increased level of incorrectly translated proteins [86, 87]. The cell gets time to adapt to the new environmental conditions by altering the properties of the ribosomes [41] and by the action of special proteins like CsdA and the Y protein in *E. coli* [88]. Recent *in vitro* studies [72] on coupled and isolated cell-free translation revealed that, at relatively high (but physiological during the initial phase of the cold shock) concentrations of the major Csps from different organisms (*B. subtilis, B. caldolyticus and T. maritima*), the protein synthesis originating from an artificial non-cold shock mRNA substrate breaks down depending on the concentration of Csp present in the assay (Fig. 2). In addition to the property of Csps keeping mRNA unfolded by binding to it, one can propose that Csps at high concentrations are able to block the general protein synthesis by completely decorating the essential parts of the mRNA for translation initiation [72]. From these data the corresponding apparent dissociation constants K_D^{app} of Csp to mRNA at cold shock temperatures can be derived (Table 1). For the studied organisms, they are rather similar and vary between 1.5 and 2.4 μM. This effect is enhanced by the property of Csps to show a large increase of affinity to ssRNA and ssDNA at decreasing temperatures [20].

The unspecific inhibition of translation requires an accumulation of Csps to rather high concentrations. It has been demonstrated that mRNAs for cold shock or cold tolerant proteins are translated more efficiently by S-30 lysate preparations from cold-shocked cells. Distinct *cis*-acting elements on the mRNA are the driving force for the selectivity of the ribosomes [41]. As described above, these elements are localized on

the 5′ UTR of the cold shock genes [35]. These elements are recognized by different factors, one of them is CspA, which has therefore been termed a *trans*-acting factor for the boost in translation of cold shock genes [41]. By this mechanism CspA can enhance the translation from Csps and from cold tolerant proteins *in vitro*.

In summary, Csps can act on translation in different ways. At low to medium concentration levels the protein can act as translational enhancer upon cold stress. When the concentration reaches a high level due to a positive feedback mechanism the translation of nearly all proteins is arrested until the cell, especially the ribosomes are adapted to the new environmental conditions (Fig. 3).

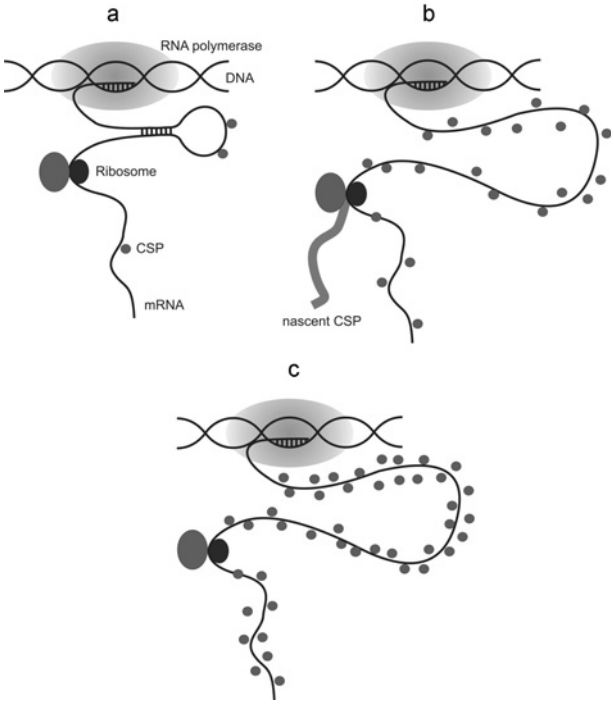

Figure 3. Schematic representation of the Csp function during acclimation phase. (*a*) Immediately after cold shock, the translation of bulk proteins is generally reduced due to increased base pairings of their mRNA (increased degradation and decreased translation initiation) and by the unspecific binding of Csp caused by an increase of the Csp affinity to RNA with decreasing temperature. (*b*) Intermediate stage in the acclimation phase: Csp level begins to increase due to the prolonged halflife of *csp* mRNA by Csps binding to its 5′ UTR and by direct interaction of its mRNA with the ribosome. Expression of bulk proteins is completely suppressed by the unspecific interaction with Csp. (*c*) Late acclimation phase: high levels of Csp suppress their own mRNA translation (and potentially also the enhanced Csp transcription). The level of Csp decreases until the translation of bulk proteins resumes with a pattern adapted to the new temperature.

Expression of other CIPs

To rescue misfolded mRNA molecules, another family of molecules can act as RNA chaperones, the DEAD box helicases [89] that unwind double-stranded nucleic acids. DEAD-box family helicases are involved in different aspects of RNA metabolism, like translation initiation, ribosome biogenesis or assembly and RNA degradation [90–94]. Some of them are shown to be cold induced [78]. CsdA (cold-shock DEAD-box protein) for example has been first isolated as a cold shock induced helicase in *E. coli* [77] and was found to be involved in ribosomal biogenesis of 50S subunits [94].

A direct interaction of the putative RNA helicases CshA and CshB with CspB from *B. subtilis* was recently detected with fluorescence energy transfer (FRET) techniques. These results suggest a more cooperative role of cold-induced proteins in "chaperoning" misfolded mRNA in cold shock response. Here, helicases could destabilize unpropitious secondary structures of mRNA and induce the formation of single-stranded regions that can then be occupied by Csps to prevent further refolding [95]. According to this model, a concerted action of these molecules could lead to an increased translation initiation after cold-shock-induced mRNA decay and a growth lag phase [96].

As already discussed, the *E. coli* helicase CsdA is involved in RNA degradation by the degradosome [97, 98], a multienzyme complex that plays a pivotal role in controlling the level of transcripts by processing and degrading mRNA in the cell as adaptation to changing growth conditions [99]. Recently, a direct interaction of cold-induced CsdA with RNaseE, PNPase, a 3'-exoribonuclease, and other components of the RNA degradosome has been detected under cold shock conditions forming the so-called "cold shock degradosome" [100]. Furthermore, CsdA is able to replace the resident RNA helicase of the RNA degradosome, RhlB. The PNPase has 3'-exoribonuclease activity and autoregulatively represses its own synthesis after RNase III cleavage in a PNP stem loop.

Structural aspects of Csps

The three-dimensional structures of a number of bacterial Csps have been solved, *i.e.*, the NMR-derived structures of Csp from the hyperthermophilic bacterium *T. maritima* (*Tm*Csp) at 303 K (30°C) [101], and at a physiologically more relevant temperature of 343 K (70°C) [102], the X-ray-derived structures of Csp from the thermophilic bacterium *B. caldolyticus* (*Bc*Csp) [103], the NMR- and X-ray-derived struc-

tures of CspB from the mesophilic bacterium *B. subtilis* (*Bs*CspB) [52, 104], and the NMR- and X-ray-derived structures of CspA from the mesophilic bacterium *E. coli* (*Ec*CspA) [105, 106]. All Csp structures show an OB (oligonucleotide/oligosaccharide-binding) fold, consisting of five antiparallel β-strands that form a Greek-key β-barrel. An example is the structure of *Tm*Csp shown in Fig. 4. *Ec*CspA was shown to exist as a monomer in solution [105] and in the crystal [106], whereas *Bs*CspB exists as a dimer in the crystal [104] and in solution as a monomer [52] depending upon the solvent conditions [107]. *Bc*Csp exists as a monomer in the crystal [103], and *Tm*Csp exists at all temperatures as a monomer in solution [101, 102].

Regarding their relative intrinsic stabilities against temperature denaturation, bacterial Csps were found to reflect the optimum growth temperatures of their mesophilic, thermophilic, or hyperthermophilic origin. The corresponding melting points T_m are: T_m (*Bs*CspB) approximately 325 K, T_m(*Ec*CspA) approximately 333 K, T_m(*Bc*Csp) approximately 345 K, and T_m(*Tm*Csp) approximately 360 K (87°C) [15, 107, 108]. The formation of specific ion clusters at the surface was suggested to be especially important for the thermostability of Csps. The structure determination of *Bc*Csp in the crystal [91] and *Tm*Csp in solution [101, 102] revealed the existence of such a cluster (Fig. 5). Mutational studies on *Bs*CspB and *Bc*Csp [109] confirmed that the clustering of the charged amino acids is responsible for the increased thermostability, only introducing the same number of charged amino acids randomly on the surface had almost no effect on the thermostability.

Temperature variation has also an influence on the three-dimensional structure of Csp. For Csp of the hyperthermophilic bacterium *T. maritima*, a low and high temperature structure was solved by NMR, which showed a temperature-dependent rearrangement of the RNA binding site (Fig. 6). In a thorough thermodynamic analysis, Schuler et al. [110] verified that entropic factors play an important role for the thermostability of *Tm*Csp relative to its homologues of mesophilic origins.

The interaction with single-stranded nucleic acid, concluded from the existence of the RNP-1 and RNP-2 motif, has been verified experimentally by chemical shift perturbation analysis of complexes between *Ec*CspA and ssDNA [105], between *Bs*CspB and ssDNA [22], and between *Tm*Csp and ssDNA and ssRNA [23], by ssRNA-binding gel-shift assays [17, 111], by tryptophan fluorescence quenching studies and by site-directed mutagenesis of the ssDNA-binding function of *Ec*CspA [112] and *Bs*CspB [27]. The binding specificity has not yet been determined for

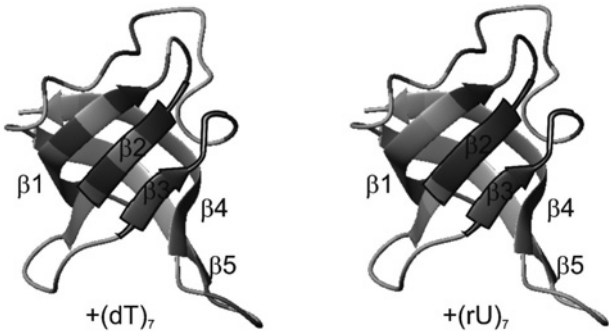

Figure 4. Three-dimensional structure of Csp from *T. maritima* and its nucleotide binding sites. Mapping of the chemical shift perturbations caused by oligonucleotide binding onto a three-dimensional model of pure *Tm*Csp. Residues that display an interaction with the ligand in the NMR assay with the deoxyribonucleotide $(dT)_7$ (left) or the ribonucleotide $(rU)_7$ (right) are depicted in dark gray. The RNA-binding sites RNP1 and RNP2 usually found in Csps are marked by boxes on the surface (details will be published elsewhere).

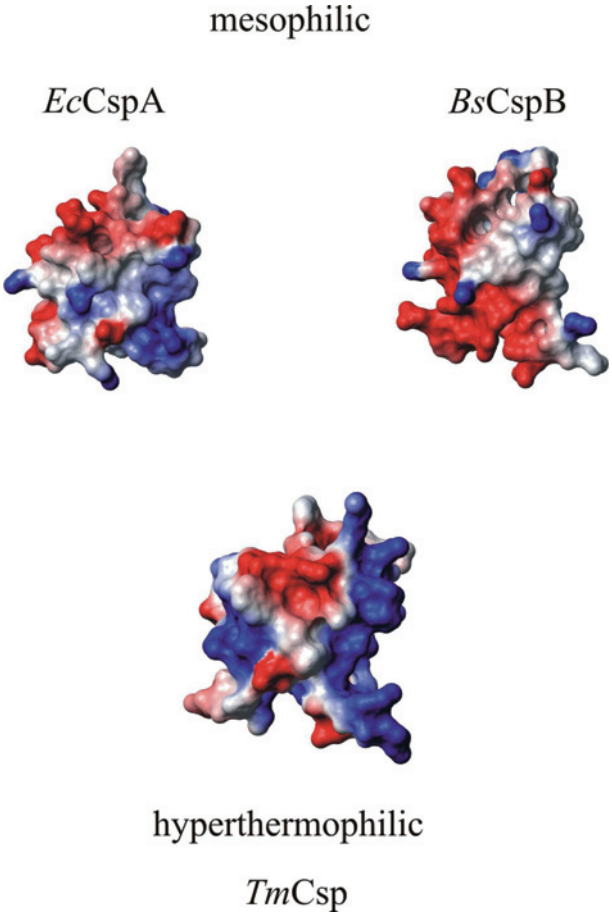

Figure 5. Surface charge distribution and ionic clusters in Csps. Comparison of the surface charge distribution of the solution NMR structures of *Ec*CspA [105] and *Bs*CspB [52] with the surface charge distribution of the solution NMR structure of *Tm*Csp [101]. Basic amino acids are shown in blue, acidic amino acids in red, and hydrophobic amino acids are shown in white. Surface charges were calculated in MOLMOL [118].

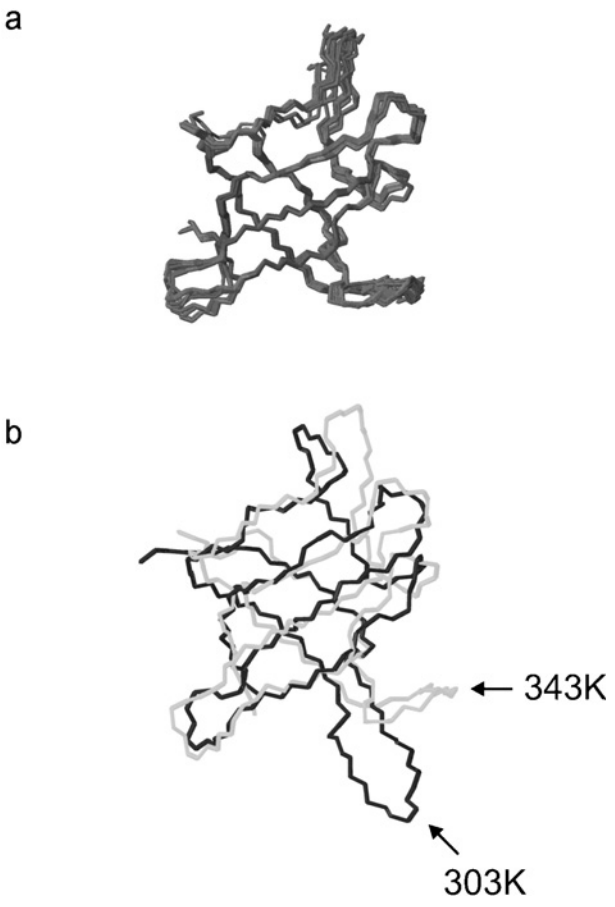

Figure 6. Low and high temperature structure of Csp from *T. maritima*. (*a*) Superposition of the backbone atoms of a family of the ten lowest energy structures of *Tm*Csp at high temperature (343 K) generated with NMR, overlaid on the mean structure of these ten structural models. (*b*) Comparison of the superposition of the backbone atoms of representative NMR-derived structures of *Tm*Csp at low (303 K, black) and high (343 K, gray) temperatures. Structures were taken from [101] and [102].

*Ec*CspA; for ssDNA the sequences CCAAT and ATTGG seem to be preferred, whereas ssRNA is bound unspecifically. An *in vitro* selection approach (SELEX) revealed the specific consensus sequences UUUUU, AGGGAGGGA and AAAUUU in the cases of *Ec*CspB, *Ec*CspC and *Ec*CspE, respectively [73]. *Ec*CspD exists exclusively as a dimer in solution and binds ssDNA and RNA noncooperatively, but not dsDNA [53].

In the case of *Bs*CspB, a thorough analysis of the interaction with ssDNA templates revealed that *Bs*CspB preferentially binds to poly-pyrimidine but not poly-purine ssDNA templates. Thymine-based ssDNA templates are bound with high affinity and binding is salt independent, whereas binding of cytosine-based ssDNA templates is strongly salt dependent, indicating a large electrostatic component involved in the interactions. Upon binding each *Bs*CspB seems to cover a stretch of six to seven thymine bases on T-based ssDNA. The binding of *Bs*CspB to T-based ssDNA template is enthalpically driven, indicating the possible involvement of interactions between aromatic side chains on the protein with the thymine bases [20, 21]. Nevertheless, chemical shift mapping on *Bs*CspB was executed with the Y-box25 DNA, ATCCTACTGATTGGCCAAGGTG-CTG, which revealed moderate cooperativity (3:1) by binding to ssDNA [22], similar to ssRNA and ssDNA binding of *Ec*CspA [17, 19].

As pointed out above, the Csps belong to the OB-fold family of proteins. Previously, all our detailed structural knowledge of OB-folded nucleoprotein complexes originated from the X-ray structures of telomere DNA-binding proteins [113–115]. However, recently a crystal structure of T-rich ssDNA in complex with *Bs*CspB showed that the sequence TTTTTT is not only bound by one single *Bs*CspB protein molecule, as seen in solution ([116] and references therein), but bridges two *Bs*CspB protein molecules, leading to a continuous arrangement in the crystal (Fig. 7). In particular, these authors found seven subsites per *Bs*CspB protein molecule and designed from the crystal structure the new ssDNA ligand TTCTTTT, which showed the highest affinity to *Bs*CspB reported so far, and represents a C as the optimal base for the subsite 3 [116]. Summing up, the affinity of *Bs*CspB to oligonucleotides does not simply depend on the abundance of thymines in a ligand molecule but rather on preferential base binding to individual subsites.

The recent X-ray data from CspB from *B. subtilis* also questions the validity of earlier models of Csp-RNA interaction [22]. A mutational analysis of CspE from *E. coli* led to a model in which the side chains of Phe17 and Phe30 first bind to the bases of the stem loop of an RNA; later, when the stem is partially melted, the side chain of His32 probably intercalates between the next nucleotide bond and propagates the melting of the double-stranded region of the RNA [38, 47].

The crystal structure of *Bs*CspB-TTTTTT shows aligned dimers. In contrast, the solution structure of *Bs*CspB bound to an ssDNA heptathymidine fragment (dT_7) [117] displays a monomeric nucleoprotein complex, where the *Bs*CspB conformation is almost invariant to oligonucleotide binding compared to the unbound conformation. Only minor reorientations were observed in loop β1-β2 and β3-β4 as well as of a few aromatic side chains involved in base stacking. Binding studies showed that the association with ssDNA is diffusion-controlled and exhibits a low sequence specificity. The ssDNA affinity is varied only by changes in the dissociation rate [117]. In fact, the authors found a strong increase of the dissociation rate of *Bs*CspB/dT_7 with increasing temperature:

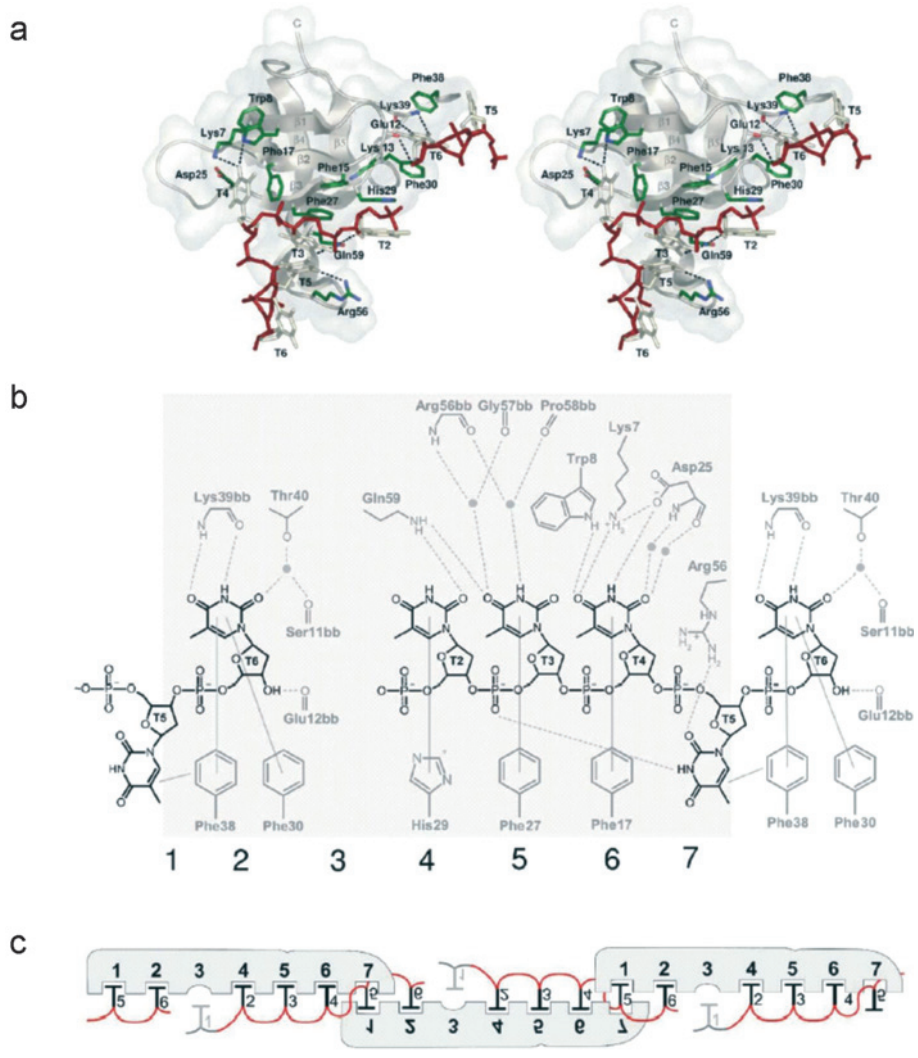

Figure 7. Interactions of CspB from *B. subtilis* with ssDNA. Hydrophobic and polar interactions between dT_6 and *Bs*CspB (modified and adapted from [116]). (*a*) The contact surface of *Bs*CspB is shown as a semitransparent gray object, protein groups involved in stacking interactions and hydrogen bonding are colored according to the CPK scheme with the exception of carbon, which is in green. Hydrogen bonds between protein and DNA groups are depicted as dotted lines. This is a stereo picture. (*b*) Representative section from the continuous arrangement of DNA (black) interacting with *Bs*CspB groups (gray), displayed as structural formulas (bb, protein backbone). All parts of a complete binding site from a single protein molecule are highlighted by a gray box. Stacking interactions between aromatic side-chains and nucleobases, originating from the centers (stack) or rims (edge-on stack) of the aromatic rings are depicted as solid gray lines. Interactions involving hydrogen bonds are displayed as dotted lines, water molecules mediating protein–ligand interactions are depicted as small spheres. Adjacent side-chains belong to symmetry related molecules. The numbers of the contact subsites for individual nucleobases are given at the bottom. (*c*) Schematic overview of *Bs*CspB·oligonucleotide assemblies. Protein molecules (gray objects) interact with nucleobases (characters) from oligonucleotide molecules (strings) at distinctive binding subsites (cavities). In the crystal, a continuous arrangement is formed. A gap between the 3′ nucleotide (bound to subsite 2) and the first structured 5′ nucleotide (bound to subsite 4) exists, which is big enough to accommodate one additional nucleotide at a postulated binding site (subsite 3). The unstructured T1 nucleotide (gray character) is expected to be located close to this position.

1.8±0.4 nM at 288 K, 60±4 nM at 298 K, and 464±31 nM at 308 K, suggesting a strong entropic contribution of the binding affinity. Generally, binding of dT_7 increases the stability of *Bs*CspB and reduces the sub-nanosecond dynamics of the entire protein.

For *Tm*Csp, the ssDNA-ligands AAAAAAA, CCCCCCC, and TTTTTTT and the ssRNA ligand UUUUUUU were characterized by gel shift measurements and high resolution NMR spectroscopy [23]. Both methods showed a specific interaction with high affinity of *Tm*Csp only with TTTTTTT and UUUUUUU, which leads to a dimerization of the *Tm*Csp protein molecules upon ligand binding. Chemical shift perturbation analysis revealed that only the postulated motif RNP1 interacted with the ligands, while no interaction of RNP2 was observed (Fig. 4). In this sense, it is remarkable that, among all Csps known, *Tm*Csp is the only one that possesses a

tryptophan residue instead of a highly conserved phenylalanine or tyrosine residue at the end of RNP2 [101]. NMR titration experiments showed that the chemical shift of this residue (W29) changed extremely when the ssDNA ligand AAAAAAA was added compared to TTTTTTT and CCCCCCC [23]. The position of W29 in *Tm*Csp is identical to the F30 in the *Bs*CspB sequence, where F30 is close to the position of the unstructured T nucleotide in the crystal structure [116]. As pointed out above, the crystal structure was used to design the new ligand TTCTTTT for *Bs*Csp that showed the highest known affinity of all ligands characterized so far. Similarly, our NMR data of *Tm*Csp [23] indicate that neither dT_7 nor rU_7 are the natural ligand and that, instead of pyrimidines only, purine bases are also required for correct binding.

In summary, we can state that in the case of the Csps, being the smallest OB-fold proteins known, many regulatory and sensory functions (*i.e.*, nucleotide binding, temperature stability and perhaps sensing) are associated with conformational entropy changes either determining the unfolding rate, as in *Tm*Csp [110], or the dissociation rate of oligonucleotides, as measured in *Bs*CspB [117].

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