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Experiments and simulations show how long-range contacts can form in expanded unfolded proteins with negligible secondary structure

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The sizes of unfolded proteins under highly denaturing conditions scale as $N^{0.59}$ with chain length. This suggests that denaturing conditions mimic good solvents, whereby the preference for favorable chain-solvent interactions causes intrachain interactions to be repulsive, on average. Beyond this generic inference, the broader implications of $N^{0.59}$ scaling for quantitative descriptions of denatured state ensembles (DSEs) remain unresolved. Of particular interest is the degree to which $N^{0.59}$ scaling can simultaneously accommodate intrachain attractions and detectable long-range contacts. Here we present data showing that the DSE of the N-terminal domain of the L9 (NTL9) ribosomal protein in 8.3 M urea lacks detectable secondary structure and forms expanded conformations in accord with the expected $N^{0.59}$ scaling behavior. Paramagnetic relaxation enhancements, however, indicate the presence of detectable long-range contacts in the denatured-state ensemble of NTL9. To explain these observations we used atomistic thermal unfolding simulations to identify ensembles whose properties are consistent with all of the experimental observations, thus serving as useful proxies for the DSE of NTL9 in 8.3 M urea. Analysis of these ensembles shows that residual attractions are present under mimics of good solvent conditions, and for NTL9 they result from low-likelihood, medium/long-range contacts between hydrophobic residues. Our analysis provides a quantitative framework for the simultaneous observation of $N^{0.59}$ scaling and low-likelihood long-range contacts for the DSE of NTL9. We propose that such low-likelihood intramolecular hydrophobic clusters might be a generic feature of DSEs that play a gatekeeping role to protect against aggregation during protein folding.

atomistic simulations | denatured proteins | paramagnetic relaxation

Quantitative descriptions of unfolded proteins are important for understanding collapse transitions (1), protein folding mechanisms (2), misfolding, aggregation (3, 4), and the effects of macromolecular crowding on protein stability (5, 6). Expanded unfolded states are sampled in high concentrations of chemical denaturants such as urea and guanidinium chloride. The sizes of these denatured proteins, quantified using hydrodynamic radii ($\langle R_h \rangle$) or radii of gyration ($\langle R_g \rangle$), scale as $N^{0.59}$ with chain length (7–9). This $N^{0.59}$ scaling arises because denatured proteins expand to make favorable contacts with the surrounding solvent, implying that high concentrations of denaturants are good solvents for generic proteins.

In good solvents, the ensemble-averaged interresidue pair interaction coefficient is positive, suggesting that the preference for favorable chain-solvent interactions leads to intrachain interactions being repulsive on average (10). The validity of this inference for denatured proteins has been demonstrated recently using a combination of single-molecule experiments and polymer theory (11). Quantitative descriptions of chain statistics for polymers in good solvents rely on the so-called excluded volume (EV) limit as an important reference state, and this is true for denatured state ensembles (DSEs) as well. EV limit ensembles are typically generated using atomistic descriptions of proteins and ignoring all non-bonded interactions excepting steric repulsions (12–15). Hence, the

ensemble-averaged interresidue interaction coefficient (related to the second virial coefficient) is, by construction, positive in the EV limit, thus affording the reproduction of $N^{0.59}$ scaling (10). Descriptions of chain statistics based on EV limit ensembles are routinely used as reference states for intrinsically disordered proteins (IDPs) and denatured proteins and have proven useful in interpreting the results of NMR and small angle X-ray scattering (SAXS) measurements for these systems (12–21).

If measured properties of DSEs can be adequately explained using EV limit ensembles, then the implication is as follows: in a typical Flory-like mean field description, the energy for each polymer configuration can be written as $U = U_{EV} + U_{non-EV-intrachain} + U_{chain-solvent}$ (22). The EV limit is reached if the non-EV-intrachain interactions are exactly counterbalanced by chain-solvent interactions. Deviations from the EV limit arise if there is imperfect compensation between non-EV-intrachain interactions and chain-solvent interactions. If imperfect compensation results from attractive non-EV-intrachain interactions, then deviations from the EV limit can lead to persistent local structure and global compaction with deviation from $N^{0.59}$ scaling, as has been the observed for proteins under mild denaturing conditions (23–33) or for proteins under folding [i.e., poor solvent (34)] conditions (35, 36). Imperfect compensation that results from long-range intramolecular electrostatic repulsions and/or favorable chain-solvent interactions will also cause deviations from $N^{0.59}$ scaling. In these cases, the deviation causes increased chain expansion, as has been observed recently for highly charged IDPs in aqueous solutions and in the presence of denaturant (18, 37).

Of interest is the possibility that imperfect compensation can be achieved between non-EV-intrachain interactions and chain-solvent interactions without causing deviations from $N^{0.59}$ scaling.

Although this is the intuitive and canonical expectation given the documented denatured state effects on protein folding (6), no quantitative evidence has been offered in support of imperfect compensation being achieved while preserving $N^{0.59}$ scaling. Here, we report an archetypal dataset for the DSE of the N-terminal domain of the L9 (NTL9) ribosomal protein in 8.3 M urea, showing chain expansion consistent with $N^{0.59}$ scaling and the presence of detectable long-range contacts despite negligible secondary structure. Paramagnetic relaxation enhancements

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(PREs) provide support for the presence of transient long-range contacts in the DSE of NTL9. The PRE data cannot be explained using EV limit ensembles and hence highlight the presence of imperfect compensation with $N^{0.59}$ scaling. To understand the observations for the DSE of NTL9 we used atomistic thermal unfolding simulations of NTL9. In the simulations, the temperature acts as a proxy for the effect of denaturant (which changes solvent quality) by modulating the balance between the preference for compact conformations (seen at low temperatures) and expanded conformations (seen at high temperatures). We identified a set of high temperatures whose ensembles yield averages that are congruent with experimental data for the DSE of NTL9 in 8.3 M urea. Analysis of these ensembles provides a quantitative reasoning for the observed properties of NTL9's DSE in 8.3 M urea. Our simulation approach does not impose any a priori assumptions of equivalence between thermal and urea denaturation. Instead, we use it to identify unfolded ensembles that yield properties consistent with the full panel of measurements, thus yielding a quantitative, albeit computationally tractable framework for interpreting the experimental observations.

Results

NTL9 Samples Predominantly Expanded Conformations with Negligible Secondary Structure in Its DSE in 8.3 M Urea. The values of R_h and R_g were measured using NMR pulsed field gradient diffusion and SAXS experiments, respectively. The measured R_h for NTL9 DSE in 8.3 M urea at 12 °C is 22.5 Å, and the measured R_g is 21.3 ± 1.5 Å at a protein concentration of 7.5 mg/mL (*SI Appendix, Figs. S1 and S2*). At a similar concentration, the measured R_g value for the native state is 12.2 Å (*SI Appendix, Fig. S3*). The measured R_h and R_g values are consistent with reported scaling laws for highly unfolded polypeptide chains (8, 9).

NMR spectra for the native and urea-induced denatured states of NTL9 are shown in *SI Appendix, Fig. S4*. Although the spectrum is less well resolved in 8.3 M urea, we obtained complete backbone ^1H , ^{13}C , and ^{15}N assignments as well as side chain $^{13}\text{C}_\beta$ assignments using standard approaches (*SI Appendix, p. S20*). In 8.3 M urea, 93% of the molecules are unfolded. Although native state resonances were detected at lower contour levels, these were in slow exchange with the denatured state. Secondary shifts (38) were used to quantify the deviation between measured and random coil chemical shifts. *SI Appendix, Fig. S5* shows a plot of the $^{13}\text{C}_\alpha$, $^{13}\text{C}_\beta$, $\Delta\delta^{13}\text{C}_\alpha - \Delta\delta^{13}\text{C}_\beta$, ^{13}CO , and $^1\text{H}_\alpha$ secondary shifts for the native state and for the NTL9 DSE in 8.3 M urea. The secondary shifts for the DSE are significantly smaller than those in the native state, with all of the ^{13}C secondary shifts being less than 1.0 ppm and the ^1H secondary shifts being less than or equal to 0.2 ppm. Residues 42–47 exhibit small positive $^{13}\text{C}_\alpha$ and ^{13}CO secondary shifts (0.31–0.37 ppm) as well as small positive values for $\Delta\delta^{13}\text{C}_\alpha - \Delta\delta^{13}\text{C}_\beta$ (maximum of 0.34 ppm). This suggests the presence of residual, albeit very small, α -helical propensity. In Fig. 1 we present a summary of the chemical shifts using secondary structure propensity (SSP) scores (39). A score of 1 indicates fully formed α -helical structure, and a score of -1 represents fully formed β -strands. The SSP scores for the NTL9 DSE in 8.3 M urea are close to zero and support the conclusion of negligible preference for α -helical and β -strand structure.

Paramagnetic Relaxation Enhancement Experiments Indicate the Presence of Long-Range Contacts for the NTL9 DSE in 8.3 M Urea.

Nitroxide spin labels cause significant broadening of NMR resonances of spins that are within 20 Å of the spin label (40–42). These effects provide a useful probe for long-range contacts (15, 19, 30, 43, 44). Spin labels were attached at positions 2, 10, 32, 49, and 51, respectively (*SI Appendix, Fig. S6*). Under native conditions all of the spin-labeled mutants are folded as judged by NMR (*SI Appendix, Fig. S7*). We used the peak intensity ratios from the ^1H - ^{15}N heteronuclear single quantum coherence (HSQC) spectra of the paramagnetic and diamagnetic proteins. Using the methods of Iwahara et al. (45) we also measured the paramagnetic relaxation enhancement (PRE) $^1\text{H}_N$ transverse relaxation rates (Γ_2) for the

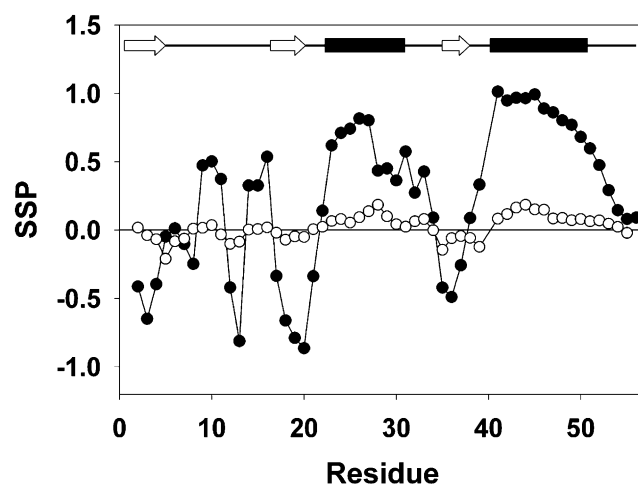


Fig. 1. SSP scores for the folded (●) and the DSE (○) of NTL9 in 8.3 M urea. A schematic diagram of the elements of secondary structure of the native state is shown at top.

NTL9 DSE in 8.3 M urea. Both measurements yield similar trends, as shown in Fig. 2.

The PRE results for the DSE (Fig. 2) show deviations from the values predicted by standard random coil models (40) for all positions. The observed PRE effects are not due to contributions from a small native fraction, because the two states are in slow exchange. We observed similar albeit reduced effects for the variants labeled at residue 49 and 51. There is an asymmetry in the PREs observed for the labels near the N terminus (K2) compared with the labels near the C terminus (A49 and K51). We propose that this reflects the differences in contact patterns between the two termini (see simulation results below) and the fact that the spin label is attached to a long side chain. For example, insertion of the spin labeled side chain of K2C into a hydrophobic cluster can enhance the relaxation of amide protons in the C terminus; conversely, the spin labeled side chains of the A49C and K51C mutants might project away from the clusters, leading to decreased relaxation enhancement for amide protons in N terminus.

The PRE data show significant deviations from the standard Gaussian chain random coil model used to benchmark PREs (40). The Gaussian random coil model does not account for EV effects nor does it account for the size and flexibility of the attached spin label, and it is possible that the observed deviations might reflect limitations of the Gaussian random coil model. To address these issues we performed two sets (with and without spin labels) of atomistic EV limit simulations (16) of NTL9. Results from both simulations differ from the Gaussian chain model, and there are small but detectable differences between the EV simulations with and without spin labels (*SI Appendix, Fig. S9*). Although the EV limit models predict more extensive PRE effects than the Gaussian chain model, neither model can reproduce the experimentally observed PREs. The observed PREs cannot be attributed solely to the simplicity of reference models used to calibrate these effects. Instead they suggest the presence of long-range contacts that need quantitative characterization.

Atomistic Simulations Help Identify Ensembles That Are Consistent with the Properties of NTL9 DSEs in 8.3 M Urea.

We performed Metropolis Monte Carlo (MC) simulations based on atomistic descriptions of NTL9, the ABSINTH implicit solvation model and underlying force field paradigm (46), and parameters from the OPLSS-AA/L molecular mechanics force field (47). For each simulation temperature between 240 K and 500 K we performed multiple independent MC simulations, each based on a different random seed and the native state as the starting conformation. We calculated ensemble averages for R_g , secondary structure propensities, and PRE data as a function of the simulation temperature. These

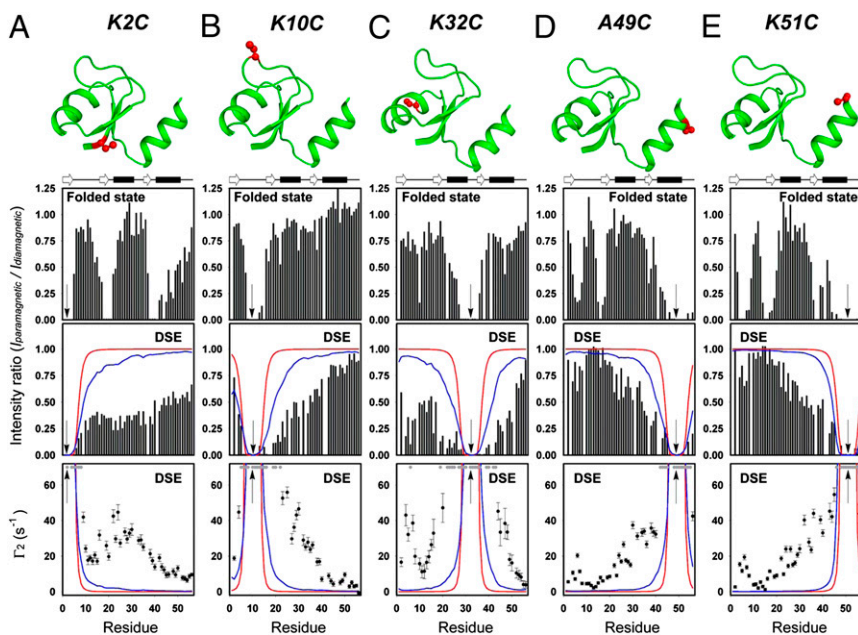


Fig. 2. Paramagnetic relaxation data for NTL9 under native state conditions and for the DSE in 8.3 M urea for (A) K2C, (B) K10C, (C) K32C, (D) A49C, and (E) K51C. The histograms display the intensity ratio of the ^1H - ^{15}N cross-peaks in the HSQC spectra in the folded and urea denatured state. (Bottom) PRE $^1\text{H}_\text{N}$ - Γ_2 rates (●) for urea denatured state are plotted. Residues for which the peaks disappeared in the paramagnetic form are indicated by gray dots. Red lines represent the values expected from the Gaussian random-coil model. The blue line represents the values calculated using simulations of atomistic ensembles in the EV limit with explicit incorporation of the spin label. Arrows indicate the location of each spin label.

were used to identify temperatures whose ensembles generated averages that are concordant with experimental data and thus serve as models for the DSE of NTL9 in 8.3 M urea.

We compared the measured PRE data with profiles calculated from simulated ensembles using the parameters Δ_1 and Δ_2 that quantify the temperature-dependent root mean square deviations from measured values of the intensity ratios and Γ_2 , respectively (Fig. 3 A and B and *SI Appendix*, Eq. S10). The smallest Δ_i values were obtained for three simulation temperatures—380 K, 390 K, and 400 K—and we refer to these as T_D temperatures. Fig. 4 and *SI Appendix*, Fig. S10 show details of the quantitative agreement between PRE profiles calculated for each of the T_D temperatures and experimental data.

Fig. 3C plots the average α -helical and β -sheet contents calculated at each of the simulation temperatures. These contents are less than 3% at T_D temperatures in agreement with estimates from NMR experiments for the DSE of NTL9 in 8.3 M urea. Fig. 3D plots the temperature dependence of the ensemble-averaged R_g . We obtained $\langle R_g \rangle = 20.50 \pm 0.08 \text{ \AA}$, $21.21 \pm 0.06 \text{ \AA}$, and $21.74 \pm 0.05 \text{ \AA}$ for 380 K, 390 K, and 400 K, respectively. These $\langle R_g \rangle$ values are in accord with expectations from scaling relations (9) and similar to the values obtained using SAXS measurements for the DSE of NTL9 in 8.3 M urea. As a reference, $\langle R_g \rangle = 28.31 \pm 0.02 \text{ \AA}$ for NTL9 in the EV limit. We used the ensembles for T_D temperatures as proxies for NTL9 DSE in 8.3 M urea.

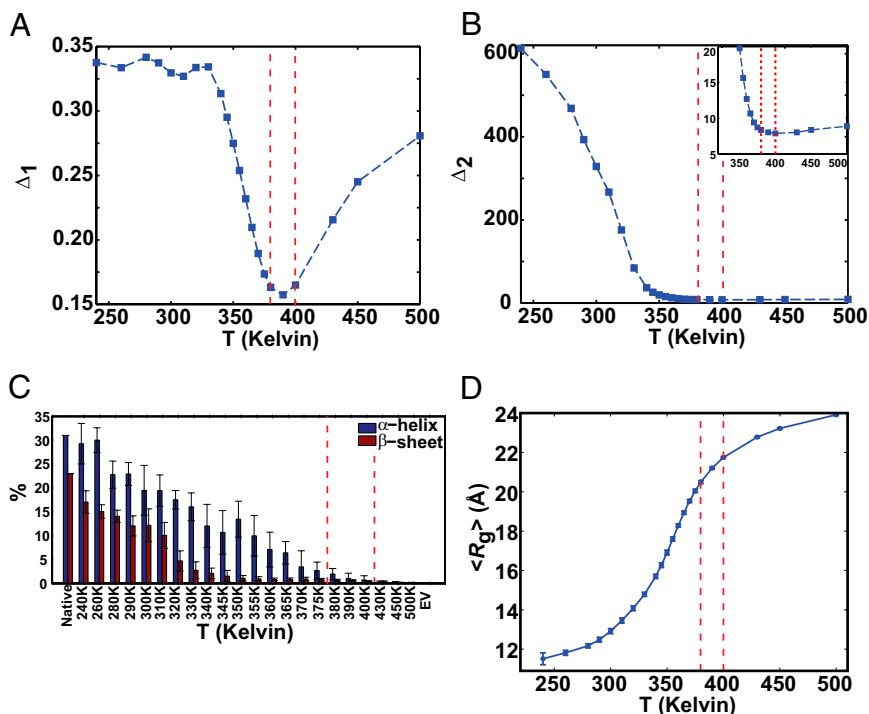


Fig. 3. Identification of the temperature interval for T_D ensembles. In all panels the dashed lines bracket the temperatures $T = 380 \text{ K}$, 390 K , and 400 K , which are the T_D temperatures. (A and B) Plots of Δ_1 (A) and Δ_2 (B) vs. temperature. (C) Plot of the temperature dependence of secondary structure contents, which were calculated using the DSSP algorithm (51). (D) Plot of the temperature-dependent $\langle R_g \rangle$ values from simulation results.

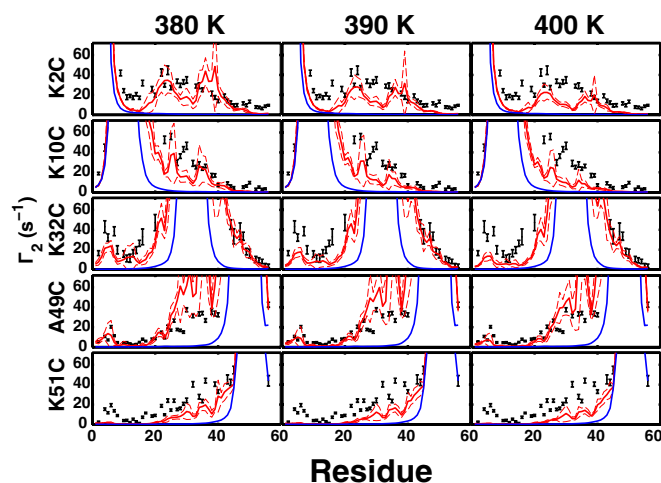


Fig. 4. Comparison between experimental data (in black symbols with error bars) and calculated PRE profiles, with comparisons shown in terms of Γ_2 . The profiles shown in blue were obtained using conformations drawn from the EV ensemble, and the profiles shown in red were obtained using conformations for T_D ensembles, with each column corresponding to a specific T_D temperature. The dashed red curves indicate the confidence intervals on calculated profiles. We calculated the latter by partitioning the simulated ensembles into 10 blocks and using the deviations of block averages from the overall mean calculated these errors.

Comparative Analysis of Contact Probabilities. In the EV limit all pairwise interactions are purely repulsive, and as a result the average percent probability p_{ij} of realizing contacts between residues i and j decreases sharply with increasing sequence separation $|i-j|$, such that $0.001\% < p_{ij} < 0.05\%$ for $|i-j| > 10$ (Fig. 5). In contrast, the corresponding probabilities for conformations drawn from the T_D ensembles ($0.01\% < p_{ij} < 1\%$) are at least two orders of magnitude larger than the EV limit for sequence separations in the range $10 < |i-j| < 40$. This indicates the presence of detectable low likelihood medium/long-range contacts in the T_D ensembles.

Do T_D Temperatures Mimic Good Solvents for NTL9? The scaling exponent is $\nu \sim 0.59$ in the EV limit and for generic proteins in high concentrations of denaturants (10, 17). For expanded conformations the average distance $\langle R_{ij} \rangle$ between any pair of residues i and j should follow a power law relationship *viz.*, $\langle R_{ij} \rangle = R_0 |j-i|^\nu$ (SI Appendix, Fig. S11) (10, 16, 20). If the T_D temperatures mimic good solvents, then ν should approach 0.59 for the scaling of $\langle R_{ij} \rangle$ as a function of $|j-i|$, providing the latter is long enough for scaling theory to apply (16, 20). For different combinations of pairs of residues (i,j) and (k,l) we used $\nu = \frac{\ln(\langle R_{ij} \rangle) - \ln(\langle R_{kl} \rangle)}{\ln(|j-i|) - \ln(|l-k|)}$, where $\langle R_{ij} \rangle$ and $\langle R_{kl} \rangle$ denote average distances, and $|j-i|$ and $|l-k|$ denote sequence separations between residues (i,j) and (k,l) , respectively. We analyzed the EV limit ensembles of NTL9 for all combinations of (i,j) , and (k,l) that satisfy the constraints $|j-i|$ and $|l-k| \geq 25$. This yields a distribution of values for ν (Fig. 6A) with an average value of $\langle \nu \rangle = 0.59 \pm 0.03$. A similar analysis was carried out for each T_D temperature, and we found $\langle \nu \rangle$ to be 0.59 ± 0.05 , 0.58 ± 0.05 , and 0.57 ± 0.04 for 380 K, 390 K, and 400 K, respectively. Therefore, T_D temperatures mimic good solvent conditions for NTL9.

Reconciling $\nu \sim 0.59$ with Deviations from the EV Limit. Dimensionless quantities such as $|j-i|^{0.59}$ are converted to distances using a multiplicative prefactor R_0 . In a good solvent, this parameter can be used to quantify the average volume excluded per residue for favorable interactions with the surrounding solvent. If the net charge per residue is ≤ 0.3 (18) and intrachain electrostatic repulsions are screened (48), then $R_0 \leq R_0^{\text{EV}}$ in a generic good solvent where R_0^{EV} denotes the value of R_0 calculated in the EV limit.

If $R_0 < R_0^{\text{EV}}$, then residual intrachain attractions persist in the good solvent. This will influence the probabilities associated with long-range contacts.

We estimated the value of R_0 for each T_D temperature and compared these with R_0^{EV} . We fit the values for $\langle R_{ij} \rangle$ to the equation $\ln(\langle R_{ij} \rangle) = \ln(R_0) + 0.59 \ln(|j-i|)$ for all $|j-i| \geq 25$, where R_0 is the free parameter. The results are shown in Fig. 6B. The maximal value of R_0 is R_0^{EV} , and these values decrease as the T_D temperatures decrease. Our analysis provides quantitative evidence for imperfect compensation between non-EV-intrachain and chain-solvent interactions. For the DSE of NTL9 our model ensembles suggest that imperfect compensation results from the presence of residual attractions.

Analysis of Contact Patterns for T_D Temperature Ensembles. SI Appendix, Fig. S12 quantifies the probabilities of interresidue contacts in the native state ensemble (240 K), the T_D temperatures, and the EV limit, respectively. Each cell in a contact map quantifies the probability that residues i and j are in contact. We define residues to be in contact if the interresidue distance between at least one pair of heavy atoms is $\leq 3.5\text{\AA}$. We observe patterns that include several low-probability native as well as nonnative contacts ($p_{ij} < 0.1$). We calculated two sets of difference contact maps to quantify the differences between the ensembles sampled at T_D temperatures and the native state and EV limit ensembles, respectively. Fig. 7 and SI Appendix, Fig. S13 show difference maps with respect to the native state ensemble and the EV limit as the reference. The colors of the cells are set by the magnitude and sign of the quantity $d_{ij} = p_{ij} - q_{ij}$, where q_{ij} denotes the probability that residues i and j are in contact in the reference ensemble (native or EV). If $d_{ij} < 0$, then the probability of realizing a contact between residues i and j is lower at the T_D temperatures compared with the reference ensembles, and the converse is true if $d_{ij} > 0$.

The prominent differences between T_D ensembles and the two reference ensembles are the higher probabilities associated with long- and intermediate-range contacts involving hydrophobic residues drawn from four specific groups labeled g_1 – g_4 , where $g_1 \equiv (M1, V3, I4, F5, L6)$, $g_2 \equiv (I18, K19, N20, V21, A22)$, $g_3 \equiv (G24, Y25, A26, N27, N28, F29, L30, F31)$, and $g_4 \equiv (G34, L35, A36, I37)$. These contacts have low probabilities (≤ 0.1), are predominantly nonnative (top row in Fig. 7 and SI Appendix, Fig. S13), and are either medium-range (contacts between residues from g_1 and g_2 , g_2 and g_3 , or g_3 and g_4) or long-range (contacts between residues from g_1 and g_3 or g_1 and g_4). SI Appendix, Fig. S14 displays a montage of conformations to illustrate how

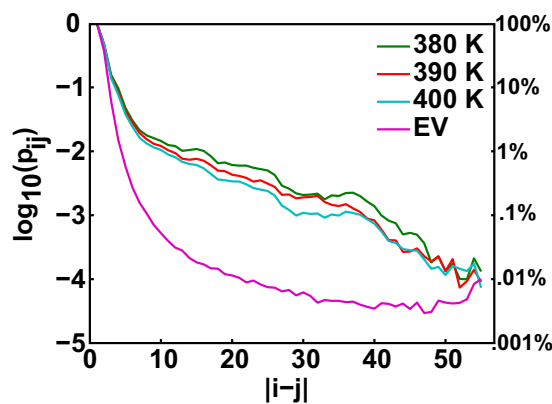


Fig. 5. Comparison of the probabilities associated with short-, intermediate-, and long-range contacts. We calculated the probability p_{ij} of realizing spatial contacts between residues i and j that are $|i-j|$ apart in the linear sequence. The figure shows plots of $\log_{10}(p_{ij})$ plotted vs. linear sequence separation $|i-j|$ for the three T_D temperatures and the EV ensemble. In all cases the p_{ij} values decrease with increasing sequence separation $|i-j|$, and the p_{ij} values are less than 1% for $|i-j| > 10$.

probe at 12 °C. The PRE $^1\text{H}_\text{N}$ - $^1\text{T}_2$ rates measurement were performed in a two-time-point approach (45) on a Bruker 700 MHz spectrometer with a conventional probe.

MC Simulations. We used the CAMPARI software package (<http://campari.sourceforge.net/>), the ABSINTH implicit solvation model (46), and the underlying force field paradigm. Parameters were taken from the abs3.2_opls.prm set. The protein was modeled in atomic detail, and the ABSINTH implicit solvation model was used to model solvent-mediated interactions.

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Details regarding the simulation setup, analysis of conformational ensembles, solution conditions, and its impact on our results are discussed in *SI Appendix*.

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