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FOR THE RECORD

Effect of pH and phosphate ions on self-association properties of the major cold-shock protein from *Bacillus subtilis*

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Abstract: The intermolecular interactions of the major cold-shock protein from *Bacillus subtilis* (CspB) in solution in the presence of different salts, including phosphate, have been studied by means of scanning calorimetry and size-exclusion chromatography. Calorimetric results indicate that, in all cases, protein unfolding can be approximated by a 2-state model, but the modes of unfolding can differ depending on the conditions. In the presence of phosphate, the cooperative folding unit is a monomer, whereas in the absence of phosphate, the cooperative unit is a dimer. The difference in the self-association of CspB in the presence and absence of phosphate was supported by size-exclusion chromatography. These results are compared with recent structural studies of CspB in crystal and in solution.

Keywords: cold-shock protein; monomer-dimer; phosphate; scanning microcalorimetry; size-exclusion chromatography

Bacterial cells produce a specific subset of proteins when temperatures are decreased to 10 °C. The dominant fraction of this subset is the so-called major cold-shock protein, identified in many bacterial species (Goldstein et al., 1990; Wistow, 1990; Wolffe et al., 1992; Schröder et al., 1994), including *Bacillus subtilis* (Willimsky et al., 1992). The major cold-shock protein from *B. subtilis* (CspB) is a small soluble protein of 67 amino acid residues and a molecular weight of 7,400 Da (Schindelin et al., 1992). Its sequence has more than 60% identity with the major cold-shock proteins from other bacteria (Willimsky et al., 1992). It has also about 40% identity with the cold-shock domain found in many eukaryotic transcription factors (Schindelin et al., 1994), which bind specifically *cis*-elements of DNA called Y-boxes. Gel-retardation experiments of CspB have demonstrated that the protein binds preferentially to the single-stranded DNA containing a CCAAT-box (Graumann & Marahiel, 1994; Schröder et al., 1994).

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The structure of CspB was solved recently both in a crystalline form (Schindelin et al., 1993) and in solution (Schuchel et al., 1993). It has been shown that the structure of CspB consists of 5 β-strands organized into an antiparallel β-barrel (Fig. 1). The X-ray structure of CspB was determined using data collected from 2 different crystal forms. In both of them the protein exists as a dimer, with the dimer interface formed by a β4-strand. The small size of CspB and the fact that it is an all β-sheet protein make it interesting for studying the thermodynamic parameters that characterize the stability of this protein using high-sensitivity scanning calorimetry.

Figure 2 presents the partial specific heat capacity profiles for the heat denaturation of CspB at different pHs in different buffers: imidazole, HEPES, glycine, Tris, and sodium phosphate. One immediately notable fact is that the heat capacity profiles of CspB in sodium phosphate are significantly different than in other buffers. The area under the heat capacity profile represents the enthalpy of the observed heat denaturation process (ΔH_{exp}). The sharpness of the heat capacity profile gives another characteristic of the observed process, the effective enthalpy of transition, usually referred to as the van't Hoff enthalpy (ΔH_{vH}). The ratio of these 2 enthalpies provides information about the mode of the observed transition. A ratio equal to 1 means that the observed transition is 2-state, proceeding from native to unfolded states without a significant population of intermediates (Privalov & Khechinashvili, 1974; Sturtevant, 1987). Deviations from unity indicate that the transition is more complicated (Privalov, 1982; Ghosaini et al., 1988). Table 1 summarizes the results of the experimental and van't Hoff enthalpies of the heat denaturation of CspB under different solvent conditions, calculated per mole of monomer. The ratios of the experimental to van't Hoff enthalpies, $\beta_{mw} = \Delta H_{vH}/\Delta H_{exp}$, reported in the last column of Table 1, are very different in the presence and absence of phosphate ion and do not depend on pH. In the presence of phosphate, β_{mw} is equal to 1, meaning that the transition can be well approximated by a 2-state model where the native monomer unfolds:

$$N \rightarrow U. \qquad (1)$$

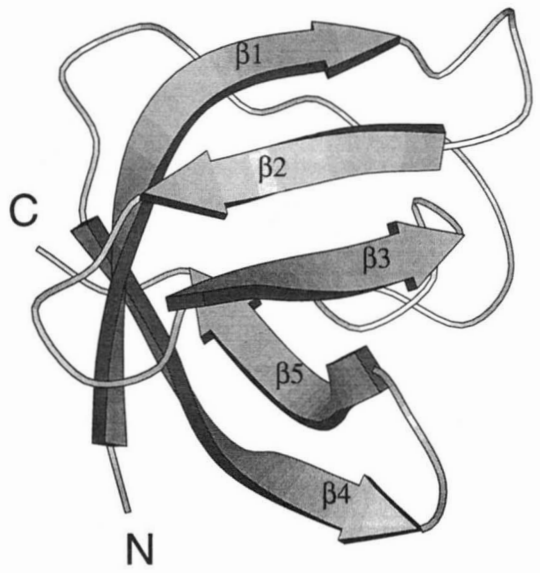

Fig. 1. Three-dimensional structure of CspB showing the 5 β-strands numbered β1 (residues 2–10), β2 (15–19), β3 (24–29), β4 (46–54), and β5 (57–65). Strand number 4 forms the dimer interface in the crystal structure. The picture is drown using MOLSCRIPT (Kraulis, 1991) and the solution NMR structure (Schuchel et al., 1993).

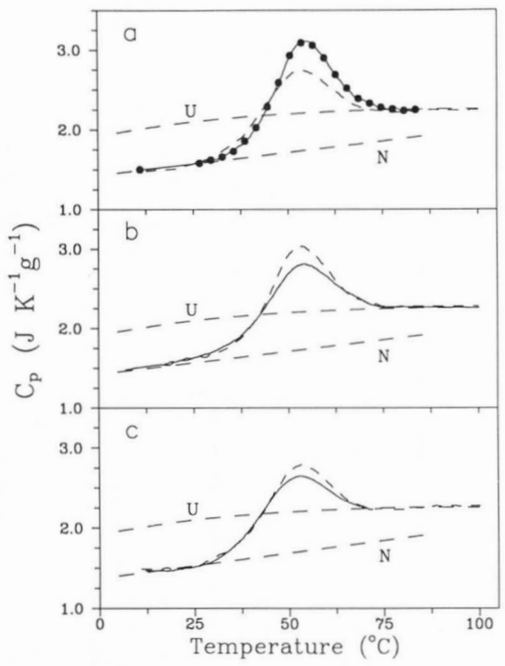

Fig. 2. Partial specific heat capacity of CspB under different solvent conditions. **A:** 50 mM imidazole, pH 7.5 (dashed line), and 50 mM Na-phosphate, pH 7.5 (solid line). The circles (●) show an example of the heat capacity profile fitted according to Equation 1. **B:** 50 mM Tris, pH 8.0 (dashed line), and 50 mM Na-phosphate, pH 8.0 (solid line). **C:** 50 mM glycine, pH 9.0 (dashed line), and 50 mM glycine/50 mM Na-phosphate, pH 9.0 (solid line). N and U (dashed-and-dotted lines) represent the partial specific heat capacity of CspB in the native and unfolded states, respectively. The heat capacity of the native state was linearly extrapolated to the transition region. The heat capacity of the unfolded state was calculated as previously described (Makhatadze & Privalov, 1990; Makhatadze et al., 1993). The heat capacity change upon unfolding, obtained as a difference between heat capacities of the unfolded and native state, was found to be 0.5 ± 0.1 J·K^{-1}·g^{-1} at T_t.

Table 1. *Thermodynamic parameters of CspB unfolding under different solvent conditions*[a]

pH	Buffer	T_t (°C)	ΔH_{exp} (kJ·mol^{-1})	ΔH_{vH} (kJ·mol^{-1})	β_{mw}
7.5	50 mM HEPES	52.8	110	209	1.9
7.5	50 mM imidazole	51.0	105	190	1.8
7.5	50 mM Na-phosphate	53.4	154	167	1.1
8.0	50 mM Tris	51.0	92	184	2.0
8.0	50 mM Na-phosphate	53.1	135	155	1.1
9.0	30 mM glycine	51.0	87	173	2.0
9.0	30 mM glycine + 50 mM Na-phosphate	51.2	125	141	1.1

[a] T_t is the transition temperature, defined as the temperature at which the reaction is half completed; ΔH_{cal} is the calorimetrically measured enthalpy of unfolding calculated per molecular weight of monomer (7,348 Da); ΔH_{vH} is the van't Hoff enthalpy obtained from the sharpness of the denaturational transition; β_{mw} is the ratio of van't Hoff to calorimetric enthalpies. The accuracy of enthalpy determination is 5–7%. The thermodynamic analysis of the excess heat capacity function obtained in the calorimetric experiments was performed by the procedure of Filimonov et al. (1982), a modification of a method described by Freire and Biltonen (1978). The quality of the fit, defined as a standard deviation between experimental and calculated heat capacity curves, in all cases was better than 1%. CspB was purified from *E. coli* strain (k38-cspB) containing the overexpression plasmid pGP1-2 (Willimsky et al., 1992) as described by Schindelin et al. (1992). The purity of the enzyme was at least 98% as estimated by silver staining of SDS-PAGE gels. Protein concentration was determined spectrophotometrically using an extinction coefficient of $\epsilon^{1\%}_{280nm} = 7.7$ cm^{-1}, calculated as described by Gill and von Hippel (1989). Correction for light scattering was taken into account as described by Winder and Gent (1971). Typical protein concentration in calorimetric experiments was 1–2.5 mg/mL. Before loading the solution into the calorimetric cell, protein samples were extensively dialyzed (molecular weight cutoff = 3,500 Da) at 4 °C against the desired buffer followed by centrifugation for 10–15 min at 5,000 × *g*. Heat capacity measurements were performed using a differential scanning microcalorimeter DSM-92 built at The Johns Hopkins University from a DASM-1M prototype (Privalov & Potekhin, 1986; Privalov & Plotnikov, 1989), with a temperature operation range of 0–120 °C, at a heating rate of 1 deg/min. The partial specific volume of protein required for the calculation of the partial heat capacity of protein was computed according to Makhatadze et al. (1990) and found to be 0.738 cm^3·g^{-1} at 25 °C.

In the absence of phosphate, the van't Hoff enthalpy is twice larger than the experimental one and the parameter β_{mw} is equal to 2. This suggests that the size of the cooperative unit involved in the transition is twice as large as a monomer (Privalov, 1982; Sturtevant, 1993). In other words, the unfolding of CspB in the absence of phosphate can be represented by the unfolding of a dimer:

$$N_2 \rightarrow 2U. \qquad (2)$$

There are several observations that support this scheme of unfolding. The partial specific heat capacities of the unfolded state in the presence and absence of phosphate are practically identical (Fig. 2), suggesting that the structure of the unfolded state is also similar in the 2 cases. These experimentally measured heat capacities are also close to the heat capacity expected for an unfolded protein of this amino acid composition (Fig. 2 and legend).

More evidence supporting the idea that the interaction of phosphate with CspB induces dimer dissociation comes from the

results of size-exclusion chromatography. Figure 3 presents a plot of the retention volume versus the molecular weight of CspB in solution in the presence and absence of phosphate. In the absence of phosphate, the retention volume of CspB in size-exclusion experiments corresponds to a molecular weight of 15.1 kDa, suggesting that the protein exists as a dimer (MW of monomer 7.4 kDa). The retention volume of CspB in a solution containing 50 mM phosphate is larger and corresponds to an apparent molecular weight of 7.8 kDa. The change of buffers does not notably affect the retention volume of standard proteins.

These results show that, in the absence of phosphate, CspB exists in solution as a dimer in the concentration range studied (1.5–3.4 μM of monomers) and, in the presence of low concentrations of phosphate (30–50 mM), CspB exists predominantly as a monomer. They are also consistent with the results of structure determination both by crystallography (Schindelin et al., 1993) and in solution (Schuchel et al., 1993). In the crystal, the protein exists as a dimer. In contrast, the NMR study showed that all observed NOEs in the spectra are intramolecular. It may be significant that the conditions used in the NMR structure determination included 50 mM phosphate, whereas those in crystal structure determination did not include phosphate.

Interestingly, the structure of the highly homologous cold-shock protein CspA from *Escherichia coli* (sequence identity 61%) exists as a monomer, as revealed by X-ray crystallography (Schindelin et al., 1994) and solution NMR (Newkirk et al., 1994) studies independently of the presence of phosphate. The overall structures of CspA and CspB seem to be similar, with RMS distances of ~0.55 Å. Differences are observed in the β4-strand and the loop connecting strands 4 and 5, which forms the dimer interface in CspB. In this region, the deviation between structures is up to 5 Å (Schindelin et al., 1994). These structural differences likely preclude dimer formation in CspA under conditions similar to those where it is observed with CspB.

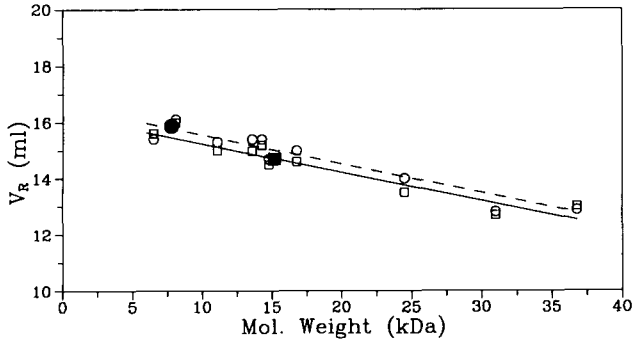

Fig. 3. Plot of the retention volume, V_R, versus the molecular weight, MW, for a variety of proteins on the Superose 12 HR 10/30 column (Pharmacia-LKB Biotechnology) in 50 mM HEPES-HCl (dashed line, $V_R = 16.2 - 0.1 \cdot MW$, $R = 0.91$) and 50 mM sodium phosphate buffers (solid line, $V_R = 16.6 - 0.1 \cdot MW$, $R = 0.92$), both at pH 7.5. Experiments were performed at 25 °C at a flow rate of 0.4 mL/min. The elution profile was monitored with a UV detector at 280 nm. The positions of CspB on the calibration plot according to the observed retention volume are shown by circles (sodium phosphate) and squares (HEPES). The proteins used for the calibration were: bovine pancreatic trypsin inhibitor, MW 6,600; eglin c, MW 8,100; ribonuclease T1, MW 11,100; ribonuclease A, MW 13,600; hen egg lysozyme, MW 14,300; intestinal fatty acid binding protein, MW 15,000; staphylococcal nuclease, MW 16,800; trypsinogen, MW 24,000; β-lactamase, MW 31,000; and β-lactoglobulin, MW 36,800.

We do not have a good molecular interpretation for the observed effect of phosphate on the self-association of CspB. One can speculate that phosphate somehow interacts with the β4-strand, sterically preventing formation of the dimer. However, the close correspondence of the β4-strand sequences in CspB (AVSFEI) and in CspA (AVSFTI) seems to rule out this possibility. An alternative explanation might be the conformational changes in the CspB molecule upon interactions with phosphate. The pair of residues Asn 55 and Arg 56 in the loop connecting strands β4 and β5 are good candidates for the potential anion binding site proposed by Chakrabarti (1993). Ongoing studies on major cold-shock proteins from different bacteria, as well as on different sets of CspB mutants, will likely shed more light on the possible role of the phosphate-dependent monomer–dimer switch of CspB in the protection of *B. subtilis* cells from damage at freezing temperatures.

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References

Chakrabarti P. 1993. Anion binding sites in protein structures. *J Mol Biol 234*:463–482.

Filimonov VV, Potekhin SA, Matveyev SV, Privalov PL. 1982. Thermodynamic analysis of scanning microcalorimetric data. 1. Algorithms for deconvolution of heat absorption curves. *Mol Biol (USSR) 16*:551–562.

Freire E, Biltonen RL. 1978. Statistical mechanical deconvolution of thermal transitions in macromolecules. I. Theory and application to homogeneous systems. *Biopolymers 17*:463–479.

Ghosaini LR, Brown AM, Sturtevant JM. 1988. Scanning calorimetric study of the thermal unfolding of catabolite activator protein from *Escherichia coli* in the absence and presence of cyclic mononucleotides. *Biochemistry 27*:5257–5261.

Gill SC, von Hippel PH. 1989. Calculation of protein extinction coefficients from amino acid sequence data. *Anal Biochem 182*:319–326.

Goldstein J, Politt NS, Inouye M. 1990. Major cold shock protein of *Escherichia coli*. *Proc Natl Acid Sci USA 87*:283–287.

Graumann P, Marahiel MA. 1994. The major cold shock protein from *Bacillus subtilis* CspB binds with high affinity to the ATTGG- and CCAAT sequence in single stranded oligonucleotides. *FEBS Lett 338*:157–160.

Kraulis PJ. 1991. MOLSCRIPT: A program to produce both detailed and schematic plots of protein structure. *J Appl Crystallogr 24*:946–950.

Makhatadze GI, Kim KS, Woodward C, Privalov PL. 1993. The thermodynamics of BPTI folding. *Protein Sci 2*:2028–2036.

Makhatadze GI, Medvedkin VN, Privalov PL. 1990. Partial molar volumes of polypeptides and their constituent groups in aqueous solution over a broad temperature range. *Biopolymers 30*:1001–1010.

Makhatadze GI, Privalov PL. 1990. Heat capacity of proteins. I. Partial molar heat capacity of individual amino acid residues in aqueous solutions. Hydration effect. *J Mol Biol 213*:375–384.

Newkirk K, Feng W, Jiang W, Tejero R, Emerson SD, Inouye M, Montelione GT. 1994. Solution NMR structure of the major cold shock protein CspA from *Escherichia coli*: Identification of a binding epitope for DNA. *Proc Natl Acad Sci USA 91*:5114–5118.

Privalov PL. 1982. Stability of proteins. Proteins which do not present a single cooperative unit. *Adv Protein Chem 35*:1–104.

Privalov PL, Khechinashvili NN. 1974. A thermodynamic approach to the problem of stabilization of globular protein structure: A calorimetric study. *J Mol Biol 86*:665–684.

Privalov PL, Plotnikov VV. 1989. Three generations of scanning microcalorimeters for liquids. *Thermochim Acta 139*:257–277.

Privalov PL, Potekhin SA. 1986. Scanning microcalorimetry in studying temperature-induced changes in proteins. *Methods Enzymol 131*:4–51.

Schindelin H, Herrler M, Willimsky G, Marahiel MA, Heinemann U. 1992. Overproduction crystallization and preliminary X-ray diffraction stud-

ies of the major cold shock protein from *Bacillus subtilis* CspB. *Proteins Struct Funct Genet 14*:120–124.

Schindelin H, Jiang W, Inouye M, Heinemann U. 1994. Crystal structure of CspA the major cold shock protein of *Escherichia coli*. *Proc Natl Acad Sci USA 91*:5119–5123.

Schindelin H, Marahiel MA, Heinemann U. 1993. Universal nucleic acid-binding domain revealed by crystal structure of the I major cold-shock protein. *Nature 264*:164–168.

Schröder K, Zuber P, Willimsky G, Wagner B, Marahiel MA. 1994. Mapping of the *Bacillus subtilis* cspB gene and cloning of its homologs in thermophilic, mesophilic and psychrotropic bacilli. *Gene 136*:277–280.

Schuchel A, Wiltscheck R, Czisch M, Herrel M, Willimsky G, Graumann P, Marahiel MA, Holak TA. 1993. Structure in solution of the major cold-shock protein from *Bacillus subtilis*. *Nature 364*:169–171.

Sturtevant JM. 1987. Biochemical applications of differential scanning calorimetry. *Annu Rev Phys Chem 38*:463–488.

Sturtevant JM. 1993. Effects of mutations on the thermodynamics of proteins. *Pure Appl Chem 65*:991–998.

Willimsky G, Bang H, Fisher G, Marahiel MA. 1992. Characterization of cspB a *Bacillus subtilis* inducible cold shock gene affecting cell viability at low temperatures. *J Bacteriol 174*:6326–6335.

Winder AF, Gent WLC. 1971. Correction of light-scattering errors in spectrophotometric protein determinations. *Biopolymers 10*:1243–1251.

Wistow G. 1990. Cold-shock and DNA binding. *Nature 344*:823–824.

Wolffe AP, Tafuri S, Ranjan M, Familari M. 1992. The Y-box factors: A family of nucleic binding proteins conserved from *Escherichia coli* to man. *New Biol 4*:290–298.