Electronic Supplementary Information for

Accessing local structural disruption of Bid protein during thermal denaturation by absorption-mode ESR spectroscopy

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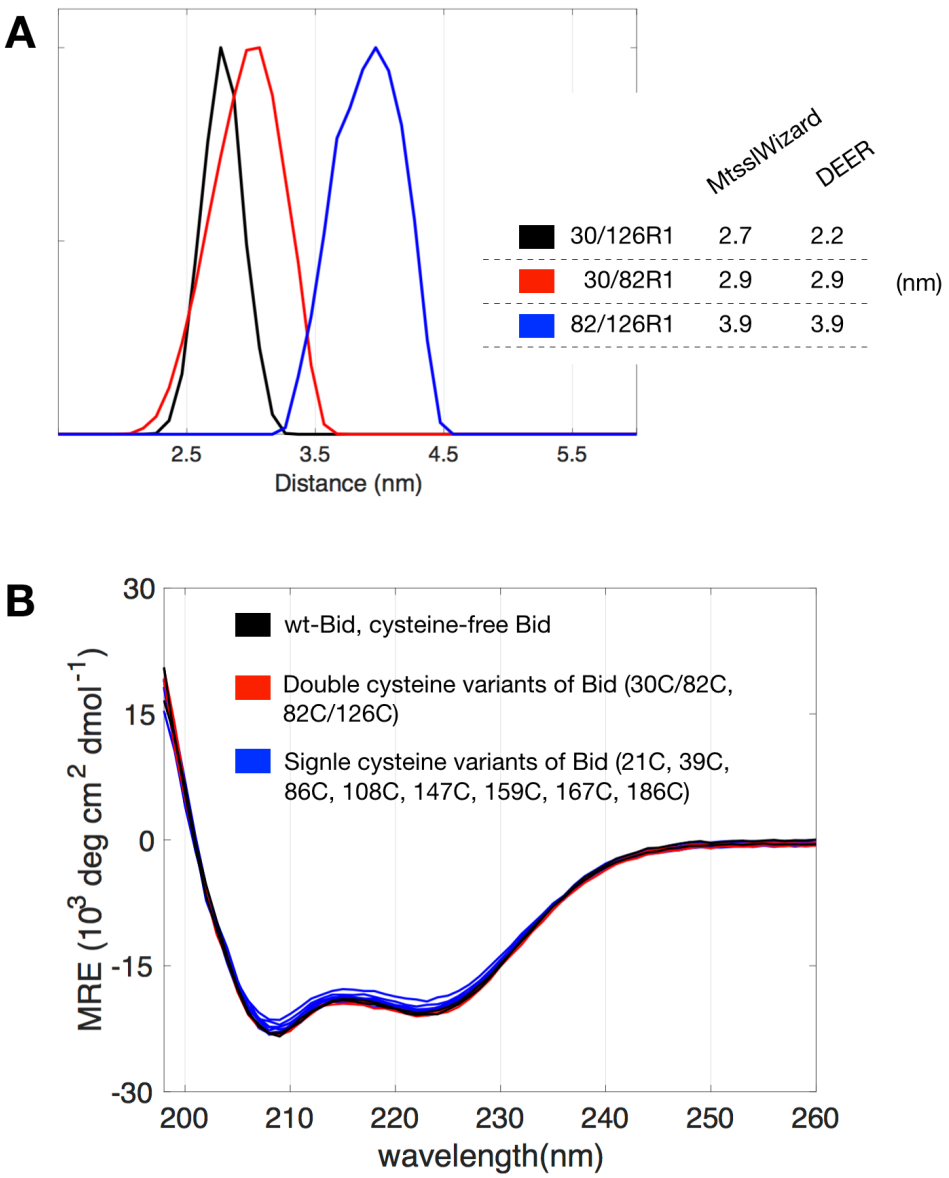


Figure S1. (**A**) Predictions of distance distributions from MtsslWizard simulations of the NMR-determined structure (PDB: 1DDB). Overall, the averages of the predicted distances are consistent with the experimental DEER results, as listed. (**B**) CD spectra of various single-cysteine variants of Bid recorded at 300 K. Wt-Bid has two native cysteine residues, C30 and C126. Cysteine-free Bid is C30S/C126S. The mutation of the two native cysteines barely affects the CD spectra. Overall, the results indicate that the structure of the Bid mutants is affected insignificantly by the single or double cysteine mutations.

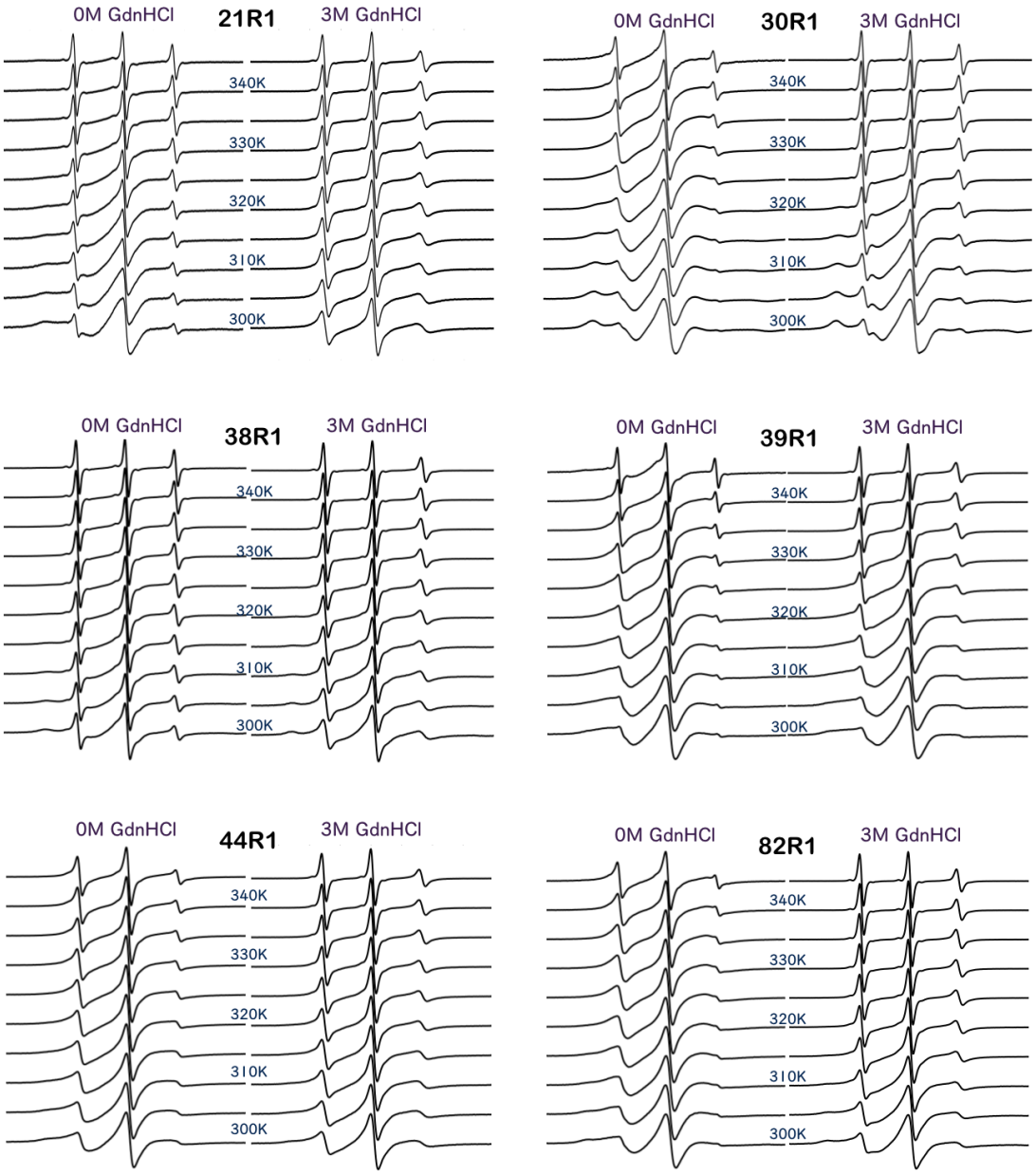


Figure S2. Cw-ESR spectra of the single-labeled Bid mutants in 0 M versus 3 M GdnHCl solutions recorded in the temperature range of 300−345 K. A total of 30 single-labeled Bid mutants are studied.

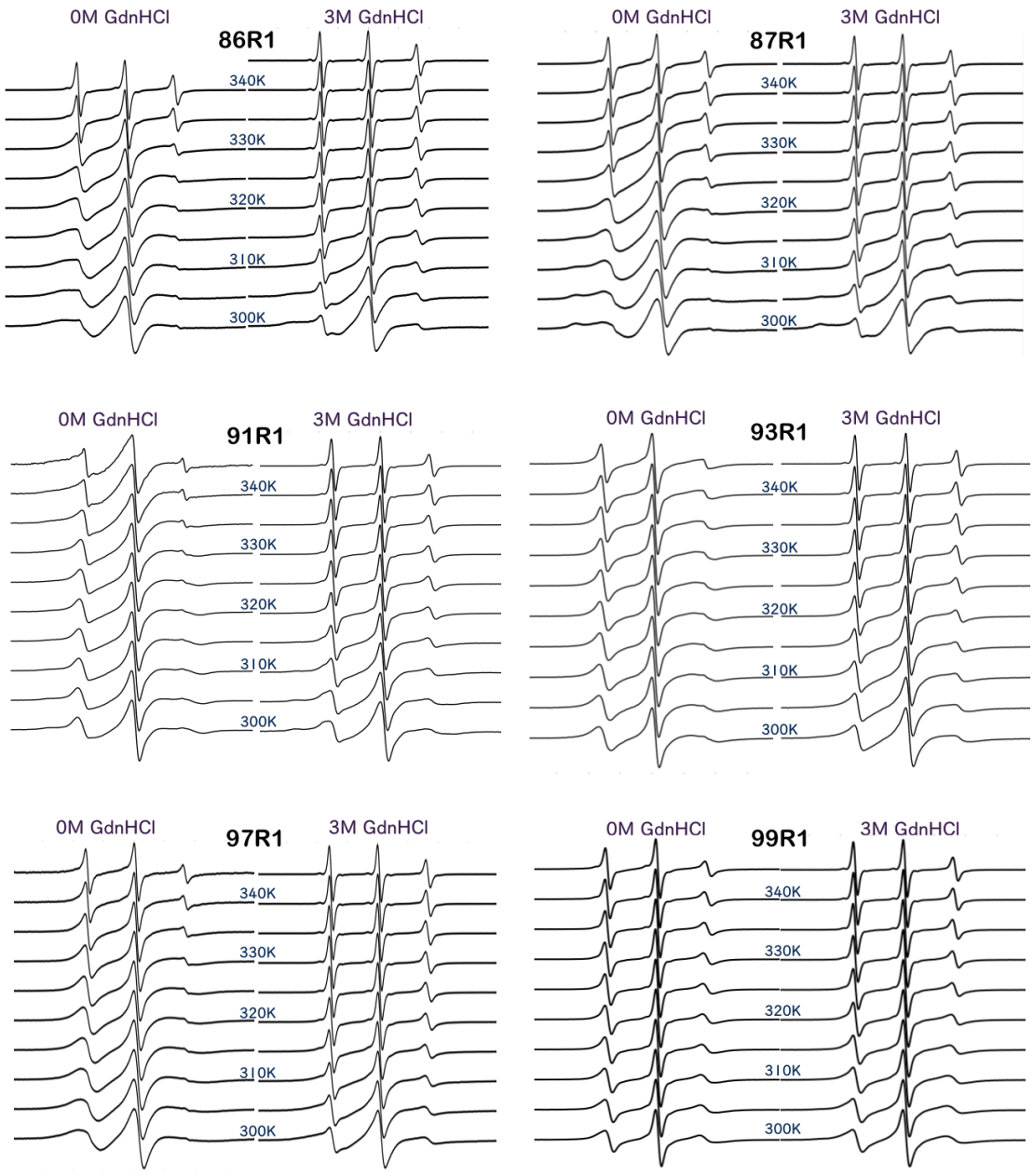


Figure S2. (continued)

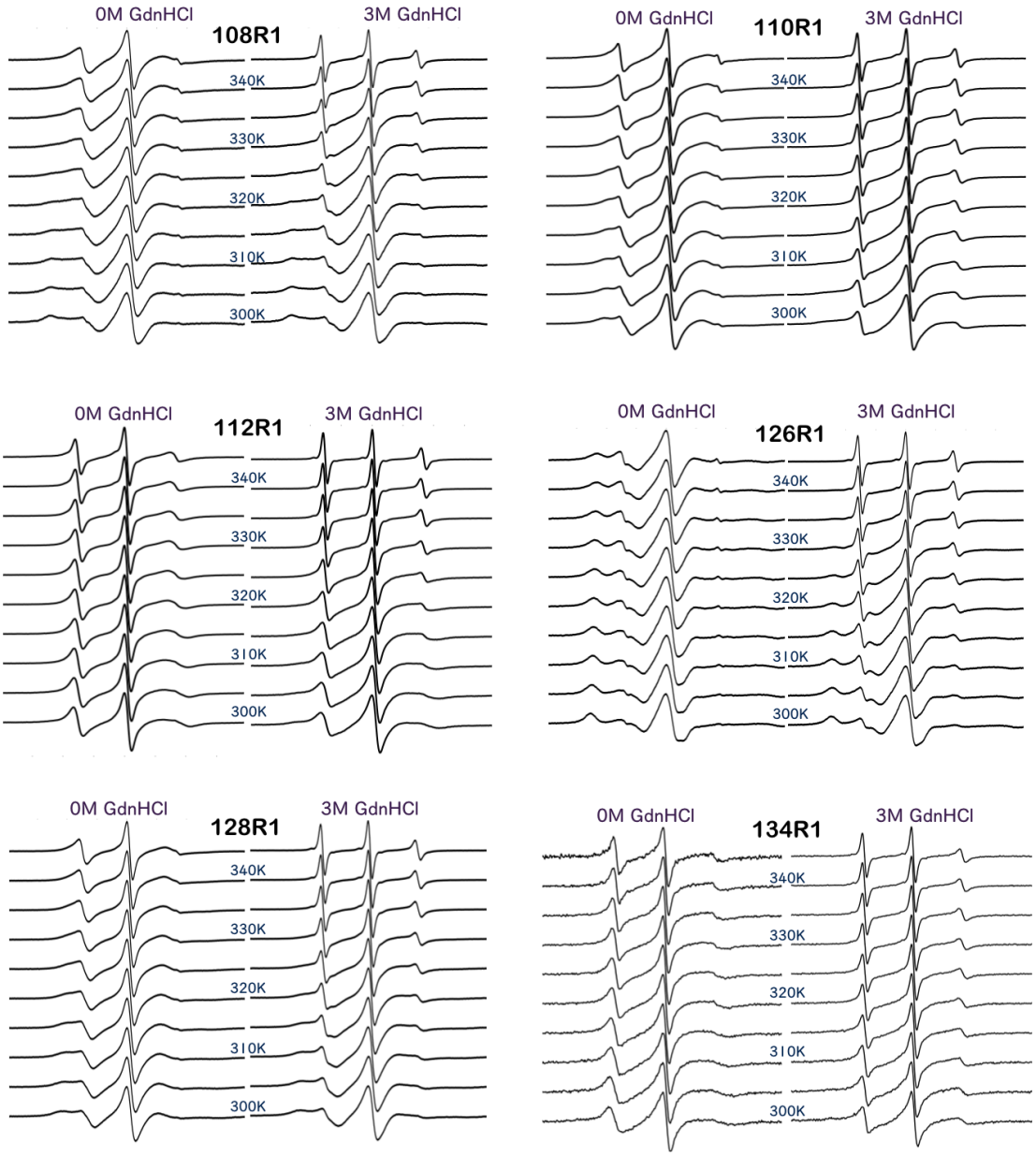
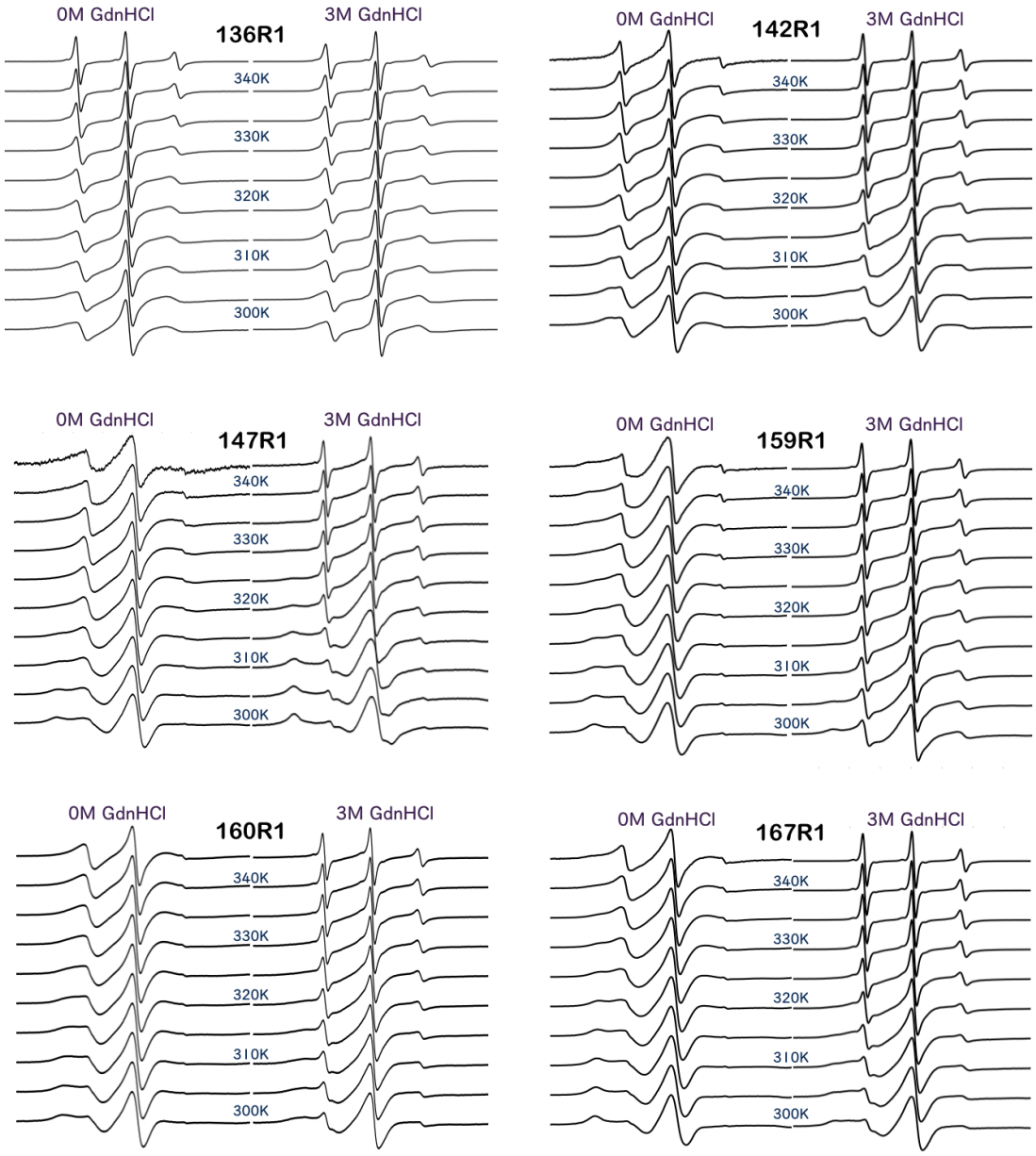


Figure S2. (continued)



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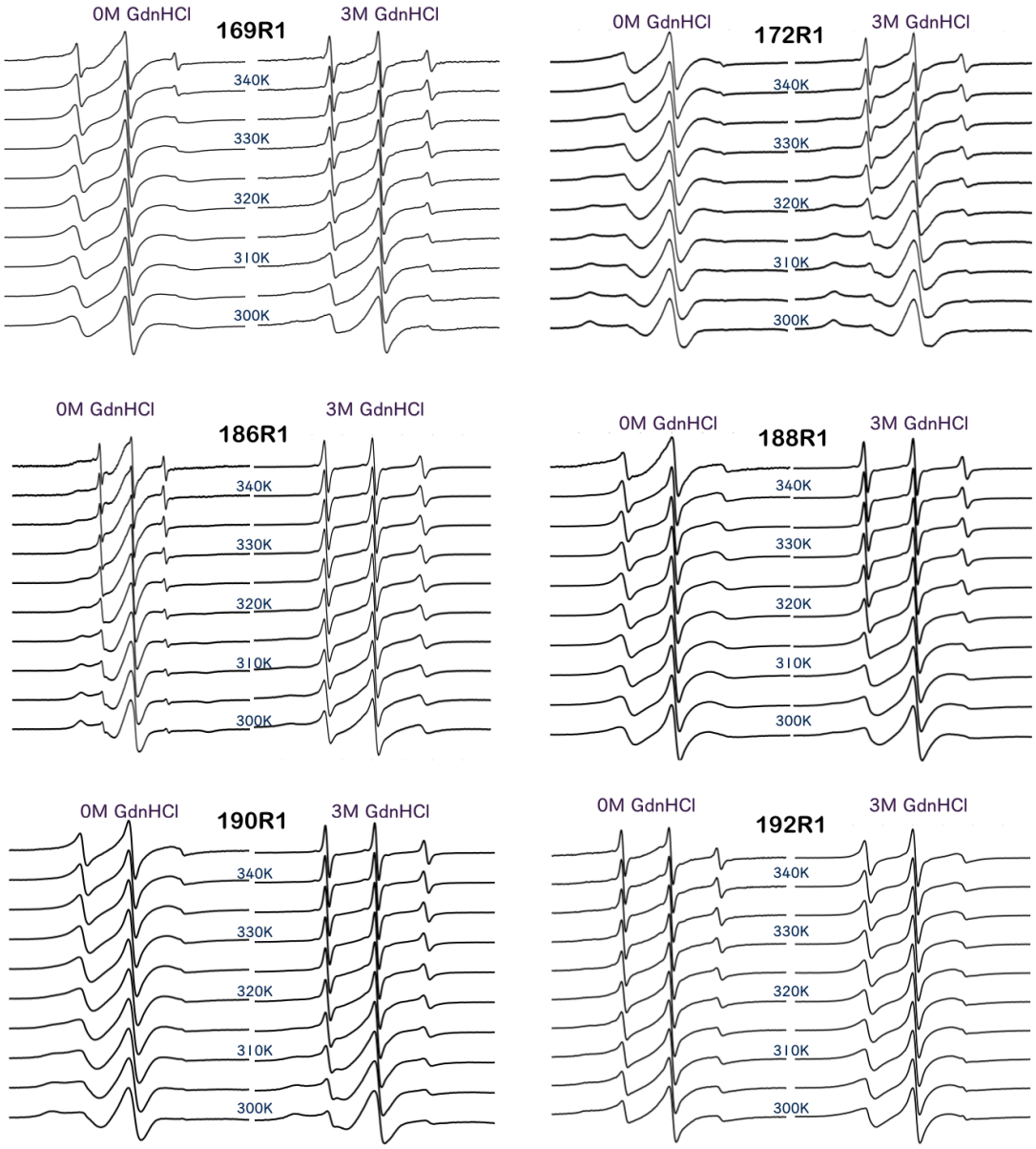

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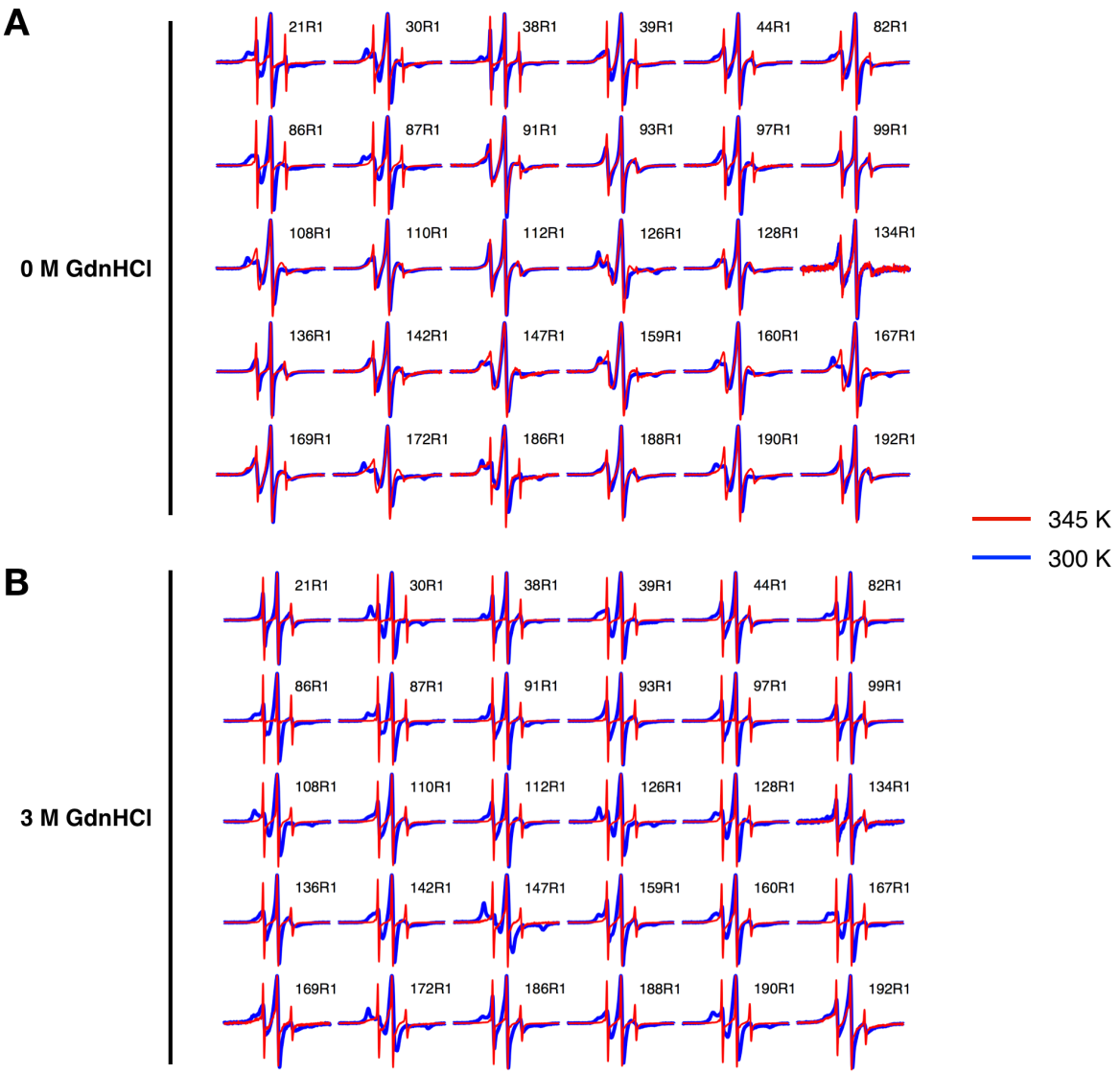

Figure S3. A comparison of cw-ESR spectra recorded at 300 K versus 345 K. (**A**) Cw-ESR spectra collected in the solution containing no GdnHCl. The spectra of 345 K are reasonably different from those of 300 K due to the temperature difference. Importantly, many of the spectra retain the lineshapes exhibiting anisotropy and low mobility, indicating that the local environments are not completely unfolded even at temperature up to 345 K, in 0 M GdnHCl. (**B**) In 3 M GdnHCl, all of the spectra for 345 K are largely different from the spectra recorded at 300 K. The spectra for 345 K all exhibit mobile and isotropic lineshapes characterized by three sharp peaks and relatively narrow linewidths. These changes indicate that the local environments in Bid in 3 M GdnHCl are unfolded and exposed to solvent at 345 K.

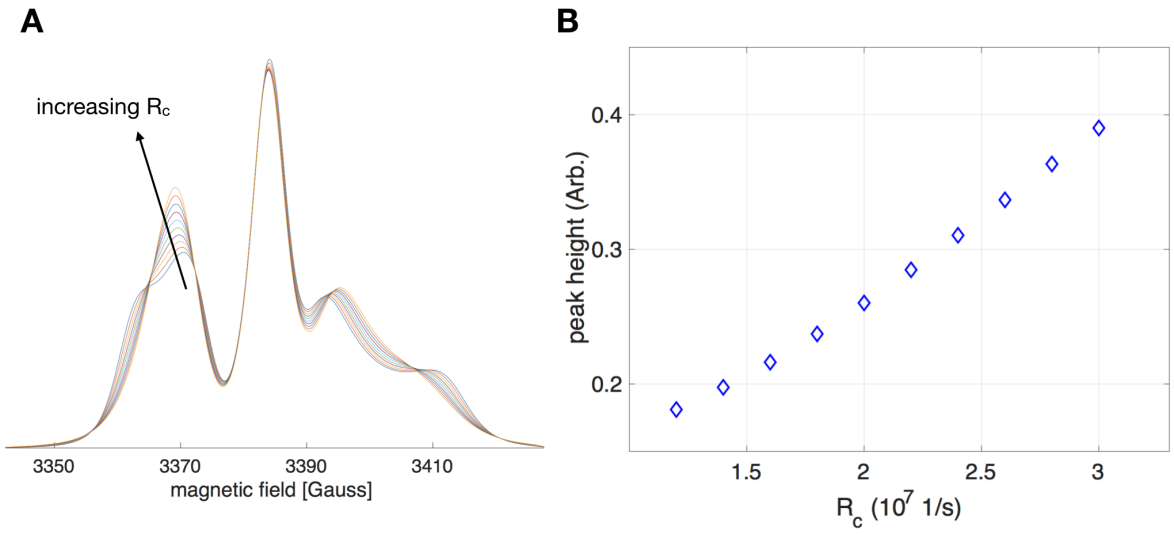

Figure S4. Theoretical investigation for the linear baselines observed in the peak-height plot. (**A**) Cw-ESR absorption spectra simulated with increasing isotropic rotational diffusion rate (R_c) from 1.2 to 3 (10^7 s^{-1}) using the slow-motional ESR function in the EasySpin software package.[52] The studied range of R_c values was confirmed to be representative of the globular tumbling rates of a spin-labeled T4L protein in the temperature range of 250−350 K, as demonstrated by multi-frequency ESR spectroscopy.[23,24] As the molecular weights of T4L and Bid are close to each other (ca. 20 kDa), it is reasonable to directly use those R_c values to simulate the absorption spectra. In other words, these simulated spectra represent the contribution from the isotropic tumbling motions of T4L, in the absence of local dynamics and orderings of spin label. (**B**) Plot of lower-field peak-height data of the simulated ESR spectra versus R_c. It shows clearly that the peak-height data changes linearly with R_c, supporting a view that in the absence of local contacts (e.g., nearby side-chain contacts, protein tertiary contacts, local structure disruption, and side-chain anisotropic orderings) a linear curve in the plot of peak-height versus R_c is indeed expected in the studied temperature range. To correlate R_c with T, we consider the following. Using Stokes-Einstein hydrodynamic theory (for which $R_c=k_BT/8\pi r^3\eta$, exhibiting a linear dependence between R_c and T for a freely tumbling macromolecule), we can, therefore, conclude that, for a freely tumbling spin-labeled Bid protein studied in the T range of 250−350 K, its corresponding peak-height data would increase linearly with T. Thus, a changeover in the slope of the peak-height data with T is caused by changes in the local environment of protein. This linear baseline was also previously demonstrated for a T4L study using ESR spectroscopy.[24] Overall, the simulation presented here lays the foundation for explaining the linear baselines observed in the present study.

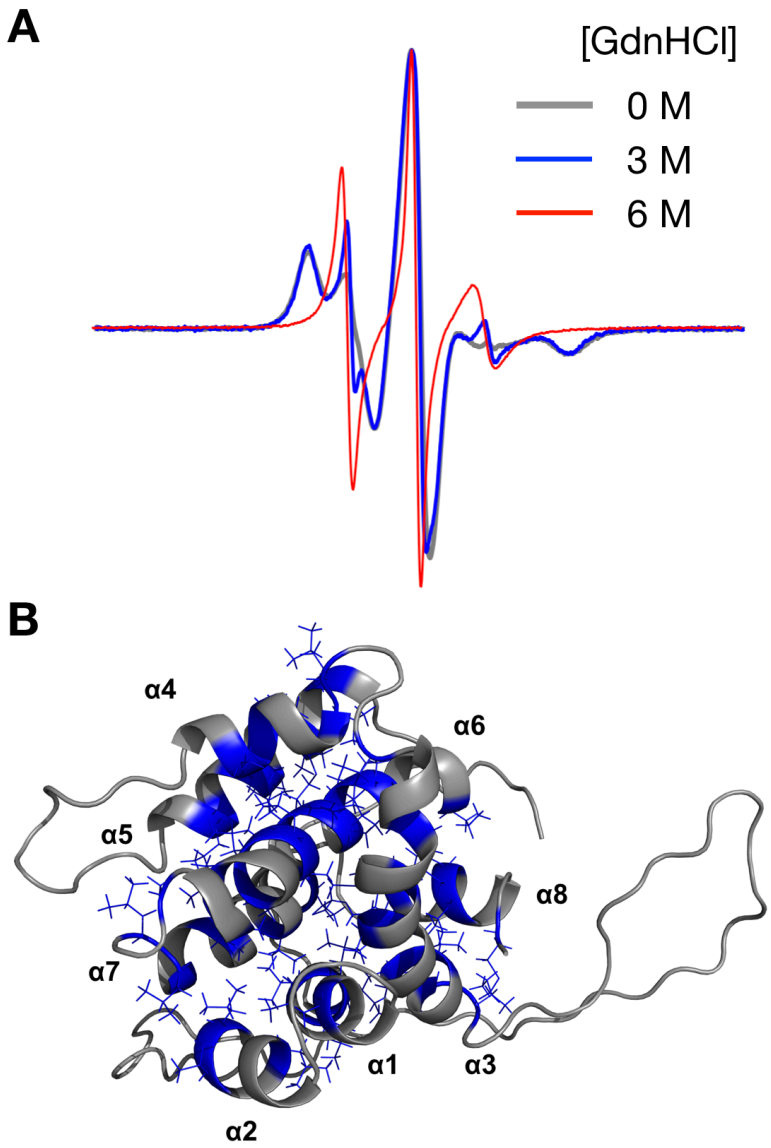

Figure S5. (**A**) Cw-ESR spectra of wt-Bid (i.e., 30R1/126R1, doubly labeled Bid) recorded at 300 K for different GdnHCl concentrations, 0, 3, and 6 M. Spectra for 0 and 3 M GdnHCl are basically similar to each other, but the lineshapes exhibit a slightly greater mobility in 3 M than 0 M GdnHCl. It indicates of little difference in the local environments of 30R1/126R1 between the two GdnHCl conditions. In 6 M GdnHCl, the spectrum is significantly different from the spectra for 0 and 3 M GdnHCl; the spectrum for 6 M GdnHCl reflects highly mobile and narrow lineshapes, which indicate of unstructured local environments. This cw-ESR study demonstrates that Bid is denatured in 6 M GdnHCl. (**B**) Cartoon model of Bid highlighting that many of its hydrophobic residues are packed within the core region. The hydrophobic residues highlighted (in blue) are Leu, Val, Ala, Met, and Ile.

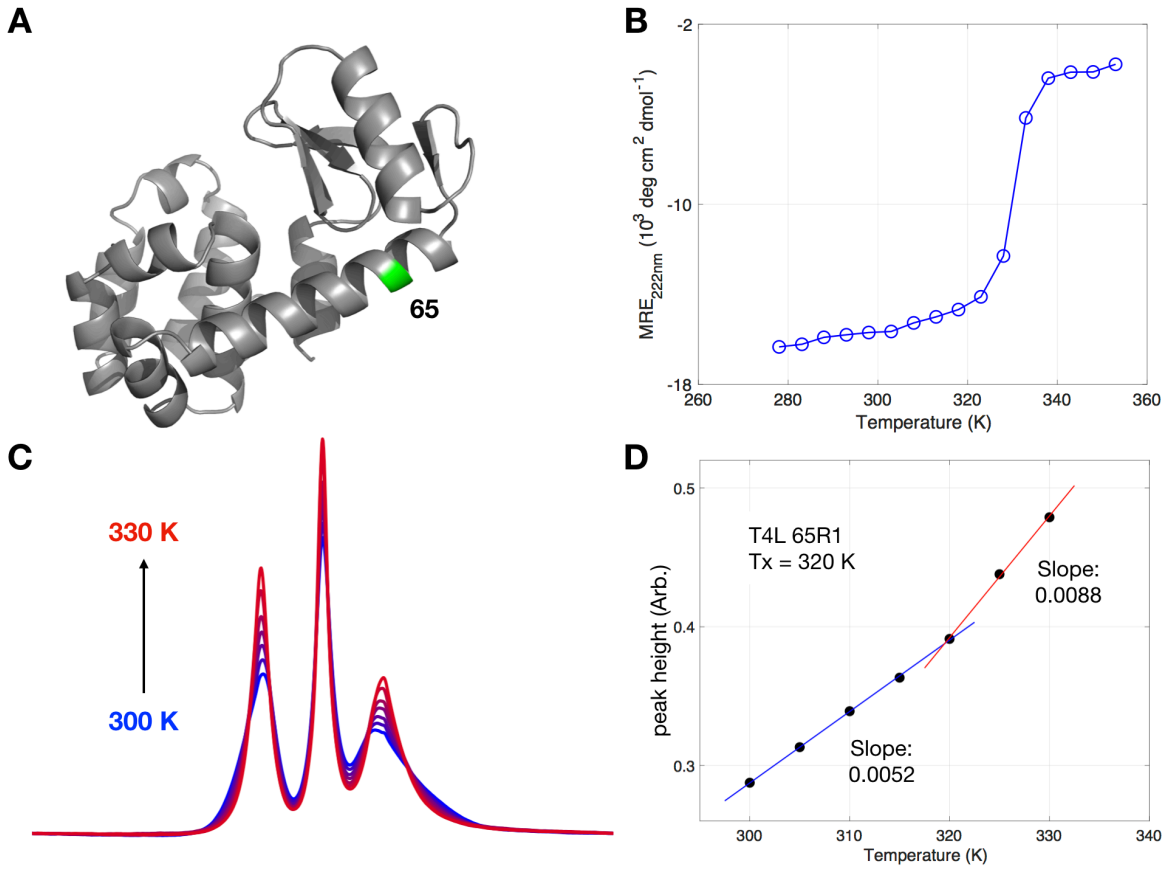

Figure S6. (**A**) Structure model of T4L protein (PDB: 3LZM) highlighting residue 65. (**B**) MRE at 222 nm for T4L 65R1 by CD spectroscopy, recorded at increasing temperatures (5−80 °C, equivalently 278−353 K, with intervals of 5 K). It reveals a melting temperature around 330 K for this mutant, characterized by a typical sigmoidal curve with the onset of growth around 320 K. (**C**) Cw-ESR absorption spectra of T4L 65R1 recorded at temperatures from 300 to 330 K. (**D**) Peak-height plot of the cw-ESR absorption spectra and the result of linear regression fits. It clearly shows an onset temperature Tx ~ 320 K, consistent with the result of CD measurement shown in **B**. Using this well-known model protein T4L, we demonstrated that our ESR absorption peak-height analysis is useful to probe the onset of local environmental disruption during thermal denaturation.

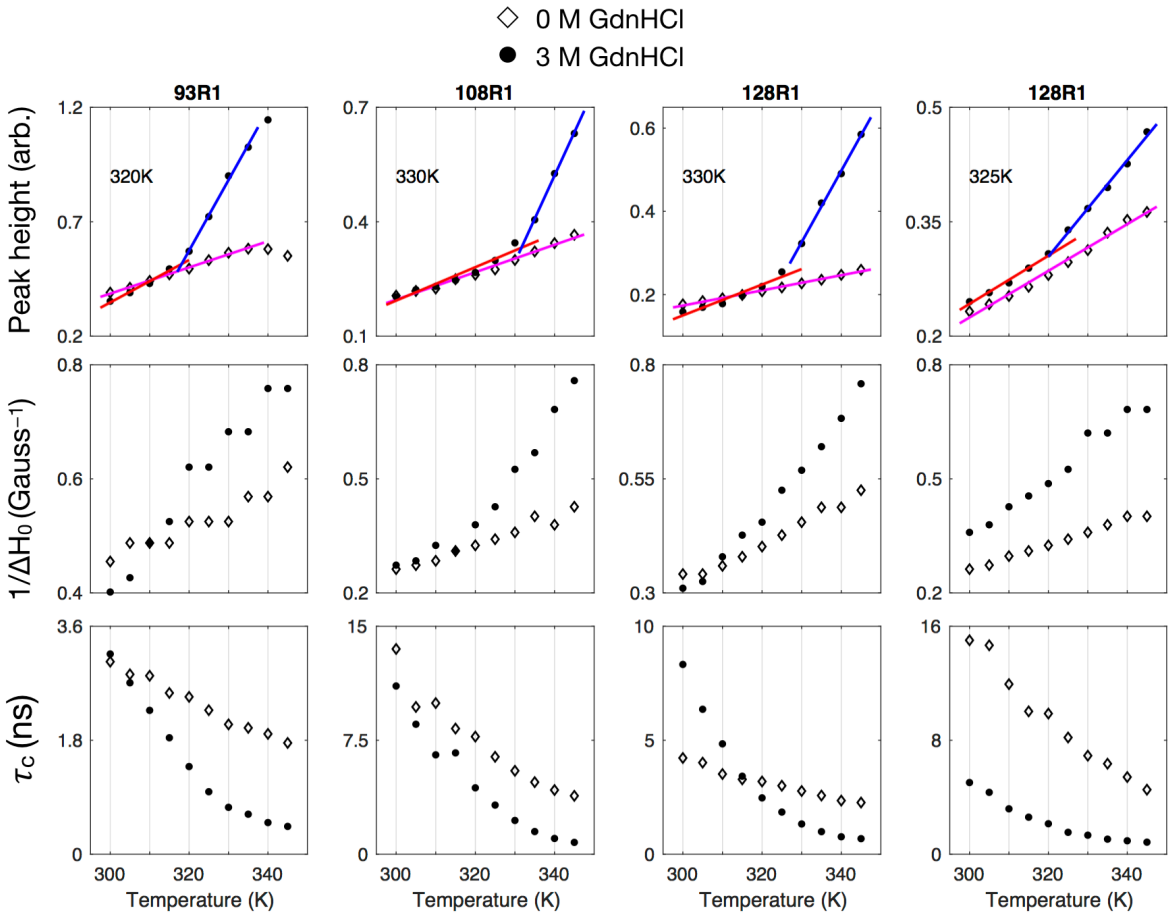

Figure S7. A comparison of the results obtained with three analysis methods, (from top to bottom) the peak-height, ΔH_0^{-1}, and τ_c analyses. The peak-height analysis is useful to distinguish the T-dependent effect from the local structural disruption. Details of the peak-height results are discussed in the main text (cf. Fig. 5). The ΔH_0^{-1} analysis focuses on the change in the center-field linewidth, which is sensitive to the side-chain mobility in the intermediate-to-fast motional regime. It is often used to explore secondary structure in a protein by the plots of ΔH_0^{-1} versus protein sequence number for a fixed T (rather than varying T). As protein secondary segment is packed asymmetrically, ΔH_0^{-1} provides a useful measure of the relative mobility to determine the secondary structure. Since the time range of the spectra in this study covers from the slow to the fast motional regime, the plots of ΔH_0^{-1} versus T are not useful to reveal the local disruption of Bid structure during thermal denaturation. Likewise, the τ_c method is suitable for studying side-chain mobility in the same time range as the ΔH_0^{-1}. Therefore, the resulting curves appear to reveal little information about the thermally-induced local disruption of Bid. In the plots of τ_c versus T, the T-dependent effect is dominant such that the onset of local disruption of protein is barely present in the curves.

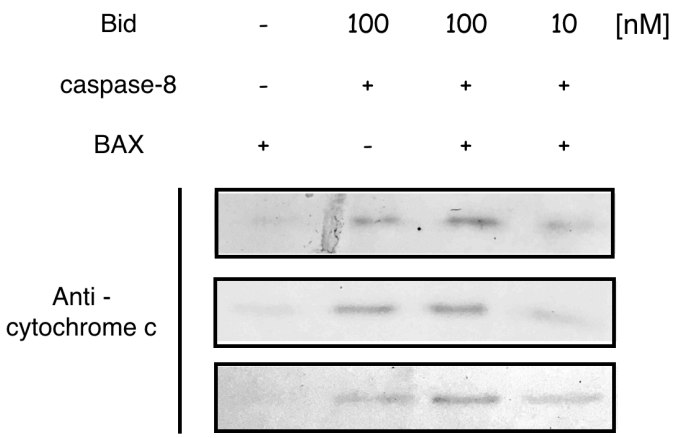

Figure S8. A control experiment for the cytochrome c release assay in Figure 7. Our CD results (Fig. 1) indicate that after the heat treatment more than 90% of Bid are denatured and aggregated in the solution, but the Bid aggregates (as shown in Fig. 7) retain the ability of promoting cytochrome c release from mitochondria. To confirm that the cytochrome c release in Figure 7 is mainly caused by the Bid aggregates rather than the minor fraction of Bid (<10%) that retain the native structure, here we performed the cytochrome c release assays with a solution containing 1/10 amount of input Bid used in Figure 7. The result shows that 1/10 amount of native Bid in the presence of BAX and the cleavage buffer is not sufficient to cause a large amount of cytochrome c release from mitochondria. This result provides support for Figure 7 that Bid aggregate induced by the heat treatment is indeed responsible for the cytochrome c release.