High-temperature solution NMR structure of *TmCsp*

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Abstract

Cold shock proteins (Csps) are assumed to play a central role in the regulation of gene expression under cold shock conditions. Acting as single-stranded nucleic acid-binding proteins, they trigger the translation process and are therefore involved in the compensation of the influence of low temperatures (cold shock) upon the cell metabolism. However, it is unknown so far how Csps are switched on and off as a function of temperature. The aim of the present study is the study of possible structural changes responsible for this switching process. ^1H - ^{15}N HSQC spectra recorded at different temperatures and chemical-shift analysis have indicated subtle conformational changes for the cold-shock protein from the hyperthermophilic bacterium *Thermotoga maritima* (*TmCsp*) when the temperature is elevated from 303 K to its physiological temperature (343 K). The three-dimensional structure of *TmCsp* was determined by nuclear magnetic resonance (NMR) spectroscopy at 343 K to obtain quantitative information concerning these structural changes. By use of residual dipolar couplings, the loss of NOE information at high temperature could be compensated successfully. Most pronounced conformational changes compared with room-temperature conditions are observed for amino acid residues closely neighbored to two characteristic β -bulges and a well-defined loop region of the protein. Because the residues shown to be responsible for the interaction of *TmCsp* with single-stranded nucleic acids can almost exclusively be found within these regions, nucleic acid-binding activity might be down-regulated with increasing temperature by the described conformational changes.

Keywords: Cold shock protein; NMR; residual dipolar couplings; thermophilic adaptation; RNA binding

The cold-shock response in microorganisms is a transient phenomenon affecting the growth rate of the cell and the

saturation of fatty acids as well as the rate of DNA, RNA, and protein synthesis at temperatures significantly lower than the normal physiological temperature (Shaw and Ingraham 1976). Whereas the expression under such specific low-temperature conditions is dramatically decreased for most proteins, so-called Cold shock proteins (Csps) show an extremely high-induction level due to the cold shock. Csps play a key role in the regulation of gene expression at low temperatures until physiological temperatures are reached again. Csps share a high similarity with respect to their amino acid sequences (Jones and Inouye 1994; Graumann and Mahariel 1996; Thieringer et al. 1998). Csps are usually folded in a Greek-key- β -barrel, made up of five β -strands, which are arranged into two antiparallel β -sheets. They belong to the OB-fold-family (Murzin 1993). Structures of the major Csps from *Escherichia coli* and *Bacillus subtilis*, Csp A and Csp B, as well as Csp B from *Bacillus caldolyticus*, were determined by X-ray crystallography

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Abbreviations: COSY, correlation spectroscopy; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy; HSQC, heteronuclear single quantum coherence; DSS, Sodium-2,2-dimethyl-2-silapentane-5-sulfonate; MOCCA, modified phase-cycled Carr-Purcell; SIAM, simultaneous acquisition of in-phase and anti-phase multiplets; rmsd, root mean square deviation; ssDNA, single-stranded DNA; Csp, cold shock protein; *Tm*, *Thermotoga maritima*; *TmCsp*, cold shock protein from *Thermotoga maritima*; T_m , melting temperature; ppb, parts per billion; ppm, parts per million; Φ , Ψ , backbone torsion angles; DMPC, DiMyristoyl Phosphatidyl Choline; DHPC, DiHexanoyl Phosphatidyl Choline; CTAB, hexadecyl (Cetyl) Trimethyl Ammonium Bromide; CNS, crystallography and NMR system; RNP, RiboNucleoProtein.

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and/or solution NMR spectroscopy (Schindelin et al. 1993, 1994; Schnuchel et al. 1993; Feng et al. 1998; Newkirk et al. 1999; Mueller et al. 2000). The structural features of Csp A from *E. coli* revealed putative RNP1 and RNP2 sequence motifs commonly found in ssDNA-binding proteins (Bandziulis et al. 1989). This suggests that Csps possibly have a regulatory role for the adaptation of the organisms to low temperature. Csp A from *E. coli* was shown to increase the synthesis of several cold stress-inducible proteins after a decrease in temperature. However, different members of the *E. coli* Csp family are regulated differently. Some appear to perform functions during cell division or during the stationary phase (Murzin 1993) at physiological temperatures. The interaction with single-stranded nucleic acids has been verified by chemical-shift perturbation analysis of CspA/ssDNA complexes (Newkirk et al. 1999), ssRNA-binding gel-shift assays (Jiang et al. 1996), and site-directed mutagenesis of the ssDNA-binding site of *E. coli* Csp A (Hillier et al. 1998) and *B. subtilis* Csp B (Schröder et al. 1995). It is believed that electrostatic, as well as hydrophobic interactions are involved in nucleic acid binding. Nevertheless, the role of these interactions in the cell biology of the cold shock response is still not fully understood (Phadtare et al. 1999).

Csps are small globular proteins consisting of 65 to 70 amino acids. The proteins do not exhibit post-translational modifications or cofactor binding. It was shown that Csps undergo reversible folding/unfolding following a two-state mechanism without slow kinetic phases (Schindler and Schmid 1996). Csps have been identified in a wide variety of microorganisms from psychrophiles to thermophiles. Even among eukaryotic proteins, a domain was found that shows 43% identity with Csp A, the so-called Y-box-domain (Wistow 1990). From the evolutionary point of view, Csps, therefore, belong to the most conserved proteins presently known. Correspondingly, they have been used as model systems for numerous folding studies and thermodynamic analyses, especially with respect to the molecular mechanisms of thermal stabilization (Frankenberg et al. 1999; Perl and Schmid 2001). The first hyperthermophilic member of the Csp-family, *TmCsp*, was cloned from *Thermotoga maritima* (Welker et al. 1999). *TmCsp* is a small protein of 66 amino acids with a molecular mass of 7474 Da. Its room-temperature structure has already been determined by solution NMR spectroscopy (Kremer et al. 2001). Although *TmCsp* shows 76% sequence homology (61% identity) to Csp from the mesophilic *B. subtilis* CspB, its thermal stability ($T_m = 361$ K) strongly exceeds that of *B. subtilis* Csp ($T_m = 325$ K) (Perl et al. 1998). It was suggested (Kremer et al. 2001) that *TmCsp* gains its high stability compared with its mesophilic and moderately thermophilic analogs from a single peripheral ion cluster around the side chain of Arg 2, as well as increased hydrophobic stacking at the protein surface. This is in agreement with the

observation that protein stability seems to be accomplished by the cumulative contributions of very few noncovalent interactions (Matthews 1996). ^{19}F NMR investigations of the thermal unfolding of *TmCsp* enriched with 5-fluorotryptophan revealed a typical two-state folding/unfolding pattern (Schuler et al. 2002). The analysis of the data suggests that the extreme thermal stability of *TmCsp* is almost exclusively due to a decrease of the unfolding rate constant. Furthermore, it is suggested that entropic factors are important for the thermal stability of this protein. The aim of the present study is the determination of the structure of *TmCsp* at a temperature close to the bacterium's optimum physiological temperature and especially its comparison with the structure at 303 K. This comparison should reveal structure-function relationships that are neither provided by the analysis of the room-temperature structure alone nor by a hypothetical structure of a corresponding protein-nucleic acid complex.

Results

Figure 1 shows the two-dimensional ^1H - ^{15}N HSQC spectrum of *TmCsp* at 328 K. An interesting effect is seen in this figure; many of the ^1H - ^{15}N cross-peaks split into at least two signals (e.g., see Gly 3, Lys 4, Glu 33, and His 61) at 328 K, indicating the existence of at least two subtly different conformations of the molecule. Often, the signals due to the different conformations overlap, resulting in asymmetric cross-peaks (e.g., see Lys 19, Val 25, or Leu 40). An analysis of the complete spectrum shows that ~50% of the ^1H - ^{15}N cross-peaks are split. At a temperature of 328 K, the average difference between the two signals is 0.03 ppm for ^1H and 0.15 ppm for ^{15}N . This behavior is not restricted to specific secondary structure elements or regions, indicating that the structural changes responsible for this effect are not limited to certain parts of the molecule. The relative intensities of the signals, that is, the population of the different states, depends on the temperature as well. At 343 and 303 K, one of the different conformations clearly dominates.

Furthermore, the ^1H NMR chemical shifts change with increasing temperature, in most cases, to smaller chemical shift values. This is a well-known phenomenon. Thermal fluctuations cause a decrease of amide proton chemical-shift values due to a higher mobility at elevated temperatures (Baxter and Williamson 1997). The described effect should be most pronounced for residues located within mobile regions of the molecule and less distinct for those in stable areas such as secondary structure elements. Therefore, negative temperature coefficients of variable size are expected, depending on the thermal stability of the individual protein segments. First-order temperature coefficients were determined for the amide proton chemical shifts of *TmCsp*

Figure 1. Two-dimensional ^1H - ^{15}N HSQC spectrum of *TmCsp* measured at 328 K. Glutamine side-chain amide correlation peak pairs are interconnected with solid lines and denoted by an asterisk.

by linear extrapolation from the chemical shifts at 303, 328, and 343 K. These temperature coefficients are plotted as a function of the amino acid residue number in Figure 2. The majority of these coefficients is negative, only a few are positive. Values higher than, that is, less negative than -5 ppb/K, indicate amide protons involved in intramolecular hydrogen-bonds (Freund et al. 1995; Rothmund et al. 1996), whereas NH protons with temperature coefficients lower than -5 ppb/K are likely to form a hydrogen bond to water. Values distinctly lower than -5 ppb/K are especially observed for amino acid residues Phe 8 (-8.9 ppb/K), Lys 19 (-8.4 ppb/K), and Asp 24 (-8.5 ppb/K). Furthermore, the average temperature coefficients for the backbone amide protons of $\beta 1$ (Arg 2–Asp 9) and $\beta 5$ (Gln 58–Val 65) in their room-temperature extent are -4.6 and -6.2 ppb/K, respectively. These values are clearly lower than for the remaining strands $\beta 2$, $\beta 3$, and $\beta 4$ (e.g., -3.0 ppb/K for $\beta 2$), indicating that possible temperature-dependent conformational changes are preferentially expected for $\beta 1$ and $\beta 5$. Within this context, it is interesting to note that these two β -strands are divided into two segments, $\beta 1'$ and $\beta 1''$, as well as $\beta 5'$ and $\beta 5''$ by two so-called β -bulges centered at

Lys 6 and Ala 60. Temperature coefficients have also been determined for side-chain protons of *TmCsp*. The chemical-shift changes caused by an increase of temperature from 303 to 343 K turned out to be particularly significant for H^δ and H^ϵ of a single aromatic amino acid residue, namely Trp 7. Its H^{E1} -chemical shift changes with a temperature coefficient of -5.5 ppb/K. In contrast, the temperature coefficients for H^{E1} -protons in other aromatic residues (Phe 8, Phe 16, Phe 26, Trp 29, Phe 37, and Phe 48), fall within the range of -1.5 to 0 ppb/K.

The described analysis of the temperature dependence of chemical shifts is, however, incapable of delivering reliable quantitative structural information. We therefore decided to determine the three-dimensional structure of *TmCsp* at 343 K, to gain a more precise insight into the structural features of the protein at the physiological temperature of the hyperthermophilic bacterium. This high-temperature structure is compared with the molecular structure at a lower temperature, that is, under conditions similar to the environment under cold shock. We have also tried to obtain structural information at temperatures up to 353 K, that is, at temperatures close to the melting temperature of *TmCsp* (361 K). Almost the complete protein backbone could be assigned. However, the decreasing signal-to-noise ratio, especially of the NOESY spectra, prevented us from determining the structure of the protein at 353 K. Note that the absence of NOE information at elevated temperatures does not necessarily indicate a higher flexibility of the molecule. More often, this effect is caused by the onset of rapid amide proton exchange, decreasing spin polarization and increasing electronic noise. It could be shown earlier (Schuler et al. 2002; see above) that *TmCsp* exhibits a characteristic two-state folding/unfolding pattern. The temperature-dependent

Figure 2. First-order temperature coefficients for the amide-proton chemical shifts of *TmCsp* determined by linear extrapolation from the chemical shifts measured at temperatures of 303 K, 328 K, and 343 K, respectively. Arrows indicate the extent of β -strands 1, 2, 3, 4, and 5, respectively, at high temperature. Regular secondary structure elements correlate with first-order temperature coefficients higher than -5 ppb/K, whereas temperature coefficients below -5 ppb/K preferentially occur for amino acid residues located in loop regions.

conformational changes described here occur well below the melting temperature. Therefore, the NMR structure determined here does not represent a classical folding intermediate.

The secondary structure elements of *TmCsp* at 343 K were determined using a combination of data, including medium-range interstrand NOEs, $^3J_{\text{HNH}\alpha}$ coupling constants as well as deviations of the chemical shifts of backbone nuclei ($\delta^{13}\text{C}^\alpha$, $\delta^{13}\text{C}'$, $\delta^1\text{H}^\alpha$) from random-coil values combined with statistical methods and homology search (TALOS; Cornilescu et al. 1999). The results were checked by examining the corresponding Ramachandran plots as described in the Materials and Methods section.

Characteristic interstrand NOEs identified at 343 K show that the global Greek-key topology is conserved at high temperature. Nevertheless, the observation of NOEs is limited to central segments of the β -strands, suggesting a partial melting of the β -sheets at 343 K. This observation is further corroborated by the results derived from TALOS analyses (see Table 1). All five β -strands ($\beta 1$ – $\beta 5$) observed at 303 K could be identified at 343 K, as well from the characteristic backbone torsion angles Φ and Ψ ; however, their length changed. The amino acid residues identified as belonging to these strands at 343 K are given as follows (the extent of the corresponding strands at 303 K is indicated in parenthesis): $\beta 1$: Gly 3–Val 5 (Arg 2–Asp 9), $\beta 2$: Tyr 14–Lys 19 (Gly 13–Lys 19), $\beta 3$: Asp 24–Trp 29 (Asp 24–Trp 29), $\beta 4$: Gln 44–Ile 50 (Gly 43–Gln 51), $\beta 5$: Gln 58–Val 64 (Gln 58–Val 65). Note that the extent of the β -strands given here was taken from the finally determined structures and is not completely identical with the TALOS predictions (Table 1). Most amino acid residues originally bordering the β -strands show torsion-angle values that do not allow them to adopt typical planar β -sheet conformation at 343 K. In agreement with the preceding analysis of temperature coefficients, the most pronounced effects can be observed for $\beta 1$. $\beta 1$ undergoes a distinct loss of regular secondary structure at high temperature. Its N-terminal part, $\beta 1'$, is conserved, whereas the original C-terminal part, $\beta 1''$, starting with Lys 6 appears to be nonstructured at 343 K. It has to be mentioned that the observed loss of secondary structure cannot be ascribed to missing information. Almost all backbone proton chemical shifts could be identified at high temperature, except for residues Ser 10, Gly 36, Lys 38, Glu 52, and Lys 55 localized within loop regions. Furthermore, it should be noted that the reliability of this TALOS analyses is attested by a good agreement between the predicted torsion angle values Φ and the experimentally determined $^3J_{\text{HNH}\alpha}$ coupling constants obtained from MOCCA-SIAM spectra.

The constraints used in the simulated annealing protocol to calculate the three-dimensional structure of *TmCsp* at 343 K are summarized in Table 2. Due to the decreasing signal-to-noise ratio at elevated temperatures, 442 NOEs could be

Table 1. Dihedral angles Φ and Ψ for amino acid residues in secondary structure elements determined by homology search using the computer program TALOS

Residue	$\Phi/^\circ$	$\Psi/^\circ$
Arg 2	–121	161
Gly 3	–124	148
Lys 4	–123	136
Val 5	–91	118
Lys 6	–72	–39
Trp 7	–87	–11
Phe 8	–82	137
Gly 13	–68	–25
Tyr 14	–93	2
Gly 15	174	148
Phe 16	–135	140
Ile 17	–110	113
Thr 18	–105	129
Lys 19	–83	147
Asp 24	–129	138
Val 25	–107	130
Phe 26	–81	121
Val 27	–109	129
Val 45	114	130
Val 46	–118	151
Glu 47	–128	142
Phe 48	–128	152
Glu 49	–130	147
Ile 50	–114	127
Gln 51	–101	139
Gln 58	–112	116
Ala 59	–96	128
Ala 60	–90	–27
His 61	–98	–3
Val 62	–125	126
Lys 63	–105	130

All residues found by TALOS to be involved in β -strands at 303 K are shown. Gray fields indicate residues found to be located in β -strands at high temperature (343 K).

identified only at 343 K (for comparison, ca. 800 NOE contacts have been assigned in two-dimensional NOESY spectra at 303 K). Whereas an average number of 14 NOE distance restraints per residue could be used for the refinement of the 303 K structure, only eight NOE distance restraints per residue were available for the determination of the 343 K structure. Nevertheless, the overall solution structure of *TmCsp* at high temperature is well defined, as demonstrated in Figure 3, obviously due to the use of residual dipolar couplings that are capable of replacing NOE information to a high extent (Clore et al. 1999). This figure shows a superposition of the backbone atoms of a family of the 10 lowest energy structures generated with the computer program CNS. A backbone-rmsd-value of 0.2 nm could be obtained for the secondary structure elements of the final

Table 2. Structural statistics at 343 K

Restraints	Number
<i>NOEs, total</i>	442
intraresidual ($i=j$)	192
sequential ($ i-j =1$)	93
intermediate range ($1< i-j <5$)	59
long range ($ i-j \geq 5$)	98
<i>dihedral angle restraints, total</i>	85
TALOS - angles	30
Φ - angles	4
Ψ - angles	26
Φ - angles from 3J -couplings (MOCCA-SIAM)	25
<i>Hydrogen bonds</i>	0
1H - ^{15}N residual dipolar couplings	33
Structural statistics for the five-lowest-energy structures (from 200 calculated structures)	
E_{total}	2335 ± 16 kJ/mole
E_{NOE}	928 ± 14 kJ/mole
E_{bond}	151 ± 5 kJ/mole
E_{ang}	545 ± 14 kJ/mole
E_{mp}	110 ± 3 kJ/mole
E_{vdw}	454 ± 15 kJ/mole
E_{edih}	40 ± 3 kJ/mole
E_{sani}	107 ± 8 kJ/mole

structural models, taking into account all backbone atoms. To evaluate the quality of the calculated structures, *R*-factors were determined as described by Gronwald et al. (2000). Values of 0.66 and 0.70 were obtained for the structures calculated with and without residual dipolar couplings, respectively. For the refined structure of *TmCsp* at 303 K, *R*-factors of 0.51 and 0.54, respectively, were calculated. This indicates that the structures determined at 303 and 343 K are of comparable quality. The *Q*-factor defined for residual dipolar couplings by Cornilescu et al. (1998) was 0.27 for the 343 K structure, revealing a good agreement between calculated and experimentally determined residual

Figure 3. Superposition of the backbone atoms of a family of the 10 lowest energy structures generated with CNS, overlaid on the mean structure of these 10 structural models. Note the extraordinarily well-defined loop between β -strand 4 and β -strand 5.

dipolar couplings. Apart from glycines and residues located in poorly defined and potentially mobile segments, 95% of Φ and ψ values occur in sterically preferred regions of the Ramachandran plot. As usual, longer loop regions, especially that in between $\beta 3$ and $\beta 4$, are less well defined. In contrast, the loop between $\beta 4$ and $\beta 5$ (amino acid residues Gln 51–Pro 57) is amazingly well defined and exhibits a rmsd value of 0.36 nm only. The latter loop undergoes the most striking backbone conformational change when the temperature is elevated from 303 to 343 K (see also Fig. 4).

Figure 4 shows representative *TmCsp*-structures at 303 and 343 K. In both cases, secondary-structure elements were identified by the algorithm of Kabsch and Sander (1983). Figure 4 illustrates the above-discussed loss of secondary structure at elevated temperature. A particularly striking shortening is observed for $\beta 1$. The second part ($\beta 1''$) of $\beta 1$ at 303 K, Lys 6–Asp 9, is not recognized to belong to $\beta 1$ at 343 K. Residue Lys 6 represents the center of a β -bulge that had been found to disrupt $\beta 1$ into its two parts, $\beta 1'$ and $\beta 1''$, at 303 K (Kremer et al. 2001), and seems to act as a breaking point for the secondary structure within the β -strand at high temperature.

Discussion

It could be observed that one of the different *TmCsp* conformations dominates at 303 and 343 K, whereas comparable populations of the different conformations are found around 328 K. Therefore, we conclude that the protein changes between different conformational states if the temperature is elevated from 303 to 343 K. It can be hypothesized that only the predominant conformer at 303 K (cold shock temperature conditions) shows enough affinity for nucleic acid binding. The conformational changes with increasing temperatures would then reflect a mechanism to down-regulate the protein's cold shock activity and prevent the bacterium from cold shock-specific gene expression at physiological temperature conditions.

As illustrated in Figures 3 and 4, *TmCsp* is compact and well defined, even at 343 K. Usually, hydrogen bonds are the only noncovalent bonds keeping the three-dimensional structure of mesophilic *Csps* intact. Additional forces, like ionic interactions, typically contribute to the stability of thermophilic and hyperthermophilic *Csps* (Kremer et al. 2001). In the final high-temperature structures, pronounced shortening of the β -strands is observed, especially close to the β -bulges. This is in line with the fact that the hydrogen-bond energy decreases with the temperature. In contrast, the secondary structure is well conserved close to the peripheral ion cluster around the side chain of residue Arg 2, including the side chains of Asp 20, Arg 2, Glu 47, and Lys 63. This ion cluster has already been found at 303 K, and its existence is now confirmed at high temperature as well. It functions like a bracket, keeping the whole Greek-key motive together, even at high temperature. The side chains of Asp

Figure 4. Comparison of the refined backbone structure (ribbon plot) of *TmCsp* at 303 K (A) and 343 K (B,C). The arrow in the 303 K structure points to the β -bulge. In the 343 K structure, the salt bridge Lys 6–Asp 24 (B) and the ion cluster around Arg 2 (C) are indicated. It can be seen that β 1 is shortened at elevated temperature; it only consists of residues Gly 3–Val 5 at 343 K, and the second part of β 1 at 303 K, residues Lys 6–Asp 9, does not belong to β 1 at 343 K. Note the pronounced change of the loop between β 4 and β 5.

20, Arg 2, Glu 47, and Lys 63 are closely neighbored in all 343 K structures. This is a prerequisite for the formation of the proposed ionic interactions. The distance from the nitrogen atom of one guanidinium group of Arg 2 to the γ -carbon atom of Asp 20 is 0.53 ± 0.01 nm in the 10 lowest energy structures, the distance from the nitrogen atom of the other guanidinium group of Arg 2 to the δ -carbon atom of Glu 47 amounts to 0.505 ± 0.015 nm, and the distance from the δ -carbon atom of Glu 47 to the ζ -nitrogen atom of Lys 63 is 0.377 ± 0.012 nm. The existence of Arg 2, which dominates the geometry of the ion cluster is, in fact, a unique and crucial feature observed for thermophilic members of the Csp family (Kremer et al. 2001).

Two salt bridges have also been discussed in the room temperature structure, Glu 49–His 61 and Lys 6–Asp 24. They coincide exactly with the centers of the two β -bulge regions, as shown for the salt bridge Lys 6–Asp 24 in Figure 4. Lys 6 and His 61 located in the β -bulges exhibit backbone angles Φ and Ψ , characteristic for residues in α -helices at 343 K, as well as at 303 K. These findings support the idea that the two salt bridges might represent the driving forces giving rise to the β -bulge formation by preventing the corresponding residues to adopt planar conformations typically found within β -strands. As the influence of inter-strand hydrogen bonds on the stability of the protein decreases with increasing temperature (Jaenicke 2000), ionic interactions, particularly the ion cluster and the two salt bridges, are likely to dominate at 343 K, provoking deformations of backbone angles. These deformations are proved by corresponding changes in the torsion angles for some characteristic residues (Trp 7, Phe 8, and Ala 60) at 303 and 343 K.

Apart from the conserved RNA-binding motifs RNP1 (Tyr 14, Gly 15, Phe 16, and Ile 17) and RNP2 (Val 25, Phe 26, Val 27, His 28, and Trp 29), it is suggested that especially Lys 6, Trp 7, Lys 12, and Lys 55 are additionally

involved into nucleic acid binding (Kremer et al. 2001). This suggestion is in close analogy to the behavior observed for Csp A from *E. coli* and to the cold shock domain of the human transcription factor YB-1 (Kloks et al. 2002). Titration of the cold shock domain of YB-1 with DNA even suggested stacking of the aromatic ring of Trp 15 (corresponding to Trp 7 in *TmCsp*) with bases of the nucleic acid. In fact, our analysis of side-chain proton temperature coefficients strongly indicates a special function of Trp 7. Interestingly, Lys 6 and Trp 7 are located in a region of the molecule that is integrated into β 1 at 303 K, but does not show regular secondary structure at high temperature. Furthermore, amino acid residue Lys 55 is located within the loop between β 4 and β 5, which undergoes the most striking backbone conformational change when the temperature is elevated from 303 to 343 K as shown in Figure 4.

The growth rate of *T. maritima* clearly decreases at temperatures below 338 K, as reported by Welker et al. (1999). Assuming that the cold shock response correlates with the

Figure 5. Different conformations of the side chains of residue Trp 7 at 303 and 343 K. These characteristic conformational changes could be observed in all of our structural models calculated for *TmCsp* at high temperature.

growth rate, *TmCsp* is not expressed until the temperature is lower than ca. 338 K. Most amino acid residues necessary for nucleic acid binding (except for Lys 12 and Lys 55) are located in β -strands. It is likely to assume that their side chains are then arranged such that the molecule is capable of binding RNA (active state). Therefore, *TmCsp* induces other proteins by RNA chaperoning under low-temperature conditions, and allows the organism to be responsive to the new environmental conditions. As the temperature rises, the activity of Csp has to be reduced continuously to prevent misregulation of the protein expression at physiological temperatures. This seems to be accomplished by the influence of ionic interactions, causing torsions of the protein backbone, in particular in β 1 and β 5, which results in conformational changes of side chains involved into nucleic acid binding as shown for Trp 7 (Fig. 5). A similar effect could be found for Lys 6 as well. These characteristic conformational changes could be observed in all structures calculated for *TmCsp* at high temperature. Furthermore, it is interesting to note that Trp 7 is an amino acid residue whose pressure dependence of the ^{15}N chemical shift strongly indicates conformational changes at high temperatures (Arnold 2002). Due to this anomaly, it is speculated that Trp 7 might be of special functional significance. This assumption is in agreement with our analysis of temperature coefficients for characteristic side-chain protons; the lowest first-order temperature coefficient was observed for amino acid residue Trp 7. An interesting hypothesis can be derived from these data. Because at least two basic side chains (Lys 6 and Lys 55) and one hydrophobic side chain (Trp 7) might no longer be available for the interaction at physiological temperature of the bacterium, due to the described conformational changes, the affinity of *TmCsp* to single-stranded nucleic acids may decrease below a threshold value necessary for sufficiently strong *in vivo* RNA binding.

In summary, ionic interactions do not only seem to be the strategy of choice for the thermophilic adaptation of cold shock proteins (ion cluster around Arg 2), there are also indications for a role in temperature-dependent down-regulation. Hydrogen bonds and ionic interactions (ion cluster around the side chain of Arg 2, salt-bridges Lys 6–Asp 24 and Glu 49–His 61) are influenced differently after a temperature raise following the cold shock; whereas hydrogen bonds are weakened due to a faster chemical exchange (loss of secondary structure), ionic interactions obviously resist the increase of temperature. Therefore, the overall fold is conserved especially close to the ion cluster. However, subtle conformational changes affecting the orientation of side chains of residues involved in nucleic acid binding occur, which are assumed to convert the molecule from its active state into the inactive state. Although other or additional regulatory mechanisms cannot be excluded, the proposed temperature-dependent protein inactivation would prevent the bacterium from cold-shock-specific gene ex-

pression under physiological conditions in a very elegant and direct way; signal (temperature) sensing and RNA chaperoning are combined in one single molecule.

Materials and methods

Protein expression and purification

TmCsp was expressed in *E. coli* BL21(DE3) as described by Welker et al. (1999) and purified according to the procedure established by Wassenberg et al. (1999). For the expression of the ^{15}N and $^{15}\text{N}/^{13}\text{C}$ isotope-enriched protein, the procedure established by Kremer et al. (2001) was used. NMR samples contained 1.5 mM protein, 10 mM sodium phosphate, 1 mM EDTA, and 100 mM NaCl.

NMR spectroscopy

NMR experiments were carried out on Bruker DMX-500 and Bruker DRX-600 spectrometers. ^1H - ^1H 2D-NOESY spectra (mixing time, 250 msec) and ^1H - ^1H 2D TOCSY spectra (isotropic mixing time, 55 msec) were recorded at 343 K and 600 MHz. Proton resonances of the side chains could be assigned from the two-dimensional TOCSY and the HCCH-TOCSY spectrum in $^2\text{H}_2\text{O}$ measured at 343 K. Chemical shifts of nuclei other than ^1H were determined on the basis of HSQC, HNCQ, and HNCA spectra recorded at 343 K. The latter spectrum also served to check the sequence-specific resonance assignment. H^{N} - H^{α} -couplings were determined with an accuracy of ± 0.2 Hz from two-dimensional MOCCA-XY16-SIAM spectra (Prasch et al. 1998) recorded at 343 K by Titman-Keeler-fit using an automated computer program (Möglich et al. 2002). For all of the thus-determined couplings, it was possible to limit the Karplus curve to a certain segment by taking into account the information provided by secondary structure prediction algorithms (Cornilescu et al. 1999). The resulting dihedral Φ angles were used directly for structure calculation. Distance restraints were obtained from homonuclear two-dimensional NOESY spectra with a 250-msec mixing time in $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$. About 20% of the NOEs were finally identified by automated knowledge-based NOE assignment using a Bayesian Algorithm (Gronwald et al. 2002). The NOE evaluation was iteratively refined by NOE back calculation on the basis of the complete relaxation matrix formalism (Görler and Kalbitzer 1997). The correlation time of reorientation for *TmCsp* amounts to 1.42 nsec at 343 K in contrast to 4.25 nsec at 303 K. Ψ and additional Φ angle information was obtained by chemical-shift analysis (^{15}N , $^1\text{H}^{\text{N}}$, $^1\text{H}^{\alpha}$, $^{13}\text{C}^{\alpha}$, and $^{13}\text{C}'$) combined with statistical methods and homology search using the program TALOS (Cornilescu et al. 1999). The ^1H NMR chemical shifts were referenced relative to DSS used as an internal reference. The ^{15}N and ^{13}C chemical shifts were indirectly referenced (Wishart et al. 1995). For exact determination of the experimental temperature, a calibration was performed using ethylene glycol (Raiford et al. 1979). Spectra analysis, peak picking, volume integration, as well as relaxation matrix back calculation were performed using the program AUREMOL (Ganslmeier 2002).

Residual dipolar couplings

A prerequisite for the measurement of residual dipolar coupling is the partial alignment of the protein molecules leading to an incomplete averaging of the magnetic dipole-dipole interaction. As shown by Losonczi and Prestegard (1998) mixtures of DMPC and DHPC are highly temperature stable if CTAB is added. The magnetically orienting bicellar solutions were prepared following the procedure described by Losonczi and Prestegard (1998) (molar

ratio DMPC : DHPC : CTAB = 3 : 1 : 0.2). Residual ^1H - ^{15}N dipolar couplings were measured as the difference of the corresponding couplings measured in oriented (10% w/v DMPC/DHPC/CTAB, 1.5 mM ^{15}N -TmCsp) and isotropic solution (1.5 mM ^{15}N -TmCsp) using ^1H -coupled HSQC experiments. A total of 57 residual dipolar ^1H - ^{15}N residual dipolar couplings could be measured. The magnitude of the axial (1.85 Hz) and rhombic (0.37 Hz) component of the alignment tensor were determined by examining the distribution of the experimental dipolar couplings.

Structure calculation

Structures were calculated by simulated annealing (Nilges et al. 1988) using the computer program CNS (Brünger et al. 1998). About 550 conformational NMR constraints were taken into account (Table 2). Approximate interproton distances were calculated from the intensities of the NOE cross-peaks in two-dimensional ^1H - ^1H NOESY spectra with an upper bound for distance restraints set to 0.5 nm. The minimum sum of the van der Waals radii (0.18 nm) was used as the lower bound for distance restraints. The initial structure was energy minimized with 1000 cycles of Powell minimization. High-temperature dynamics was run for 30 psec at an initial temperature of 1000 K. The system was then cooled down slowly to a temperature of 100 K in steps of 25 K. At 100 K, a second stage of Powell minimization was performed to receive the final low-energy structure. Figures were generated using the computer program MOLMOL (Koradi et al. 1996).

Structure quality

Apart from the rmsd, other criteria have also been used to evaluate the quality of the determined structures. The computer program RFAC (Gronwald et al. 2000) provided automated estimation of residual indices (R -factors) for the calculated structures using a Bayesian analysis of the data. Furthermore, Q -factors have been calculated according to Cornilescu et al. (1998), which are a measure for the agreement between the experimental residual dipolar couplings and the residual dipolar couplings calculated from the determined three-dimensional structure. Q -factors lower than 0.3 are indicative for structures of sufficient quality (Cornilescu et al. 1998). Residual dipolar couplings were included in the CNS protocol to optimize the obtained structure by an iterative process of using further coupling data and subsequent structure calculation and determination of the corresponding R -value. Residual dipolar couplings were only taken into account for residues localized within regular secondary structure elements in those structures that had been determined without any orientation data. To check the stereochemical quality of the structures, Ramachandran plots (Ramachandran et al. 1963) were generated by the computer programs MOLMOL (Koradi et al. 1996) and PROCHECK (Laskowski et al. 1996). These diagrams also served to confirm the secondary structure elements determined by the algorithm of Kabsch and Sander (1983).

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