Supplementary Figures for

Tuning Protein Autoinhibition by Domain Destabilization

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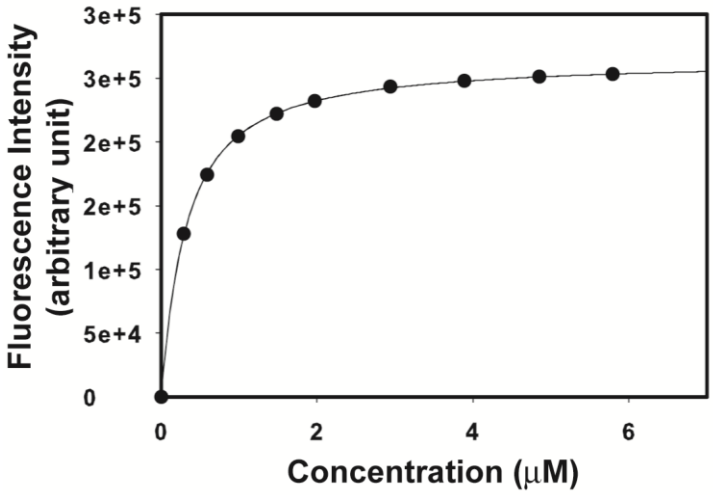
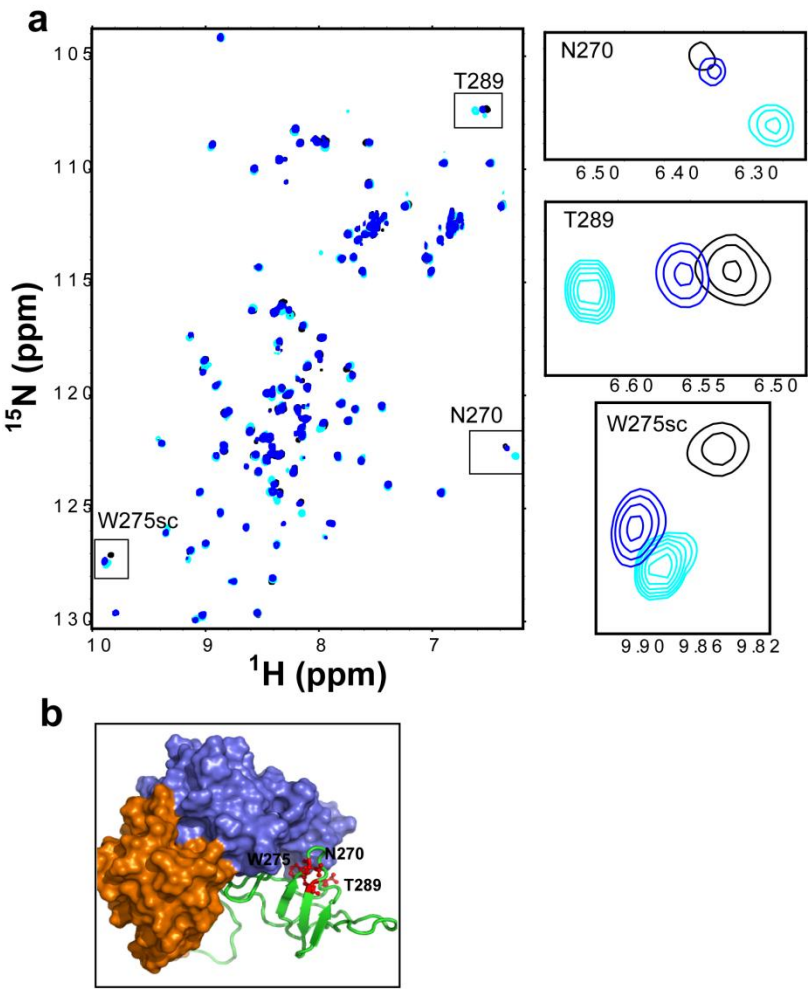
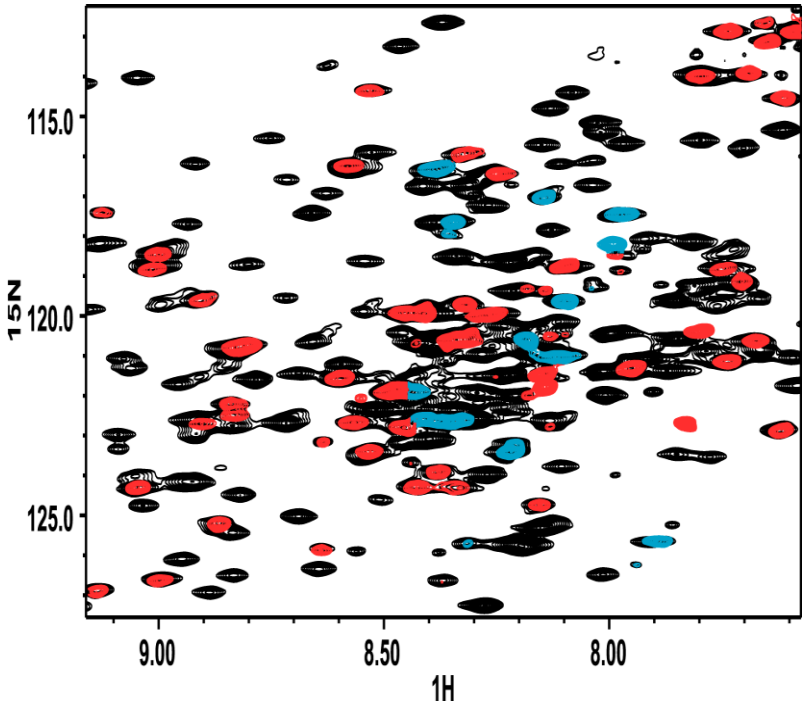


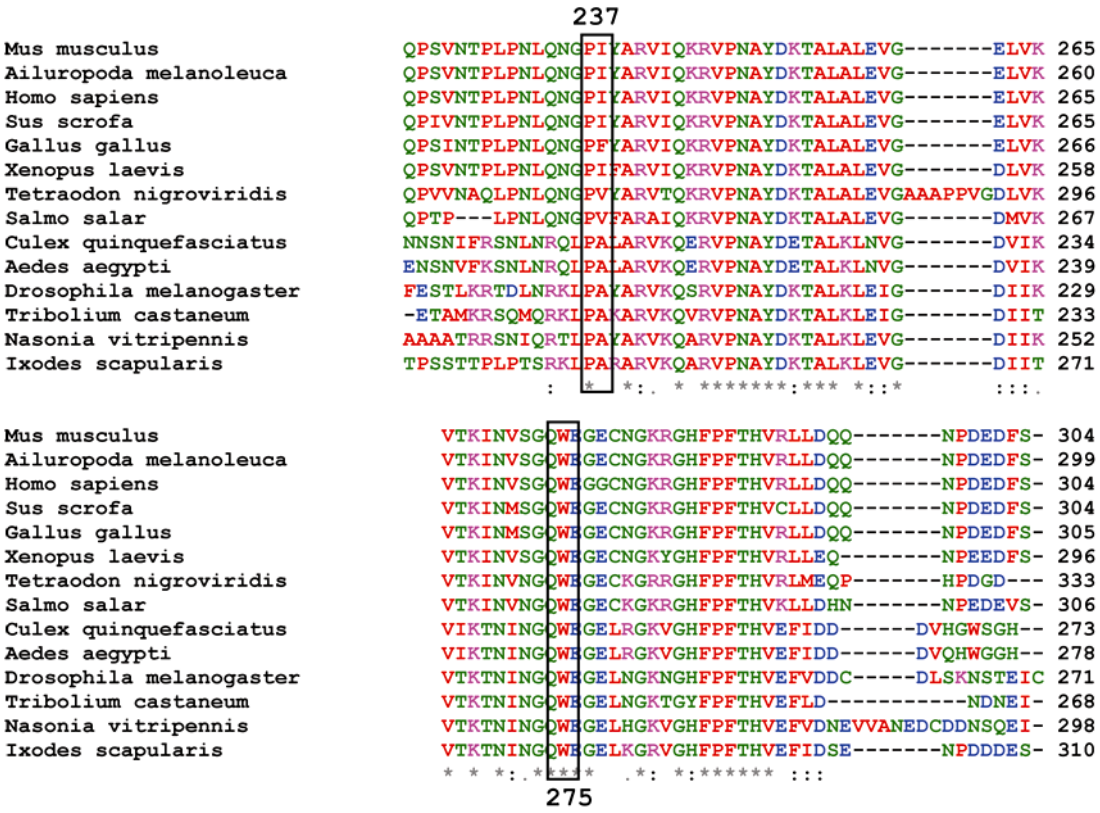
Supplementary Figure 1. Fluorescence-detected titration curve of C3G-derived ligand binding to apoCrk2.



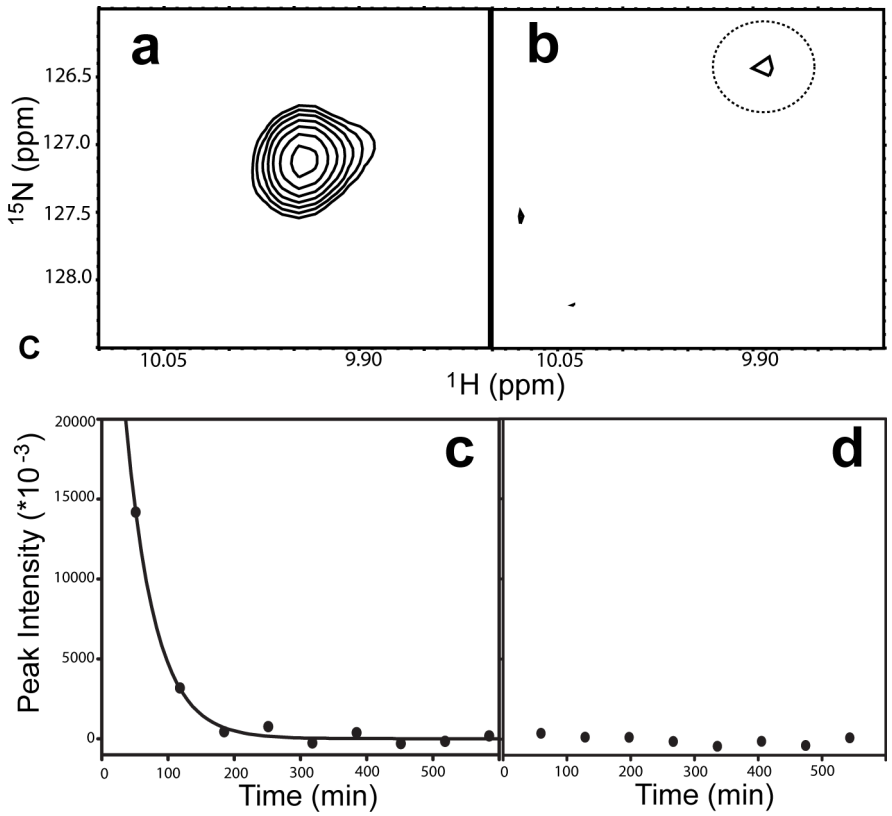
Supplementary Figure 2. Conformational change of the cSH3 domain in the context of different states of full-length Crk2. **(a)** Superimposed ^{1}H-^{15}N HSQC spectrum of the cSH3 domain in the context of isolated (cyan), Crk2 (black), Crk2/C3G (blue). Insets show an expanded view of three most affected residues, N270 (top), T289 (middle) and W275sc (bottom). **(b)** The positions of three most affected residues (N270, W275sc and T289) are shown in the aCrk2 structure.



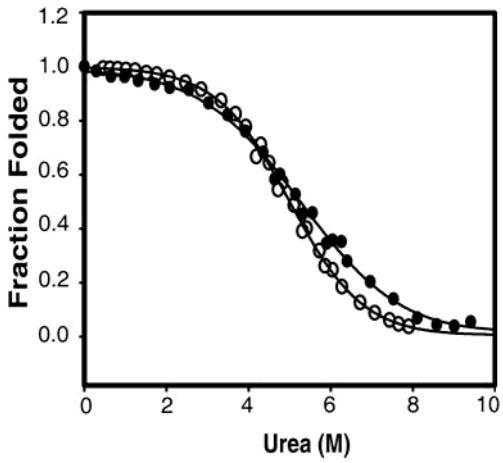
Supplementary Figure 3. Superimposed ^{1}H-^{15}N HSQC spectra of segmentally labeled full-length Crk2 in which residues from 208 to 304 are ^{15}N-labeled (red; cSH3 domain and cyan; linker) and uniformly ^{15}N-labeled Crk2 (black). The chemical shifts for linker region (residue 208 – 237, colored cyan) show little dispersion, so uniformly labeled Crk2 is not suitable for quantitative measurements of NMR spin relaxation rate constants, such as R_2 and heteronuclear NOE.



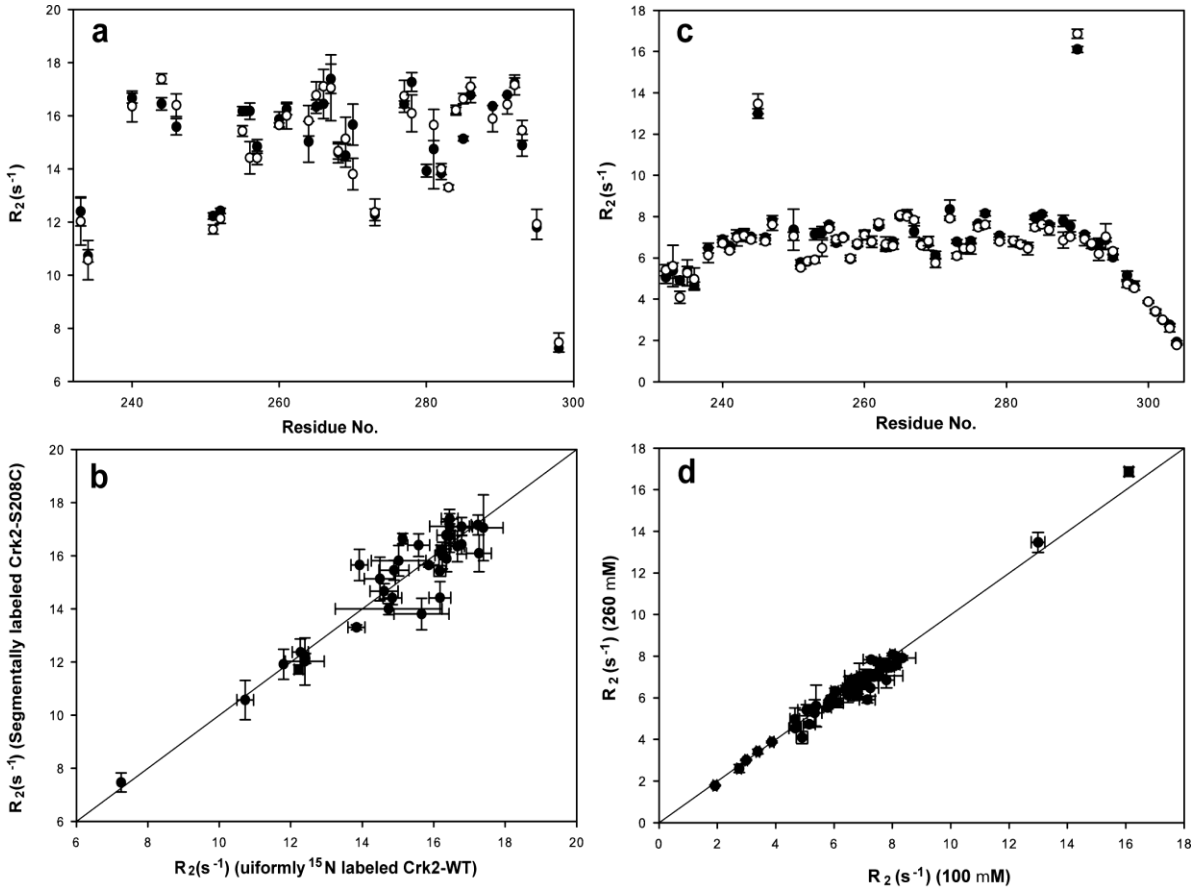
Supplementary Figure 4. Amino acid sequence alignment of the cSH3 domain in Crk2. W275 is highlighted in bold character. The sequence alignment was performed by ClustalW2 (Larkin, M.A. et al. ClustalW and ClustalX ver 2. *Bioinformatics* **23**, 2947-2948). The color coding represents the amino acid type with red, hydrophobic; blue, acidic; magenta, basic; and green, amine and hydroxyl groups). The symbols in the bottom row represent the degree of sequence conservation as the indicated position in the alignment: "*" indicates complete conservation, ":" indicates that conserved substitutions are observed, and "." indicates that semi-conserved substitutions are observed. The sequence of *Mus musculus* (mouse) listed first because it is used for the experiments described in main text.



Supplementary Figure 5. First HSQC spectra of side chain resonance (^{1}H-$^{15}N^{\varepsilon 1}$) of W275 in the series of H/D exchange experiments for isolated cSH3 domain (**a**) and for full-length Crk2 (**b**). The dotted circle in panel B indicates the position of the peak for the indole ring of W275 in the full-length Crk2. The peak intensity decay curve of side chain resonance (^{1}H-$^{15}N^{\varepsilon 1}$) of W275 for isolated cSH3 domain (**c**) and for the full-length Crk2 (**d**). The solid line in panel **c** represents the best fit curve using single exponential decay function.



Supplementary Figure 6. Urea-induced unfolding curves of the wild type isolated cSH3 (open circles) and the isolated cSH3-7AW mutant (closed circles). The experiments were performed by monitoring the fluorescence intensity of W275 for wild-type and 7AW for the cSH3-7AW mutant cSH3 domain change upon increasing the concentration of urea



Supplementary Figure 7. Comparisons of NMR spin transverse relaxation rate constants, R_2. (**a**) R_2's of uniformly ^{15}N labeled Crk2 (with wild type sequence; shown with open circles in panel **a**) and segmentally labeled Crk2 (S208C; shown with closed circles in panel **a**). Only data for residues with well-resolved peaks in the spectrum of uniformly ^{15}N labeled Crk2 were compared with data for segmentally labeled Crk2. (**b**) Correlation plot of R_2 between segmentally labeled and uniformly labeled Crk2. (**c**) R_2's of isolated cSH3 domain measured for two different protein concentrations: 100 µM (closed circles in panel **c**) 260 µM (open circles in panel **c**). (**d**) Correlation plot of R_2 between two different concentrations of isolated cSH3 domain.

Supplementary Methods for

Tuning Protein Autoinhibition by Domain Destabilization

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Protein Expression and Purification

Isolated ^{15}N labelled cSH3 domain (residues 232-304 of Crk2) was expressed in *E. coli* BL21(DE3) cells with a His-tag and cleaved using Tev-protease after Ni-column purification. cSH3-7AW (^{15}N) was expressed in *E. coli* CY15077 (Trp auxotrophic strain, Yale University *E. coli* Genetic Stock Center) cells with M9 minimal media containing D,L-7AW (Sigma). Segmentally-labeled Crk2 was produced by chemical ligation of Crk1-α-thioester (1-207) and the ^{15}N-cSH3 (208-304), containing the S208C mutation. Crk1-thioseter was produced by expressing Ckr1 as a fusion protein with the Gyrase A intein and a chitin binding domain (Crk1-GyrA-CBD). The fusion protein was purified using a chitin bead column (Novagen). The Crk1-thioester was cleaved from GyrA-CBD by incubating the fusion protein in a buffer containing 100 mM sodium phosphate (pH 7.2), 100 mM NaCl and 2% ethanthiol. After cleavage, the Crk1-thioester was further purified by RP-HPLC and characterized by ESMS. The isolated ^{15}N-cSH3 (residues 208-304) was expressed in BL21(DE3) cells. The His-tagged protein was purified by Ni-Sepharose column (GE-Healthcare) and Factor Xa protease (Roche) was used to cleave the His-tag. After cleavage, cSH3 was purified further by RP-HPLC. The ligation reaction was initiated by dissolving the purified, lyophilized proteins to a final concentration of 1 mM each in a buffer containing 100 mM sodium phosphate (pH 7.0), 100 mM NaCl, 6 M guanidinium chloride, 2% MESNA, 2% ethanthiol. Crk2 (^{15}N-cSH3) was purified by RP-HPLC and characterized by ESMS.

Equilibrium unfolding measurements of isolated cSH3 domains

Urea-denaturation experiments were performed at 298 K by monitoring the change in fluorescence signal intensity recorded at 340 nm and 398 nm for wild type cSH3 and cSH3-7AW, respectively. The sample buffer contains 20 mM sodium phosphate (pH 7.2) and 50 mM NaCl.

Protein unfolding was monitored by the change in the fluorescence signals of Trp and 7AW for wild type and mutant cSH3 domain, respectively. Signals were averaged for 60 s at each urea concentration after a 5 min equilibration. Urea concentrations were measured by measuring the refractive index of the solution. The measured fluorescence signals were fit to the two-state unfolding model[32]. To confirm that isolated cSH3 domain is monomer, R_2 relaxation rate constants were measured at 100 μM and 260 μM (Supplementary Fig. 7). Both samples showed identical R_2 values demonstrating that the isolated cSH3 domain is monomer in our experimental condition.

Determination of the unfolding free energy of the two conformations of cSH3-7AW

Both fluorescence quenching experiments and the ^{1}H-^{15}N HSQC spectrum showed that two conformations (**B**; buried and **E**; exposed) are populated in the native state (**N**) of the variant of the cSH3 domain in which W275 is replaced by 7AW, (**cSH3-7AW**). Thus, the unfolding equilibrium constant (K_{eq}^{7AW}) of the cSH3-7AW can be expressed as:

$$N\ (B \rightleftharpoons E) \overset{K_{eq}^{7AW}}{\rightleftharpoons} U$$

(Scheme S1)

$$K_{eq}^{7AW} = \frac{[U]}{[N]} = \frac{[U]}{([B]+[E])} = \frac{[U]/[E]}{([B]/[E]+1)} = \frac{K_{eq,E}^{7AW}}{([B]/[E]+1)} \qquad \text{(S.1)}$$

where K_{eq}^{7AW} = 0.015 ± 0.007 kcal mol^{-1} is obtained from the measured stability of cSH3-7AW (ΔG° = 2.5 ± 0.4 kcal mol^{-1}) and $K_{eq,E}^{7AW} = [U]/[E]$ is the unfolding equilibrium constant for the cSH3-7AW in which the 7AW moiety is exposed to solvent. The measured population ratio of **B** and **E** obtained using fluorescence quenching experiments is,

$$\frac{[E]}{[B]} = \frac{0.12 \pm 0.01}{0.88 \pm 0.01} = 0.136 \pm 0.011 \quad \text{(S.2)}$$

Therefore,

$$K_{eq,E}^{7AW} = ([B]/[E] + 1)K_{eq}^{7AW} = 0.12 \pm 0.05 \quad \text{(S.3)}$$

The unfolding free energy ($\Delta G^{\circ}_{7AW,E}$) of the cSH3-7AW domain in which the 7AW is exposed is given by,

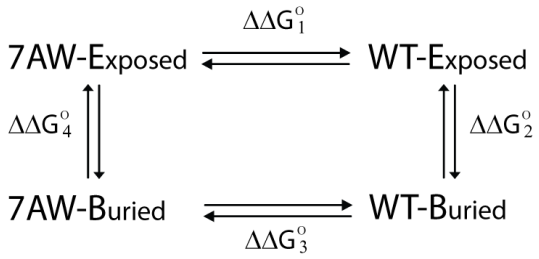
$$\Delta G^{o}_{7AW,E} = -RT \ln K_{eq}^{7AWE} = 1.26 \pm 0.21 \text{kcal mol}^{-1} \quad \text{(S.4)}$$

The unfolding free energy of the cSH3-7AW in which the 7AW is buried can be calculated similarly:

$$\Delta G^{o}_{7AW,B} = -RT \ln K_{eq}^{7AWB} = 2.43 \pm 0.25 \text{kcal mol}^{-1} \quad \text{(S.5)}$$

Calculation of the energetic penalty upon exposure of the indole ring of W275 in cSH3-WT

The free energy penalty upon exchange of the indole ring of W275 from the buried to the exposed conformation in cSH3-WT can be estimated using the hypothetical double mutant cycle shown in Scheme-S2.



(Scheme S2)

$\Delta\Delta G_x^\circ$ represents the free energy difference between two states of one side of the thermodynamic cycle. The free energy difference associated with the two conformations of the indole ring in the wild type SH3 domain ($\Delta\Delta G_2^\circ$) can be calculated when $\Delta\Delta G_1^\circ$, $\Delta\Delta G_3^\circ$, and $\Delta\Delta G_4^\circ$ are known using the relationships

$$\Delta\Delta G_1^\circ + \Delta\Delta G_2^\circ = \Delta\Delta G_3^\circ + \Delta\Delta G_4^\circ \qquad \text{(S.6)}$$

Using the results of eqs. S.4 and S.5 in combination with the measured free energy of folding of cSH3-WT and cSH3-7AW (Table 2 in Main text), $\Delta\Delta G_3^\circ$ and $\Delta\Delta G_4^\circ$ can be estimated as:

$$\Delta\Delta G_3^\circ = \Delta G_{\mathrm{WT}}^\circ - \Delta G_{7\mathrm{AW,B}}^\circ = 0.87 \mathrm{kcal\,mol^{-1}}$$

$$\Delta\Delta G_4^\circ = \Delta G_{7\mathrm{AW,B}}^\circ - \Delta G_{7\mathrm{AW,E}}^\circ = 1.17 \mathrm{kcal\,mol^{-1}} \qquad \text{(S.7)}$$

The value of $\Delta\Delta G_1^\circ$ cannot be measured directly, but is assumed to be small because it represents the energetic cost of replacing an exposed indole side chain with an exposed 7-azaindole side chain.

Finally, $\Delta\Delta G_2^\circ$ is calculated by combining the results of eq. S.7 with eq. S.6:

$$\Delta\Delta G_2^\circ = \Delta\Delta G_3^\circ + \Delta\Delta G_4^\circ - \Delta\Delta G_1^\circ = 0.87 + 1.17 - 0 = 2.04 \mathrm{kcal\,mol^{-1}} \qquad \text{(S.8)}$$

This value actually *underestimates* the energetic penalty if the assumption $\Delta\Delta G_1^\circ \approx 0$ is not satisfied. The slightly higher polarity of a 7-azaindole ring compared to an indole ring may actually make the 7AW-E_{xposed} form more stable than wild type cSH3 domain in which the W275 side chain is exposed. In this case, the sign of $\Delta\Delta G_1^\circ$ is negative and $\Delta\Delta G_2^\circ$ will be slightly larger than the above estimate. However, this result only strengthens the conclusion that the conformational change of W275 yields a significant energetic penalty that offsets favorable interdomain interactions. The estimated free energy penalty for exposing the W275 side chain in the wild type cSH3 domain predicts a negligible population of this conformation in the *isolated* domain and is consistent with that both the fluorescence and NMR experiments on the isolated domain.