ChemBioChem

Supporting Information

Insights into Protein Stability in Cell Lysate by ^{19}F NMR Spectroscopy

Hannah Welte and Michael Kovermann*

Supporting Information

Table of Contents

Experimental Section

Expression and purification of fluorinated *Bs*CspB

Fluorinated variants of the cold shock proteins B from *Bacillus subtilis* were expressed *in E. coli* cells (DSMZ 12779 strain has been used for the phenylalanine variant whereas the strain CAG 18455 7371 has been used for the tryptophan variant) by using pET24a CspB and pAR1219 vectors as described before [1]. Protein purification was done as outlined previously [1].

Preparation of cell lysate samples

An optical density (OD) of 0.55 has been used for the growth of 5-^{19}F-Trp-*Bs*CspB whereas OD = 0.71 has been used for the growth of 4-^{19}F-Phe-*Bs*CspB regarding harvesting the cells. A volume of 50,125, and 250 mL of cell culture (Table S4) was centrifuged at 6500 rpm for t = 15 min at T = 4 °C, respectively. The cell pellet was resuspended in V = 3.5-5 mL TRIS buffer (50 mM TRIS, pH 8) and disrupted by sonication (sonifier W-250, Branson) for 1600 s/100 mL of cell-dispersion with an amplitude of 25 % in cycles of t = 2 s work and t = 3 s break on ice (for details, see Table S4). The cell lysate was centrifuged at 4500 rpm for t = 15 min. From the supernatant two samples were prepared and placed in two separated 5 mm NMR tubes. Both samples contain 95 % H_2O and 5 % D_2O (*v*/*v*) and trimethylsilylproanoic acid (TMSP) for referencing. Urea was added to one of the two tubes possessing a concentration in the range between about 6 and 7 M. Next, the two samples were mixed among each other to obtain concentrations of urea ranging between 0 and about 7 M and keeping concentrations of all other molecules constant. The following scheme illustrates this experimental setup for probing the folding-to-unfolding reaction of 5-^{19}F-Trp-*Bs*CspB under dilute conditions:

data point	c^{urea} / M	sample
1	5.86	Tube 1 (start volume: 1500 µL)
2	0.00	Tube 2 (start volume: 1500 µL)
3	5.52	Tube 1 + 50 µl of Tube 2
4	0.13	Tube 2 + 50 µl of Tube 1
5	5.39	Tube 1 + 55 µl of Tube 2
6	0.23	Tube 2 + 55 µl of Tube 1
7	5.20	Tube 1 + 55 µl of Tube 2
8	0.59	Tube 2 + 55 µl of Tube 1
9	5.05	Tube 1 + 70 µl of Tube 2
10	0.82	Tube 2 + 70 µl of Tube 1
11	4.84	Tube 1 + 70 µl of Tube 2
12	1.07	Tube 2 + 70 µl of Tube 1
13	4.58	Tube 1 + 80 µl of Tube 2
14	1.36	Tube 2 + 80 µl of Tube 1
15	4.32	Tube 1 + 80 µl of Tube 2
16	1.57	Tube 2 + 80 µl of Tube 1
17	4.12	Tube 1 + 90 µl of Tube 2
18	1.80	Tube 2 + 90 µl of Tube 1
19	3.88	Tube 1 + 100 µl of Tube 2
20	2.05	Tube 2 + 100 µl of Tube 1
21	3.64	Tube 1 + 110 µl of Tube 2
22	2.35	Tube 2 + 110 µl of Tube 1
23	3.45	Tube 1 + 130 µl of Tube 2
24	2.52	Tube 2 + 130 µl of Tube 1
25	3.26	Tube 1 + 165 µl of Tube 2
26	2.70	Tube 2 + 165 µl of Tube 1
27	3.05	Tube 1 + 220 µl of Tube 2
28	2.88	Tube 2 + 220 µl of Tube 1
29	2.98	Tube 1 + 300 µl of Tube 2
30	2.98	Tube 2 + 300 µl of Tube 1

NMR spectroscopy

All one-dimensional ^{1}H and ^{19}F NMR spectra were acquired on an 800 MHz Bruker Avance NEO NMR spectrometer equipped with a TCI cryogenically cooled probe at T = 298 K. Two-dimensional ^{1}H-^{15}N HSQC NMR spectrum of cell lysate were collected on the 800 MHz spectrometer, too. Exceptionally, the one-dimensional ^{1}H NMR spectra of $c^{5\text{-}^{19}F\text{-}Trp\text{-}BsCspB}$ = 8 μM as well as the two-dimensional ^{1}H-^{15}N HSQC NMR spectrum (under dilute conditions) of this protein variant were acquired on a 600 MHz Bruker Avance III NMR spectrometer also equipped with a TCI cryogenically cooled probe at T = 298 K. The proton resonance frequency of Trimethylsilylproanoic acid (TMSP) was used for direct referencing of the ^{1}H dimension and for indirect referencing of the ^{19}F dimension. Data processing of one-dimensional spectra was done by using TOPSPIN 4.0.3 software (Bruker Biospin, Germany). Processing of two-dimensional NMR data was done by using NMRPipe [2].

The overall thermodynamic stability of fluorinated *Bs*CspB has been determined at different cell lysate concentrations by using one-dimensional ^{19}F NMR spectra obtained at different concentrations of urea ranging between c = 0 M and c = 7 M by mixing two NMR tubes among each other (see above). Furthermore, the overall thermodynamic stability of fluorinated *Bs*CspB was determined under dilute conditions using a protein concentration of c = 100 µM for 4-^{19}F-Phe-*Bs*CspB and c = 200 µM for 5-^{19}F-Trp-*Bs*CspB applying both one-dimensional ^{1}H and ^{19}F NMR spectroscopy, respectively. Additionally, the overall thermodynamic stability of 5-^{19}F-Trp-*Bs*CspB was determined under dilute conditions at a protein concentration of c = 8 µM using one-dimensional ^{1}H NMR spectroscopy. Specific ranges of chemical shifts were used to determine the fraction of native protein, f_N. In detail, one range of chemical shifts representing resonance signals seen for the folded state, I_N, and a second range of chemical shifts representing resonance signals seen for folded state and unfolded protein ensemble, I_{N+U}, were used for the analysis of one-dimensional ^{1}H NMR spectra acquired for 4-^{19}F-Phe-*Bs*CspB and 5-^{19}F-Trp-*Bs*CspB and of one-dimensional ^{19}F NMR spectra acquired for 4-^{19}F-Phe-*Bs*CspB. Contrary, a range of chemical shifts comprising native state signals, I_N, and resonance signals seen solely for the unfolded protein ensemble, I_U, were used for the analysis of ^{19}F NMR spectra of 5-^{19}F-Trp-*Bs*CspB. Calculating the ratio $I_N/(I_N+I_{N+U})$ or $I_N/(I_N+I_U)$ makes it possible to determine the population of the folded state, f_N, of 4-^{19}F-Phe-*Bs*CspB and 5-^{19}F-Trp-*Bs*CspB at any concentration of urea, respectively.

The fraction of folded protein, f_N, was then used in equation (S1) to determine, firstly, the difference in free energy between the folded state and the unfolded protein ensemble of *Bs*CspB, ΔG^0, and, secondly, the cooperativity of protein unfolding, m:

$$f_{\mathrm{N}} = \frac{(g_{\mathrm{N}} + \mathrm{m}_{\mathrm{N}} * c_{\mathrm{urea}}) + (g_{\mathrm{U}} + m_{\mathrm{U}} * c_{\mathrm{urea}}) * \left(\exp\left(-\frac{\Delta G^{0}}{RT}\right) + \frac{m * c_{\mathrm{urea}}}{RT}\right)}{1 + \left(\exp\left(-\frac{\Delta G^{0}}{RT}\right) + \frac{m * c_{\mathrm{urea}}}{RT}\right)}, \qquad \text{(S1)}$$

where f_N represents the fraction of folded protein, $g_{N/U}$ and $m_{N/U}$ account for the baselines of the folded state and the unfolded protein ensemble, R is the universal gas constant, c_{urea} is the concentration of urea and T is the absolute temperature [3]. The folding cooperativity, m, was used as a global parameter analyzing seven ^{19}F NMR and two ^{1}H NMR data sets following folding-to-unfolding transition. Thus, m was determined to m = -3.3 ± 0.1 kJ/(molM). This value was then utilized to analyze the folding-to-unfolding transition of 5-^{19}F-Trp-*Bs*CspB using a protein concentration of c = 8 µM.

Trifluoroacetic acid (TFA) with known concentration was added to the respective protein sample enabling the determination of the concentration of the protein present in cell lysate by integration of resonance signals present in an one-dimensional ^{19}F NMR spectrum (Figure S10).

The concentration of urea, c^{urea}, was determined by measuring the refractive index applying dilute conditions [4]. This method is not applicable to samples at cell lysate condition due to the presence of a large signal background. Nevertheless, since the initial concentrations of c^{urea} in cell lysate samples was known respective c^{urea} values determining the x-coordinate in a folding-to-unfolding transition could be determined using sample volumes which have been used during the mixing of the two NMR tubes among each other.

Results and Discussion

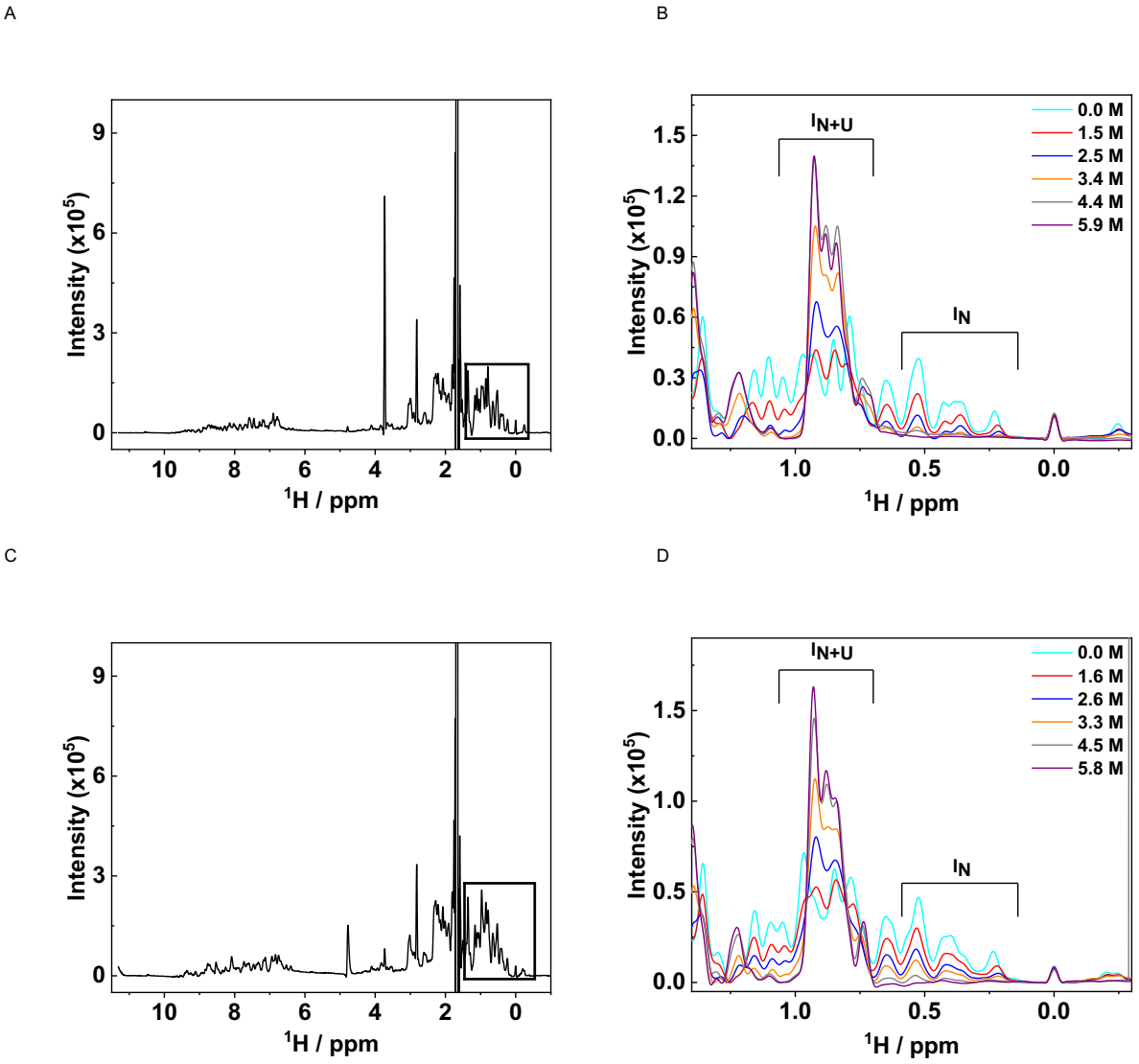
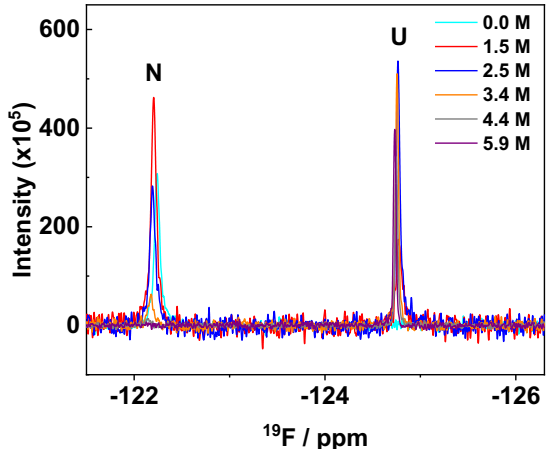


Figure S1

One-dimensional ^{1}H NMR spectrum of 5-^{19}F-Trp-*Bs*CspB acquired at c^{urea}= 0 M under dilute conditions at T = 298 K and B_0 = 18.8 T. The spectral range shown in (B) is indicated by using a box in (A). For the determination of the fraction of native protein, f_N (see equation (S1)), two spectral ranges differing in chemical shifts were used. The first range, I_N, represents resonance signals observed solely for the native state and covers chemical shifts between 0.59 ppm and 0.14 ppm ((B) and (D)). The second range, I_{N+U}, covers chemical shifts between 0.697 ppm and 1.064 ppm and represents resonance signals for both the native state and the unfolded protein ensemble, respectively ((B) and (D)). Both chemical shifts and resonance signal intensities of aliphatic protons of 5-^{19}F-Trp-*Bs*CspB are directly dependent on the concentration of urea which has been applied: c^{urea}= 0 M is colored in cyan, c^{urea}= 1.5 M is colored in red, c^{urea}= 2.5 M is colored in blue, c^{urea}= 3.4 M is colored in orange, c^{urea}= 4.4 M is colored in gray and c^{urea}= 5.9 M is colored in magenta. (C) One-dimensional ^{1}H NMR spectrum of 4-^{19}F-Phe-*Bs*CspB acquired at c^{urea}= 0 M under dilute conditions at T = 298 K and B_0 = 18.8 T. The spectral range shown in (D) is indicated by using a box in (C). Both chemical shifts and resonance signal intensities of aliphatic protons of 4-^{19}F-Phe-*Bs*CspB are directly dependent on the concentration of urea which has been applied: c^{urea}= 0 M is colored in cyan, c^{urea}= 1.6 M is colored in red, c^{urea}= 2.6 M is colored in blue, c^{urea}= 3.3 M is colored in orange, c^{urea}= 4.5 M is colored in gray and c^{urea}= 5.8 M is colored in magenta.

A



B

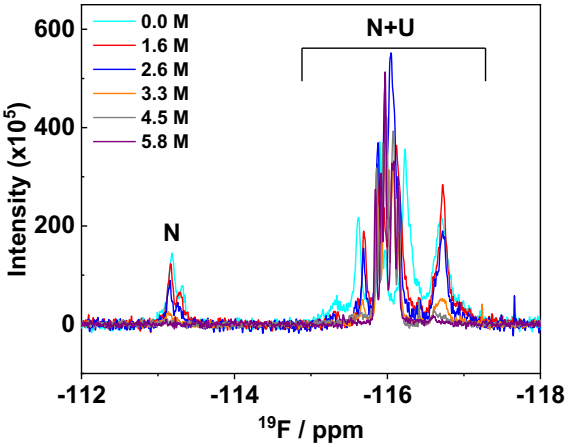
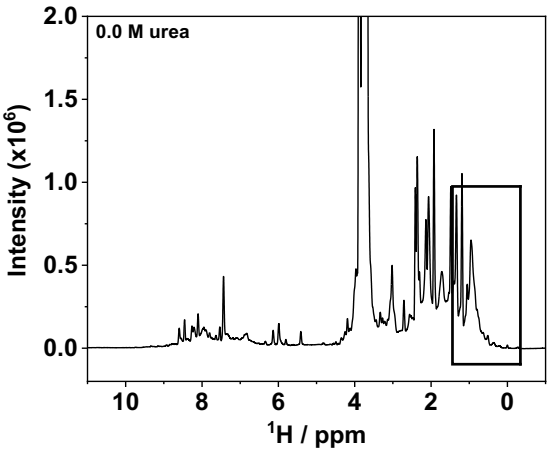


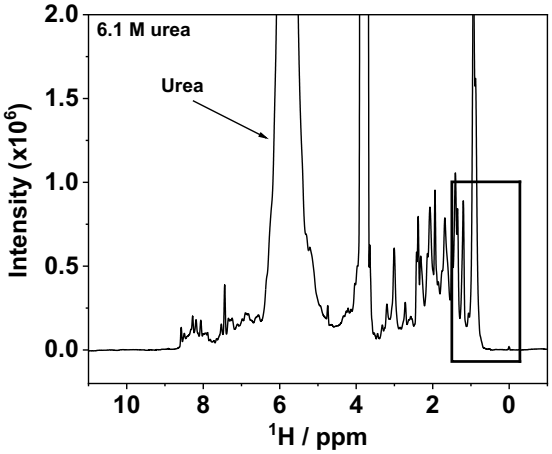
Figure S2

(A) One-dimensional ^{19}F NMR spectra of 5-^{19}F-Trp-*Bs*CspB comprising a single fluorinated site at sequence position W8 acquired at different concentrations of urea (c^{urea}= 0 M colored in cyan, c^{urea}= 1.5 M colored in red, c^{urea} = 2.5 M colored in blue, c^{urea}= 3.4 M colored in orange, c^{urea}= 4.4 M colored in gray and c^{urea}= 5.9 M colored in magenta) under dilute conditions at T = 298 K and B_0 = 18.8 T. The fluorine resonance signal representing the native state of 5-^{19}F-Trp-*Bs*CspB is highlighted by "N" whereas the resonance signal of the unfolded protein ensemble is indicated by "U". (B) One-dimensional ^{19}F NMR spectra of 4-^{19}F-Phe-*Bs*CspB comprising seven fluorinated sites at sequence positions F9, F15, F17, F27, F30, F38, and F49 acquired at different concentrations of urea (c^{urea}= 0 M colored in cyan, c^{urea}= 1.6 M colored in red, c^{urea}= 2.6 M colored in blue, c^{urea}= 3.3 M colored in orange, c^{urea}= 4.5 M colored in gray and c^{urea}= 5.8 M colored in magenta) under dilute conditions at T = 298 K and B_0 = 18.8 T. Fluorine resonance signals representing the native state of 4-^{19}F-Phe-*Bs*CspB – which are spectrally separated - are highlighted by "N" whereas resonance signals representing the native state and the unfolded protein ensemble are indicated by "N+U".

A



B



C

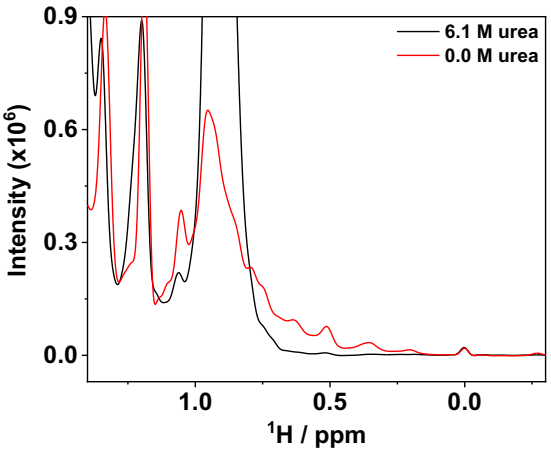
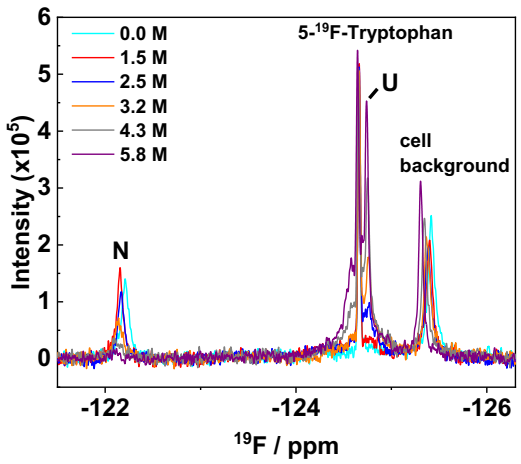


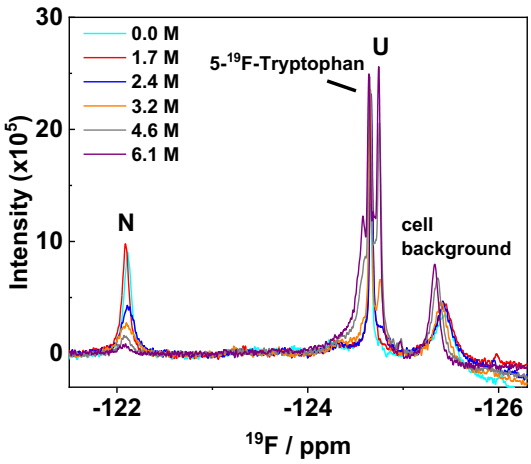
Figure S3

One dimensional ^{1}H NMR spectra of 5-^{19}F-Trp-*Bs*CspB in cell lysate with *c* = 0.0 M urea (A) and *c* = 6.1 M urea (B). Cell lysate concentration of *c* = 260 g/L was used. The spectral range shown in (C) is indicated by using a box in (A) and (B). The large signal intensity at about 6 ppm in (B) originates from urea.

A



B



C

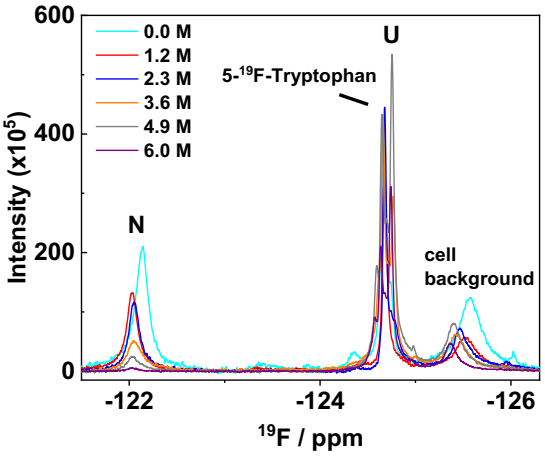
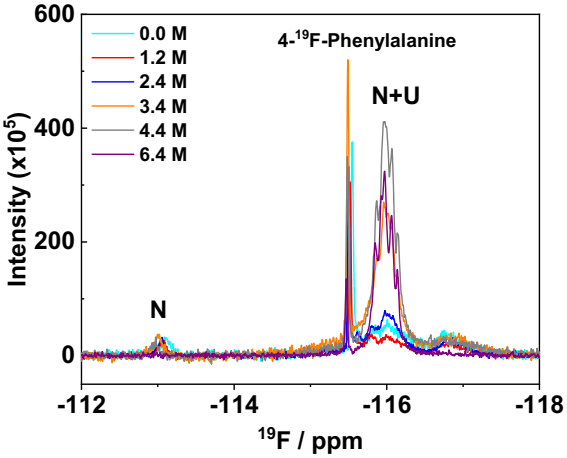


Figure S4

One-dimensional ^{19}F NMR spectra of 5-^{19}F-Trp-*Bs*CspB acquired at different concentrations of urea in cell lysate at T = 298 K and B_0 = 18.8 T. The following concentrations of cell lysate have been used: c = 120 g/L (A), c = 260 g/L (B) and c = 450 g/L (C). The increasing concentration of urea is represented by applying a color code used for plotting the NMR spectra ranging from cyan to red, blue, orange, gray and finally to magenta. The resonance signals of free 5-^{19}F-Trp, of native state ("N") and the unfolded protein ensemble ("U") of 5-^{19}F-Trp-*Bs*CspB are indicated. Resonance signals arising from the background of the cells have been labelled, too.

A



B

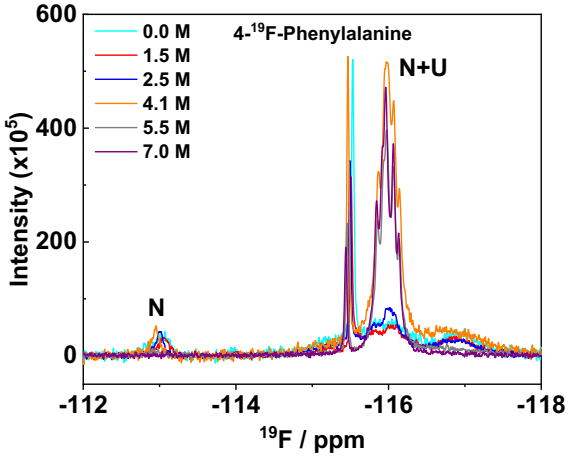


Figure S5

One-dimensional ^{19}F NMR spectra of 4-^{19}F-Phe-*Bs*CspB acquired at different concentrations of urea in cell lysate at T = 298 K and B_0 = 18.8 T. The following concentrations of cell lysate have been used: c = 150 g/L (A) and c = 450 g/L (B). The increasing concentration of urea is represented by applying a color code used for plotting the NMR spectra ranging from cyan to red, blue, orange, gray and finally to magenta. The resonance signals of free 4-^{19}F-Phe, of native state ("N") and native state plus the unfolded protein ensemble ("N+U") of 4-^{19}F-Phe-*Bs*CspB are indicated.

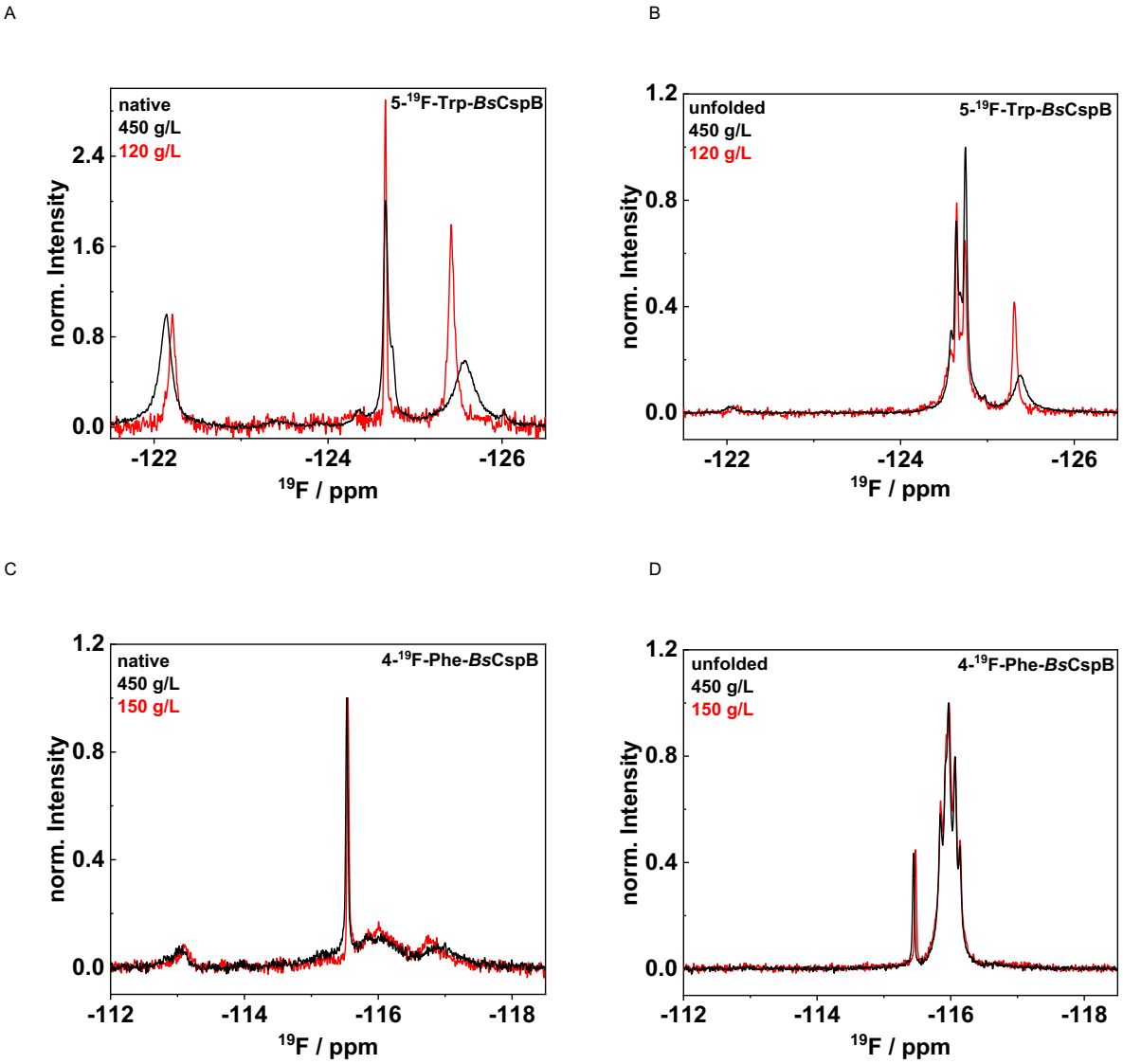


Figure S6

(A) One-dimensional ^{19}F NMR spectra representing the folded state of 5-^{19}F-Trp-*Bs*CspB present at c^{urea} = 0.0 M and $c^{cell\ lysate}$ = 120 g/L (colored in red) or c^{urea} = 0.0 M and $c^{cell\ lysate}$ = 450 g/L (colored in black). (B) One-dimensional ^{19}F NMR spectra representing the unfolded protein ensemble of 5-^{19}F-Trp-*Bs*CspB present at c^{urea} = 5.5 M and $c^{cell\ lysate}$ = 120 g/L (colored in red) or c^{urea} = 5.5 M and $c^{cell\ lysate}$ = 450 g/L (colored in black). (C) One-dimensional ^{19}F NMR spectra representing the folded protein ensemble of 4-^{19}F-Phe-*Bs*CspB present at c^{urea} = 0.0 M and $c^{cell\ lysate}$ = 150 g/L (colored in red) or c^{urea} = 0.0 M and $c^{cell\ lysate}$ = 450 g/L (colored in black). (D) One-dimensional ^{19}F NMR spectra representing the unfolded protein ensemble of 4-^{19}F-Phe-*Bs*CspB present at c^{urea} = 5.2 M and $c^{cell\ lysate}$ = 150 g/L (colored in red) or c^{urea} = 5.3 M and $c^{cell\ lysate}$ = 450 g/L (colored in black).

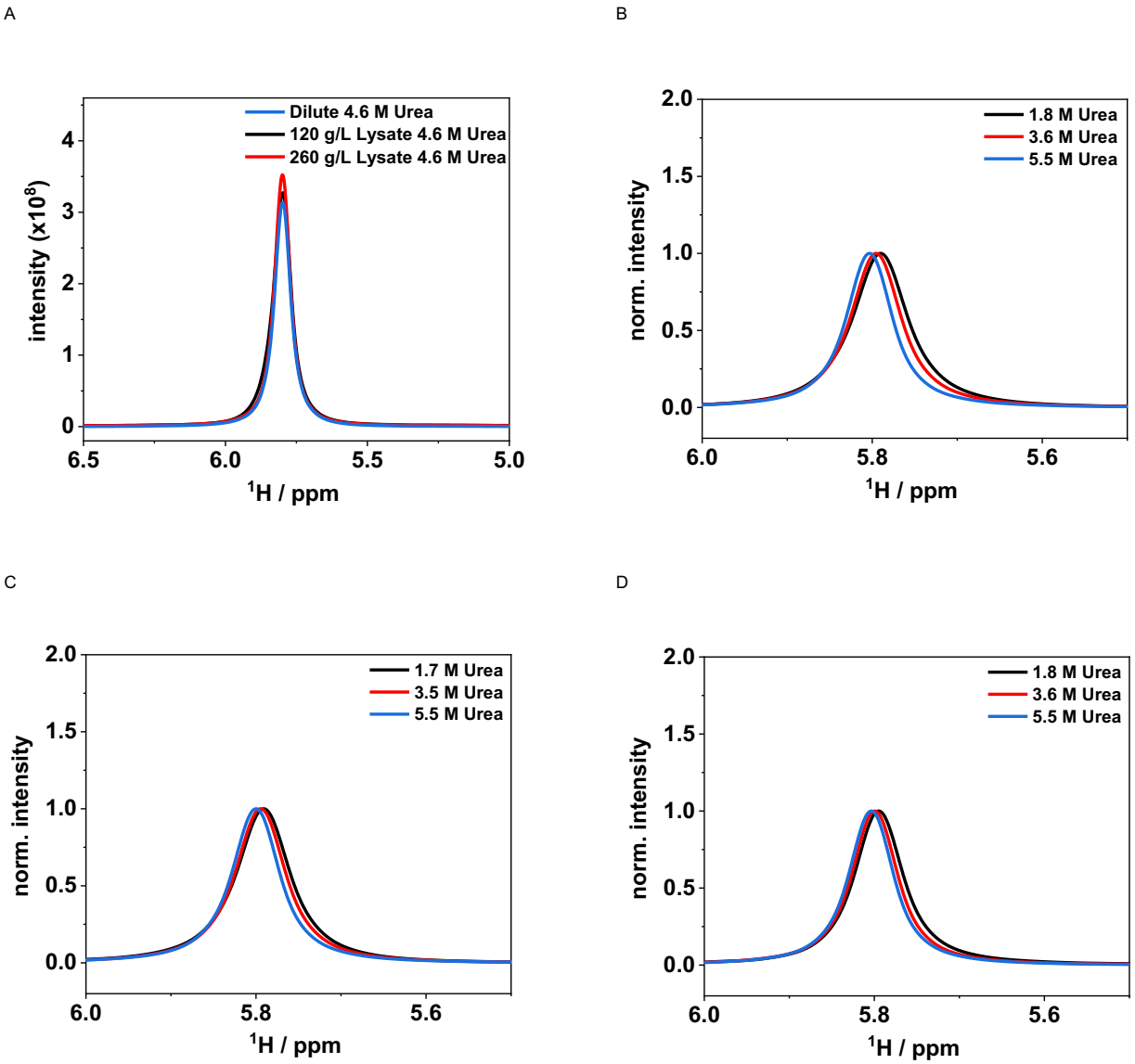


Figure S7

Analysis of the chemical shift arising from urea in one-dimensional proton NMR spectra. All data have been acquired at T = 298 K. (A) Proton resonance signal originating from solutions containing a concentration of 4.6 M urea applying different experimental conditions while all acquisition parameters have been kept constant. Data acquisition has been done under dilute conditions (colored in blue), 120 g/L cell lysate (colored in black), and 260 g/L cell lysate (colored in red) concentration. (B) Proton resonance signal originating from solutions containing different urea concentrations under dilute conditions. Data acquisition has been done in presence of c^{urea} = 1.8 M ($\omega^{1H} \simeq$ 5.790 ppm, colored in black), c^{urea} = 3.6 M ($\omega^{1H} \simeq$ 5.795 ppm, colored in red), c^{urea} = 5.5 M ($\omega^{1H} \simeq$ 5.803 ppm, colored in blue). (C) Proton resonance signal originating from solutions containing different urea concentrations in presence of 120 g/L cell lysate. Data acquisition has been done in presence of c^{urea} = 1.7 M ($\omega^{1H} \simeq$ 5.791 ppm, colored in black), c^{urea} = 3.5 M ($\omega^{1H} \simeq$ 5.795 ppm, colored in red), c^{urea} = 5.5 M ($\omega^{1H} \simeq$ 5.800 ppm, colored in blue). (D) Proton resonance signal originating from solutions containing different urea concentrations in presence of 450 g/L cell lysate. Data acquisition has been done in presence of c^{urea} = 1.8 M ($\omega^{1H} \simeq$ 5.795 ppm, colored in black), c^{urea} = 3.6 M ($\omega^{1H} \simeq$ 5.8000 ppm, colored in red), c^{urea} = 5.5 M ($\omega^{1H} \simeq$ 5.804 ppm, colored in blue). These experimental data have been obtained during the acquisition of folding-to-unfolding transitions of 5-^{19}F-Trp-*Bs*CspB (c = 200 μM (dilute conditions), c = ~5 μM (120 g/L cell lysate), c = ~25 μM (260 g/L cell lysate), c = ~70 μM (450 g/L cell lysate)).

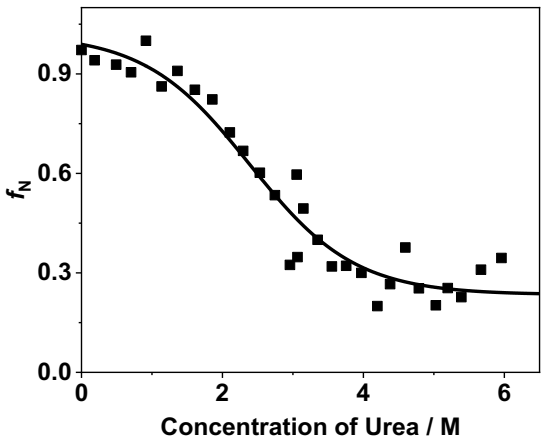


Figure S8

Folding-to-unfolding transition determined for 5-^{19}F-Trp-*Bs*CspB induced by an increasing concentration of urea under dilute conditions acquired at T = 298 K by using a protein concentration of c = 8 μM only. The overall thermodynamic stability ΔG^0 has been determined to ΔG^0 = (7.8 ± 0.5) kJ/mol using a folding cooperativity, m, of m = -3.3 ± 0.1 kJ/(molM).

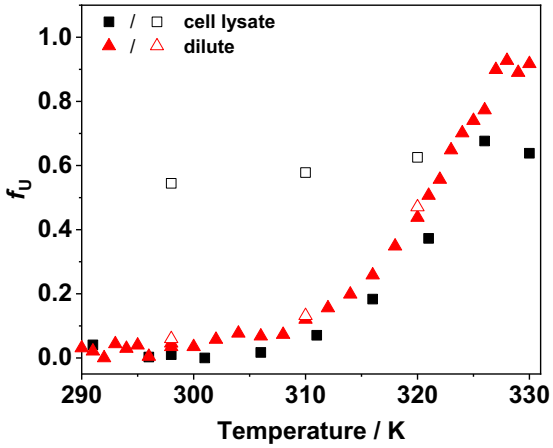


Figure S9

Folding-to-unfolding transition determined for 5-^{19}F-Trp-*Bs*CspB induced by increasing temperature under dilute conditions (triangles, colored in red) and in cell lysate (rectangles, colored in black). The direction of the increase in temperature is represented by using closed symbols whereas lowering the temperature is represented by open symbols. The concentration of cell lysate was set to 240 g pellet per L lysis buffer. Experimental data have been obtained by acquiring one-dimensional fluorine NMR spectra and the fraction of the unfolded protein ensemble, f_U, has been calculated as outlined in the Materials and Methods part.

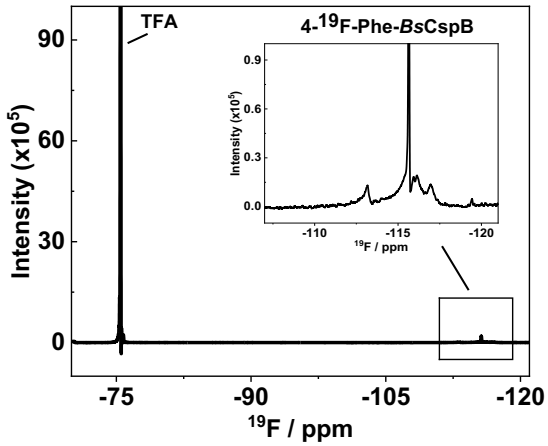


Figure S10

One-dimensional ^{19}F NMR spectrum comprising resonances of Trifluoroacetic acid (TFA) and 4-^{19}-Phe-*Bs*CspB acquired in cell lysate at T = 298 K and B_0 = 18.8 T. The resonance signal of TFA originating from a concentration of 2 mM was used here to determine the concentration of 4-^{19}-Phe-*Bs*CspB in cell lysate. The resonance signal at about ω^{19F} = -115.5 ppm indicates free 4-^{19}F-Phe present in cell lysate. The results obtained for the concentration of 4-^{19}-Phe-*Bs*CspB and 5-^{19}F-Trp-*Bs*CspB in cell lysate by using this methodology are presented in Tables S1, S2.

Table S1. Estimation of the concentration of 4-^{19}F-Phe-*Bs*CspB present at different concentrations of cell lysate determined by applying one-dimensional ^{19}F NMR spectroscopy and using the resonance signal of TFA (see Figure S9). In absence of cell lysate, the concentration of 4-^{19}F-Phe-*Bs*CspB has been determined using absorbance.

$c^{\text{cell lysate}}$ / g cell pellet/ L lysis buffer	$c^{\text{4-19F-Phe-}BsCspB}$ / µM
0	100.0
150	~ 5
450	~ 10

Table S2. Estimation of the concentration of 5-^{19}F-Trp-*Bs*CspB variant present at different concentrations of cell lysate determined by applying one-dimensional ^{19}F NMR spectroscopy and using the resonance signal of TFA (see Figure S9). In absence of cell lysate, the concentration of 5-^{19}F-Trp-*Bs*CspB has been determined using absorbance.

$c^{\text{cell lysate}}$ / g cell pellet/ L lysis buffer	$c^{\text{5-19F-Trp-}BsCspB}$ / µM
0	200.0
120	~ 5
240	~ 20
260	~ 25
450	~ 70

Table S3. Relation of the weight of dry cell pellet to the weight of wet cell pellet per litre of lysis buffer [5].

$c^{\text{cell lysate}}$ / g wet cell pellet / L lysis buffer	$c^{\text{cell lysate}}$ / g dry cell pellet / L lysis buffer
120	30
150	35
240	55
260	60
450	100

Table S4. Volume and mass of used cell culture, cell pellet and lysis buffer and resulting cell lysate concentration.

V cell culture / mL	*m* cell pellet / g	*V* lysis buffer / mL	*c* cell lysate / g cell pellet / L lysis buffer
50	0.36	3.0	120
125	1.20	5.0	240
125	1.29	5.0	260
125	1.66	3.7	450
125	0.75	5.0	150
250	1.96	4.4	450

References

[1] H. Welte, M. Kovermann, *Protein Expres Purif* **2019**, *157*, 86-91.
[2] F. Delaglio, S. Grzesiek, G. W. Vuister, G. Zhu, J. Pfeifer, A. Bax, *J Biomol Nmr* **1995**, *6*, 277-293.
[3] M. M. Santoro, D. W. Bolen, *Biochemistry* **1988**, *27*, 8063-8068.
[4] J. R. Warren, J. A. Gordon, *J Phys Chem* **1966**, *70*, 297-&.
[5] J. Glazyrina, E.-M. Materne, T. Dreher, D. Storm, S. Junne, T. Adams, G. Greller, P. Neubauer, *Microbial Cell Factories* **2010**, *9*, 42.

Author Contributions

HW and MK designed research. HW performed research. HW analyzed data. HW and MK wrote the manuscript.