SUPPORTING INFORMATION FOR

# The Temperature Dependence of the Hofmeister Series:

# Thermodynamic Fingerprints of Cosolute-Protein Interactions**

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## S.1 Analysis of DSC data

### S.1.1 Calculation of partial heat capacity

To correct for heat capacity differences between the sample and reference cell a buffer scan (buffer vs. buffer) was subtracted from the protein scan (protein vs. buffer) to obtain $\Delta C_{\mathrm{p}}^{\mathrm{app}}(T)$. The partial heat capacity of RNase A $C_{\mathrm{p,pr}}^{\mathrm{exp}}(T)$ was then calculated according to eq. 1, where $T$ is the absolute temperature, $C_{\mathrm{p,water}}$ the heat capacity of water, $V_{\mathrm{pr}}^{\mathrm{part}}$ is the partial specific volume of RNase A in the sample, $V_{\mathrm{tot}}$ is the total volume of the sample solution and $n$ is the amount of protein in mol. $V_{\mathrm{pr}}^{\mathrm{part}}$ of RNase A was calculated based on the partial molar volumes of the amino acids at 25 °C as described by Makhatadze *et al.*[1]

$$C_{\mathrm{p,pr}}^{\mathrm{exp}}(T) = \left( \Delta C_{\mathrm{p}}^{\mathrm{app}}(T) + C_{\mathrm{p,water}}(T) \frac{V_{\mathrm{pr}}^{\mathrm{part}}}{V_{\mathrm{tot}}} \right) / n \qquad 1$$

### S.1.2 Two-state transition model for DSC analysis

All DSC scans proved to be reversible indicating an equilibrium between folded and unfolded state at any temperature. This allows a meaningful extraction of equilibrium thermodynamic data from the thermograms. In the two-state protein folding model the melting temperature, $T_{\mathrm{m}}$, is defined as the temperature at which the populations of the folded ($F_{\mathrm{N}}$) and unfolded state ($F_{\mathrm{U}}$) are equal. Both populations can be related to the equilibrium constant, $K$ (eq. 2), where $R$ is the gas constant and $\Delta G_{\mathrm{u}}$ the Gibbs free energy change of unfolding,

$$K = e^{-\Delta G_{\mathrm{u}}/RT} \qquad 2$$

yielding

$$F_{\mathrm{N}}(T) = 1/(1+K) \qquad 3$$

$$F_{\mathrm{U}}(T) = K/(1+K) \qquad 4$$

Standard thermodynamics relates $\Delta G_{\mathrm{u}}$ to the heat capacities of the folded and unfolded protein ($C_{\mathrm{p,N}}$ and $C_{\mathrm{p,U}}$, respectively) by the integrated form of the Gibbs-Helmholtz equation (eq. 5). Assuming a temperature-independent heat capacity change upon unfolding, $\Delta C_{\mathrm{p}} = C_{\mathrm{p,U}} - C_{\mathrm{p,N}}$, the integrated Gibbs-Helmholtz equation is given by

$$\Delta G_{\mathrm{u}} = T_{\mathrm{m}} - T/T_{\mathrm{m}} * \Delta H_{\mathrm{u,fit}}(T_{\mathrm{m}}) + \Delta C_{\mathrm{p}} * (T - T_{\mathrm{m}}) + T * \Delta C_{\mathrm{p}} * \ln(T_{\mathrm{m}}/T), \quad 5$$

Where $\Delta H_{\mathrm{u,fit}}(T_{\mathrm{m}})$ is the enthalpy change upon unfolding at $T_{\mathrm{m}}$. Likewise, the temperature dependence for the enthalpy reads

$$\Delta H_{\mathrm{u}} = \Delta H_{\mathrm{u,fit}}/(T_{\mathrm{m}}) + \Delta C_{\mathrm{p}} * (T - T_{\mathrm{m}}) , \quad 6$$

resulting in an excess heat capacity $C_{\mathrm{p}}^{\mathrm{exc}}$:[2, 3]

$$C_{\mathrm{p}}^{\mathrm{exc}}(T) = [\Delta H_{\mathrm{u}}(T)^2/(R * T^2)] * [K/(1 + K)^2], \quad 7$$

Taken together, we obtain the final expression for the two-state model

$$C_{\mathrm{p,pr}}(T) = F_{\mathrm{N}}(T) * C_{\mathrm{p,N}}(T) + C_{\mathrm{p}}^{\mathrm{exc}}(T) + F_{\mathrm{U}}(T) * C_{\mathrm{p,U}}(T), \quad 8$$

which can be fitted to $C_{\mathrm{p,pr}}^{\mathrm{exp}}(T)$ of RNase A to obtain $T_{\mathrm{m}}$, $\Delta H_{\mathrm{u,fit}}(T_{\mathrm{m}})$, and $\Delta C_{\mathrm{p}}(T_{\mathrm{m}})$. Here, $C_{\mathrm{p,N}}(T)$ and $C_{\mathrm{p,U}}(T)$ of RNase A are represented by linear equations (eqs. 9&10).

$$C_{\mathrm{p,N}}(T) = A_{\mathrm{N}} * (T - 273.15) + B_{\mathrm{N}} \quad 9$$

$$C_{\mathrm{p,U}}(T) = A_{\mathrm{U}} * (T - 273.15) + B_{\mathrm{U}} \quad 10$$

### S.1.3 Calculation of excess heat capacity profile

The so-called excess heat capacity function $< C_\mathrm{p}(T) >^\mathrm{exc}$ is caused solely by the heat required to unfold the protein. It is calculated by subtracting the progress heat capacity function $C_\mathrm{p,pr}^\mathrm{prg}(T)$. The respective equations are

$$C_\mathrm{p,pr}^\mathrm{prg}(T) = F_\mathrm{N}(T) * C_\mathrm{p,N}(T) + F_\mathrm{U}(T) * C_\mathrm{p,U}(T), \quad 11$$

and

$$< C_\mathrm{p}(T) >^\mathrm{exc} = C_\mathrm{p,pr}^\mathrm{exp}(T) - C_\mathrm{p,pr}^\mathrm{prg}(T), \quad 12$$

The excess heat capacity of RNase A is then used to calculate the calorimetric enthalpy change at $T_\mathrm{m}$, $\Delta H_\mathrm{u,cal}(T_\mathrm{m})$ (eq. 13), and the van't Hoff enthalpy change at $T_\mathrm{m}$, $\Delta H_\mathrm{u,vH}(T_\mathrm{m})$ (eq. 14), where $< C_\mathrm{p}(T_\mathrm{m}) >^\mathrm{exc,max}$ is the maximum of $< C_\mathrm{p}(T) >^\mathrm{exc}$. Note, $\Delta H_\mathrm{u,fit}$ is a van't Hoff enthalpy as well.

$$\Delta H_\mathrm{cal}(T_\mathrm{m}) = \int_0^\infty < C_\mathrm{p}(T) >^\mathrm{exc} \, \mathrm{d}T \quad 13$$

$$\Delta H_\mathrm{vH}(T_\mathrm{m}) = \frac{4RT_\mathrm{m}^2 < C_\mathrm{p}(T_\mathrm{m}) >^\mathrm{exc,max}}{\Delta H_\mathrm{cal}(T_\mathrm{m})} \quad 14$$

### S.1.4 Calculation of excess thermodynamic parameters

The thermodynamic excess parameters $\Delta\Delta G_\mathrm{u}$, $\Delta\Delta H_\mathrm{u}$, and $T\Delta\Delta S_\mathrm{u}$ are defined as $\Delta\Delta X_\mathrm{u} = \Delta X_\mathrm{u,cosolute} - \Delta X_\mathrm{u,buffer}$ with $X = G, H, S$. The excess parameters were calculated at $T_\mathrm{m,buffer}$ as a common temperature. $\Delta H_\mathrm{u,fit}$ of each dataset was used for the calculations. No significant changes of $\Delta C_p$ in the presence of cosolutes could be observed experimentally (see S.2 for details). Therefore, the reported temperature dependence (278-398 K) of $\Delta C_\mathrm{p}$ in a cosolute-free solution by Privalov and Makhatadze[4] was fit to a second order polynomial ($\Delta C_p(T) = -35.26 + 0.2603T - 0.0004190T^2$) and used for the calculation of $\Delta\Delta G_\mathrm{u}$, $\Delta\Delta H_\mathrm{u}$, and $T\Delta\Delta S_\mathrm{u}$. The uncertainties of the excess functions were calculated according to Gaussian error

propagation using the systematic error estimation of $T_m$ and $\Delta H_{u,fit}$ from three repeated DSC measurements in buffer (Table S1). For a few scans the statistical error of $T_m$ and $\Delta H_{u,fit}$ was larger than the systematic error estimated from the buffer scans. In these cases the larger statistical error was used for the error propagation.

**Table S1: $T_m$ and $\Delta H_{u,fit}(T_m)$ obtained from the fit of eq. 8 to $C_{p,pr}^{exp}(T)$ and the results for $\Delta H_{u,cal}(T_m)$ and $\Delta H_{u,vH}(T_m)$ (eqs. 13&14, respectively) are listed. Different buffer scans were used as a reference because of different experimental conditions (VP-DSC instrument[a,d], Capillary-DSC instrument[b,c], protein concentration, buffer, and scan rate). For all scans the respective reference measurement (a-d) is given with a superscript. For reference a, errors of $T_m$ and $\Delta H_{fit}$ are representative of the 90% confidence interval of the mean. This error of the mean was derived from three repeated measurements using a Student's t-distribution, which considers the limited repetition of measurements leading to slightly larger errors in comparison to a Gaussian distribution. For all other measurements, errors of $\Delta H_{u,fit}$ are the statistical errors obtained from the fit. The statistical error of most of the melting temperatures were in the region of 0.01-0.05 K and therefore not listed. [e]: Enthalpy could not be estimated reliably from the first protein scan. The given value was extrapolated (linear function or second-order polynomial) based on the data of the subsequent protein scans. Because of that an error estimation (Gaussian error propagation of the standard errors of the fit) is given for $\Delta H_{cal}$ and $\Delta H_{vH}$ in these cases as well.**

| Solution | Conc. (M) | $T_m$, (K) | $\Delta H_{fit}$, (kJ/mol) | $\Delta H_{cal}$, (kJ/mol) | $\Delta H_{vH}$, (kJ/mol) |
|---|---|---|---|---|---|
| 50 mM citrate | (a) | 338.89 ± 0.08 | 514 ± 12 | 509 ± 9 | 515 ± 16 |
| buffer, pH 5.0, | (b) | 338.7 | 505.4 ± 0.6 | 499 | 517 |
| [pr.]= 0.5 mg mL$^{-1}$, | (c) | 338.6 | 473.6 ± 0.6 | 467 | 496 |
| Scan rate: 90 K h$^{-1}$ | | 338.5 | 477.1 ± 0.6 | 471 | 501 |

| | | | | | |
|---|---|---|---|---|---|
| 10 mM phosphate buffer, pH 5.5 [pr.]= 5.0 mg $mL^{-1}$, Scan rate: 60 K $h^{-1}$ | (d) | 335.7 | 459.9 ± 0.9 | 461 | 478 |
| LiCl[c] (Sigma-Aldrich, St. Louis, USA) | 0.25 | 335.8 | 432.9 ± 0.8 | 429 | 456 |
| | 0.5 | 335.0 | 448.9 ± 0.9 | 444 | 464 |
| | 1 | 334.5 | 442.8 ± 1.3 | 440 | 456 |
| | 2 | 334.4 | 441.1 ± 0.6 | 440 | 444 |
| | 4 | 326.8 | 374.9 ± 2.2 | 368 | 391 |
| NaCl[a] (Carl Roth, Karls-ruhe, Germany) | 0.25 | 337.1 | 491.2 ± 1.3 | 488 | 492 |
| | 0.5 | 336.8 | 485.5 ± 1.3 | 481 | 486 |
| | 1 | 337.8 | 485.7 ± 1.8 | 481 | 487 |
| | 2 | 339.5 | 456.1 ± 0.8 | 446 | 479 |
| | 4 | 342.7 | 467.2 ± 3.4 | 461 | 475 |
| KCl[a] (Mallinckrodt Baker, Deventer, Netherlands) | 0.25 | 337.0 | 488.7 ± 1.2 | 486 | 489 |
| | 0.5 | 336.6 | 486.2 ± 1.4 | 483 | 485 |
| | 1 | 337.2 | 472.9 ± 2.4 | 468 | 476 |
| | 2 | 339.8 | 457.0 ± 2.2 | 454 | 462 |
| CsCl[a] (Sigma-Aldrich, St. Louis, USA) | 0.25 | 336.3 | 477.1 ± 4.2 | 470 | 487 |
| | 0.5 | 335.1 | 446.4 ± 0.9 | 436 | 476 |
| | 1[e] | 335.8 | 461.3 ± 8.5 | 459 ± 15 | 458 ± 15 |
| | 2[e] | 337.6 | 445.3 ± 4.7 | 442 ± 8 | 455 ± 8 |
| | 4[e] | 339.9 | 425.5 ± 6.3 | 423 ± 10 | 435 ± 10 |
| NaBr[a] (ABCR, Karlsruhe, Germany) | 0.25 | 335.5 | 487.2 ± 2.1 | 478 | 489 |
| | 0.5 | 334.1 | 459.0 ± 0.9 | 455 | 461 |
| | 1 | 332.9 | 446.1 ± 1.8 | 442 | 448 |
| | 2 | 332.0 | 423.2 ± 0.6 | 420 | 424 |
| | 4 | 324.8 | 360.1 ± 4.9 | 355 | 367 |
| $NaClO_4$[a] | 0.25 | 332.4 | 457.5 ± 0.9 | 453 | 460 |

| | | | | | |
|---|---|---|---|---|---|
| (Sigma-Aldrich, St. Louis, USA) | 0.5 | 329.2 | 433.3 ± 0.7 | 430 | 435 |
| | 1 | 324.3 | 401.0 ± 1.2 | 398 | 405 |
| | 2 | 316.4 | 360.5 ± 0.8 | 359 | 366 |
| | 4 | 301.9 | 300.1 ± 1.6 | 279 | 360 |
| [chol]Cl[a] | 0.25 | 336.8 | 489.6 ± 1.3 | 485 | 492 |
| (TCI, Tokyo, Japan) | 0.5 | 335.8 | 476.2 ± 1.0 | 472 | 478 |
| | 1 | 335.6 | 470.9 ± 1.1 | 467 | 473 |
| | 2 | 337.0 | 482.0 ± 2.0 | 476 | 484 |
| | 4 | 339.1 | 480.5 ± 1.0 | 477 | 481 |
| $[Me_4N]Cl$[a] | 0.25 | 336.7 | 496.0 ± 1.6 | 490 | 499 |
| (Sigma-Aldrich, St. Louis, USA) | 0.5 | 335.6 | 476.3 ± 1.6 | 463 | 500 |
| | 1 | 336.2 | 485.8 ± 1.6 | 480 | 487 |
| | 2 | 337.5 | 491.0 ± 1.9 | 485 | 491 |
| | 4 | 337.6 | 488.3 ± 0.7 | 453 | 488 |
| K[dhp][a,b] | 0.125[a] | 340.2 | 511.9 ± 1.8 | 507 | 516 |
| (VWR, Leuven, Belgium) | 0.25[a] | 341.5 | 527.6 ± 3.6 | 518 | 536 |
| | 0.5[a] | 343.9 | 536.1 ± 2.3 | 532 | 537 |
| | 1[b,e] | 347.4 | 507.0 ± 1.5 | 507 ± 3.5 | 517 ± 3.5 |
| Ethanol[a,b] | 0.25[b] | 338.1 | 513.8 ± 1.1 | 509 | 520 |
| (Sigma-Aldrich, St. Louis, USA) | 0.5 [b] | 337.4 | 521.6 ± 1.2 | 516 | 528 |
| | 1[a] | 335.6 | 502.9 ± 0.9 | 493 | 526 |
| | 2[a] | 333.6 | 546.5 ± 3.7 | 538 | 552 |
| | 4[a] | 328.6 | 556.1 ± 4.5 | 542 | 565 |
| Ethylene glycol[a] | 0.25 | 338.7 | 519.9 ± 2.2 | 512 | 523 |
| (Sigma-Aldrich, St. Louis, USA) | 0.5 | 338.6 | 520.7 ± 2.1 | 513 | 523 |
| | 1 | 338.5 | 519.8 ± 1.6 | 515 | 520 |
| | 2 | 338.3 | 531.0 ± 1.8 | 525 | 534 |
| | 4 | 337.7 | 546.8 ± 4.3 | 539 | 553 |
| Glycerol[a] | 0.25 | 339.1 | 509.7 ± 3.0 | 504 | 514 |

| | | | | | |
|---|---|---|---|---|---|
| (Sigma-Aldrich, St. Louis, USA) | 0.5 | 339.1 | 501.7 ± 2.4 | 497 | 509 |
| | 1 | 339.6 | 522.6 ± 2.6 | 520 | 523 |
| | 2 | 340.0 | 519.5 ± 2.4 | 502 | 548 |
| | 4 | 342.9 | 555.2 ± 2.0 | 550 | 557 |
| [emim]Br[d] (IoLiTec, Denz-lingen, Germany) | 0.25 | 331.9 | 448.3 ± 1.2 | 442 | 484 |
| | 0.75 | 326.8 ± 0.3 | 412 ± 13 | 402 | 457 |
| | 1 | 324.7 ± 0.4 | 398 ± 13 | 388 | 448 |
| [bmpyrr]Br[d] (IoLiTec, Denz-lingen, Germany) | 0.25 | 331.7 | 460.7 ± 1.4 | 462 | 475 |
| | 0.5 | 328.2 ± 0.2 | 428.6 ± 5.1 | 418 | 472 |
| | 1 | 323.1 ± 0.2 | 407 ± 11 | 392 | 471 |
| | 1.5 | 319.9 ± 0.2 | 426.3 ± 4.9 | 419 | 469 |
| [bmim]Br[d] (IoLiTec, Denz-lingen, Germany) | 0.25 | 330.9 | 490.0 ± 0.7 | 483 | 507 |
| | 0.5 | 326.5 | 473.1 ± 2.2 | 462 | 498 |
| | 1 | 319.5 | 448.7 ± 1.5 | 437 | 485 |
| | 1.5 | 316.6 | 438.9 ± 1.3 | 428 | 477 |
| [$Bu_4N$]Br[d] (IoLiTec, Denz-lingen, Germany) | 0.25 | 327.0 | 472.0 ± 1.1 | 473 | 493 |
| | 0.5 | 320.1 | 477.6 ± 1.6 | 478 | 502 |
| | 1 | 310.2 | 400.9 ± 1.9 | 389 | 432 |
| [hmim]Br[d] (IoLiTec, Denz-lingen, Germany) | 0.25 | 328.3 | 440.6 ± 1.2 | 440 | 460 |
| | 0.5 | 320.8 | 398.2 ± 1.6 | 395 | 438 |
| | 0.75 | 316.0 | 387.2 ± 4.1 | 379 | 430 |
| | 1 | 311.9 | 376.9 ± 2.3 | 371 | 415 |
| | 1.5 | 305.39 | 353.9 ± 1.9 | 355 | 392 |
| [bmim][$BF_4$][d] (IoLiTec, Denz-lingen, Germany) | 0.5 | 326.6 | 452.5 ± 0.8 | 446 | 477 |
| | 1 | 322.1 | 426.7 ± 1.2 | 425 | 448 |
| | 1.5 | 317.8 | 398.5 ± 1.0 | 386 | 444 |
| [emim]Cl[d] (IoLiTec, Denz-lingen, Germany) | 0.5 | 331.5 | 458.5 ± 1.4 | 457 | 476 |
| | 1 | 328.5 ± 0.2 | 424.9 ± 5.1 | 414 | 471 |

| | | | | | |
|---|---|---|---|---|---|
| [emim][EtSO4][d] | 0.25 | 332.8 | 454.1 ± 0.9 | 453 | 474 |
| (IoLiTec, Denz-lin- | 0.5 | 330.5 | 451.7 ± 1.0 | 452 | 472 |
| gen, Germany) | 0.75 | 328.9 | 447.4 ± 1.1 | 456 | 461 |
| | 1 | 327.6 | 447.9 ± 1.1 | 447 | 469 |
| [emim][dca][d] | 1 | 314.8 | 349.5 ± 2.1 | 345 | 388 |
| (IoLiTec, Denz-lin- | | | | | |
| gen, Germany) | | | | | |
| [emim][SCN][d] | 0.25 | 328.5 | 443.6 ± 0.7 | 434 | 480 |
| (IoLiTec, Denz-lin- | 0.5 | 322.4 | 398.6 ± 0.7 | 387 | 456 |
| gen, Germany) | 0.75 | 318.2 | 392.3 ± 0.8 | 387 | 420 |
| | 1 | 314.7 ± 0.2 | 373.4 ± 4.3 | 365 | 410 |
| | 1.5 | 308.5 | 363.7 ± 1.7 | 351 | 409 |
| [chol][dhp][d] | 0.5 | 341.4 | 511.4 ± 3.8 | 499 | 563 |
| (IoLiTec, Denz-lin- | 1 | 344.2 | 518.9 ± 1.6 | 523 | 526 |
| gen, Germany) | 1.5 | 347.5 | 528.0 ± 1.2 | 527 | 537 |
| | 3 | 353.2 | 519.4 ± 3.9 | 522 | 536 |
| | 4 | 356.6 ± 0.2 | 501.4 ± 6.4 | 495 | 537 |

## S.2 Estimation of $\Delta C_p$ in the presence of cosolutes

### S.2.1 Estimation of $\Delta C_p$ of RNase A from linear extrapolation of $C_{p,\mathrm{folded}}$ and $C_{p,\mathrm{unfolded}}$

The success of measuring $\Delta C_p$ depends largely on the data quality of the partial molar heat capacity of the folded and unfolded protein. High concentrations of cosolute can cause small distortions of the baseline making it impossible to reliably determine $\Delta C_p$ from the fit described in S.1.2. A more accurate way is to determine $\Delta C_p$ independently from the fit of the two-state model to the $C_{p,\mathrm{part.mol.}}$. $C_{p,\mathrm{folded}}$ and $C_{p,\mathrm{unfolded}}$ can be described by linear equations (eqs. 9&10). The difference between both linear functions results in a linear function for $\Delta C_p(T)$ (Fig. SI). Since $C_{p,\mathrm{unfolded}}$ has a nonlinear temperature dependence[5]

the linear extrapolation holds only for a small temperature range and should therefore only be used to estimate $\Delta C_p(T_\mathrm{m})$ and not to estimate the full temperature dependence of $\Delta C_p$.

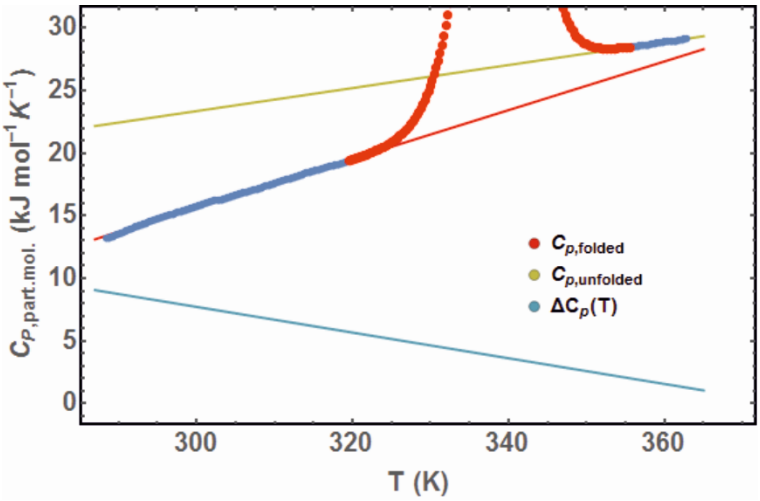


Figure SI: Estimation of $\Delta C_p$ from linear extrapolations of $C_{p,\mathrm{folded}}$ and $C_{p,\mathrm{unfolded}}$. $C_{p,\mathrm{folded}}$ and $C_{p,\mathrm{unfolded}}$ are linear fits to the blue data points. Data points shown in red describe the excess heat capacity due to the unfolding of the protein and where not used here to determine $\Delta C_p$.

Using this approach, we determined $\Delta C_p(T_\mathrm{m})$ for all DSC scans for which the quality of the data of $C_{p,\mathrm{folded}}$ and $C_{p,\mathrm{unfolded}}$ allowed a linear extrapolation. Data for $\Delta C_p(T_\mathrm{m})$ are shown in Table S1. We observed no significant trends of $\Delta C_p(T_\mathrm{m})$ with increasing cosolute concentration or type of cosolute. $\Delta C_p(T_\mathrm{m})$ remained rather constant with respect to the buffer solution considering the relatively high uncertainty of estimating $\Delta C_p$.

**Table S2: Estimation of $\Delta C_p(T_\mathrm{m})$ using a linear extrapolation of $C_{p,\mathrm{folded}}$ and $C_{p,\mathrm{unfolded}}$.**

| Solution | Conc. (M) | $\Delta C_p(T_\mathrm{m})$, (kJ mol$^{-1}$ K$^{-1}$) |
|---|---|---|
| 50 mM citrate | | 3.8 |
| buffer, pH 5.0 | | 3.7 |
| | | 4.2 |
| | | 3.7 |

| | | |
|---|---|---|
| 10 mM phosphate buffer, pH 5.5 | | 4.3 |
| Privalov[4] | | |
| 323 K | | 5.3 |
| 348 K | | 4.3 |
| NaCl | 0.25 | 3.9 |
| | 0.5 | 3.8 |
| | 1 | 3.7 |
| | 2 | 4.5 |
| | 4 | 5.0 |
| KCl | 0.25 | 3.5 |
| | 0.5 | 3.4 |
| | 1 | 3.4 |
| | 2 | 3.0 |
| NaBr | 0.25 | 4.3 |
| | 0.5 | 3.6 |
| | 1 | 3.6 |
| | 4 | 3.2 |
| $NaClO_4$ | 0.25 | 4.8 |
| | 0.5 | 3.7 |
| | 1 | 6.1 |
| | 2 | 4.6 |
| [chol]Cl | 0.25 | 4.5 |
| | 0.5 | 3.4 |
| | 1 | 3.9 |
| | 2 | 3.8 |
| | 4 | 2.3 |
| $[Me_4N]Cl$ | 0.25 | 5.8 |
| | 0.5 | 3.6 |

| | | |
|---|---|---|
| | 1 | 3.9 |
| | 2 | 4.4 |
| K[dhp] | 0.125 | 3.9 |
| | 0.25 | 3.8 |
| | 0.5 | 4.2 |
| Ethylene glycol | 0.25 | 3.6 |
| | 0.5 | 4.7 |
| | 1 | 2.9 |
| | 2 | 4.0 |
| glycerol | 0.25 | 4.0 |
| | 0.5 | 4.5 |
| [bmim]Br | 0.25 | 5.4 |
| $[Bu_4N]Br$ | 0.25 | 4.6 |
| [hmim]Br | 0.25 | 4.0 |
| $[emim][EtSO_4]$ | 0.25 | 5.3 |
| | 0.5 | 6.4 |
| | 0.75 | 6.2 |
| | 1 | 5.3 |
| [emim][dca] | 1 | 4.5 |
| [emim][SCN] | 0.75 | 4.4 |

### S.2.2 Estimation of $\Delta C_p$ of ubiquitin in ethanol solutions

To further check the influence of cosolutes on $\Delta C_p$ we choose ubiquitin as a model protein because it has a very high $\Delta C_p$ in comparison to its number of amino acids (0.075 kJ $mol^{-1}$ $K^{-1}$ $N^{-1}$ at 25°C).[6] In contrast, RNase A is at the lower end of this scale (0.042 kJ $mol^{-1}$ $K^{-1}$ $N^{-1}$ at 25°C).[4] Therefore, changes of $\Delta C_p$ are easier detectable using ubiquitin compared to RNase A. We have chosen 4 M ethanol to test

the influence of a hydrophobic cosolute on $\Delta C_p$ since $\Delta C_p$ is related to the hydration of apolar residues.[5] Thus, it might be influenced by the presence of a hydrophobic cosolute. Since $\Delta C_p$ in the presence of 4 M ethanol could not be reliably determined from the fit of the two-state transition model to the data or from the linear extrapolations of $C_{p,\mathrm{folded}}$ and $C_{p,\mathrm{unfolded}}$, we used a different procedure to obtain $\Delta C_p$. Running DSC scans to very high temperatures (> 120°C) increases the irreversibility of the folding reaction. Thus, after a series of scans only the partial molar heat capacity of the unfolded protein is measured (Fig. SIIa). Subtraction of $C_{p,\mathrm{folded}}$ (measured at the first scan) from $C_{p,unfolded}$ results in $\Delta C_p(T)$ (Fig. SIIb). Interestingly, even in 4 M ethanol $\Delta C_p$ seems not to be affected by the presence of the cosolute (at least in the temperature range shown in Fig. SIIb).

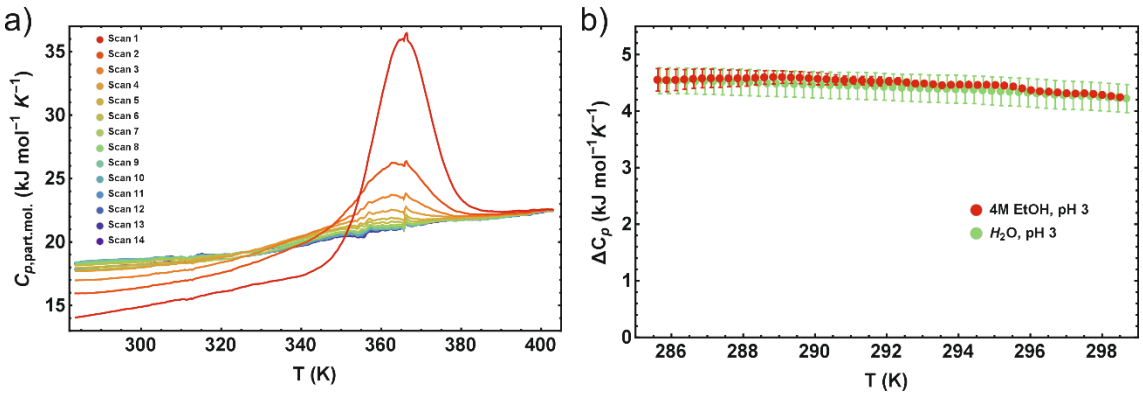


Figure SII: a) DSC scans of ubiquitin (1.0 mg mL$^{-1}$) in H2O at pH 3. b) $\Delta C_{p,\mathrm{water}}$ and $\Delta C_{p,4\mathrm{M\ EtOH}}$ of ubiquitin as a function of temperature in the low temperature regime where the protein is folded. $\Delta C_{p,\mathrm{water}}$ and $\Delta C_{p,4\mathrm{M\ EtOH}}$ are within the error bars of each other (standard error of the mean of three repeated measurements).

### S.3 Correlation between $\Delta\Delta G_\mathrm{u}$ and $\Delta T_\mathrm{m}$

Fig. S4 shows a plot of $\Delta\Delta G_\mathrm{u}$ versus $\Delta T_\mathrm{m}$ for all cosolute data listed in Table S1. A linear regression of 97 data points imposing a zero intercept resulted in a slope of 1.39 kJ mol-1 K-1 with a $R^2$ of 0.996. Thereby, the slope corresponds to $\Delta H(T_\mathrm{m})/T_\mathrm{m}$ of RNase A in the cosolute-free solution which follows from the derivative of $\Delta G(T)$ with respect to $T$ at $T_\mathrm{m}$ (eq. 15).

$$\left(\partial G(T)/\partial T\right)_{T_\mathrm{m}} = -\Delta H(T_\mathrm{m})/T_\mathrm{m} \qquad (15)$$

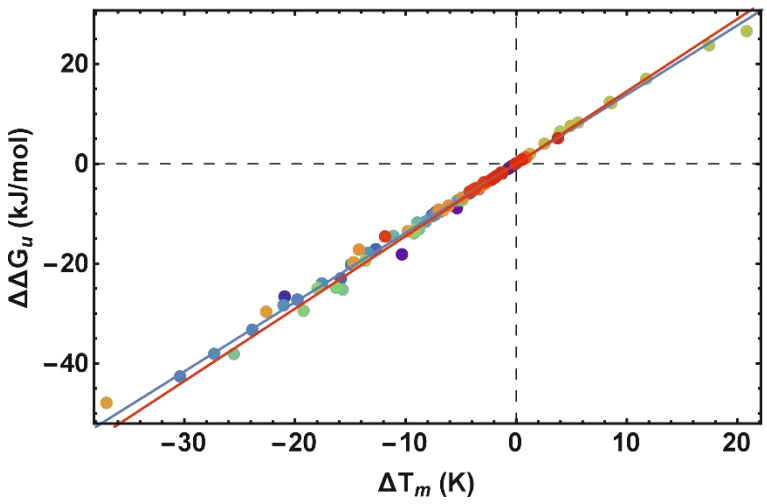


Figure S1: Correlation plot of $\Delta\Delta G_\mathrm{u}$ vs. $\Delta T_\mathrm{m}$ for all cosolute data listed in Table S1. The blue line corresponds to the linear fit to all data points ($y = 1.39x$). The red line corresponds to a linear curve with a slope of $\Delta H_\mathrm{u}(T_\mathrm{m})/T_\mathrm{m}$ of RNase A in the cosolute-free solution ($y = 1.45x$, calculated via a weighted average of all buffer data (Table S1)).

### S.4 Influence of the temperature dependence of $\Delta C_p$ on the stability curve

While the heat capacity of the folded state, $C_{p,\mathrm{folded}}$, can be described by a linear equation, the heat capacity of the unfolded state, $C_{p,\mathrm{unfolded}}$, is best described by a second-order polynomial.[5] Therefore, $\Delta C_p$ can be described by a second order polynomial as well. However, extrapolation of the second-order polynomial $\Delta C_p$ to extreme temperatures leads to a strong decrease of $\Delta C_p$ at low and high temperatures and eventually to negative $\Delta C_p$ values. This leads to an increase of protein stability at very low and very high temperatures (Fig. S2b) which is not physically meaningful. In order to estimate the temperature of maximum stability, $T_\mathrm{s}$, and the temperature of cold denaturation, $T_\mathrm{c}$, $\Delta C_p = \mathrm{const.}$ has to be used to calculate $\Delta G_\mathrm{u}(T)$ at these low temperatures. In a previous study, we have shown that using $\Delta C_p = \mathrm{const.}$ can lead to large errors for $\Delta\Delta H_\mathrm{u}$, and $T\Delta\Delta S_\mathrm{u}$.[7] However, because of strong enthalpy-entropy compensation

effects (errors cancel out to a large extent) the simplification of $\Delta C_p = \text{const.}$ leads to meaningful results for $\Delta G_\text{u}(T)$ over wide temperature range (Fig. S2) as also observed by Privalov.[8]

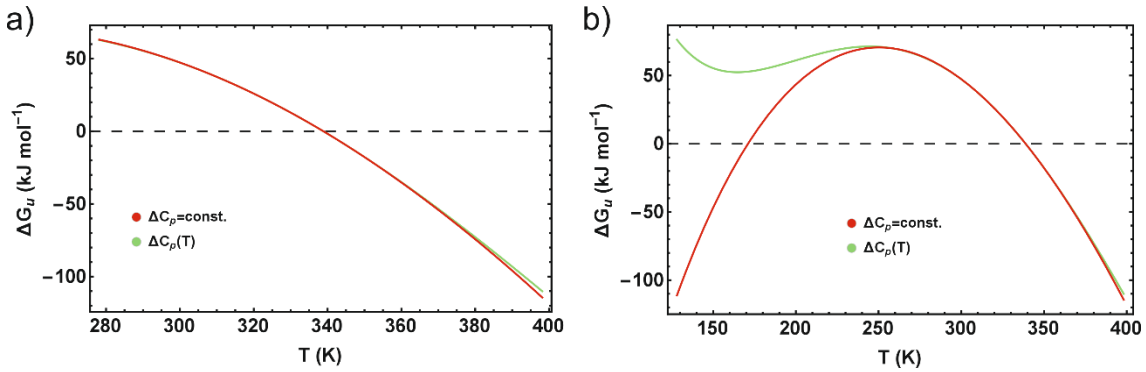


Figure S2: Protein stability curves of RNase A in 50 mM citrate buffer (pH 5.0) calculated with $\Delta C_p(T) = -35.26 + 0.2603T - 0.0004190T^2$ (fit of second-order polynomial to the $\Delta C_p$ data published by Privalov and Makhatadze[4]) and $\Delta C_p = 5$ kJ mol$^{-1}$K$^{-1}$. a) Stability curve shown in the measured temperature range of $\Delta C_p$ (5-125 °C). b) Extrapolation to extreme temperatures leads to an increase in protein stability at very low temperatures using $\Delta C_p(T)$ because $\Delta C_p(T)$ turns negative.

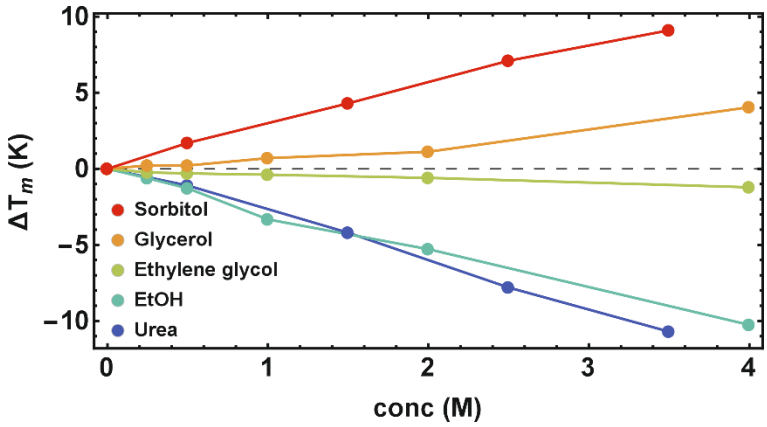


Figure S3: Concentration dependence of $\Delta T_\text{m}$ for nonelectrolytes. Error bars were calculated by Gaussian error propagation of the primary error estimates (see S.1) and are smaller than the point size. The data for sorbitol and urea are taken from Ravindra and Winter.[9]

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