Stabilization of folded peptide and protein structures via distance matching with a long, rigid cross-linker.

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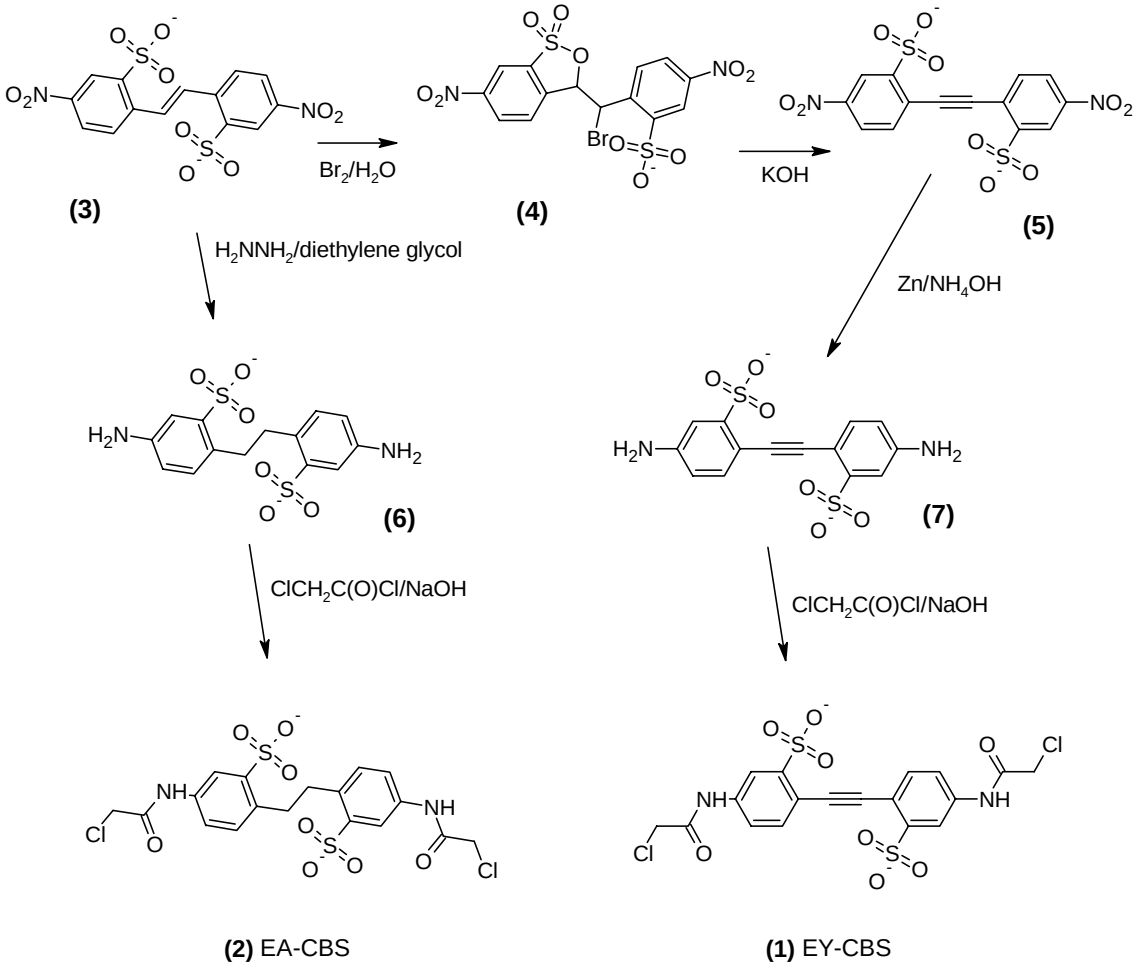
Supporting Information

Synthesis of the cross-linkers.

Synthesis was performed as outlined in the scheme below. Syntheses were adapted with major modifications from the work of Temkina et al[1] and Ruggli & Peyer.[2]



(2) EA-CBS **(1)** EY-CBS

Compound (**3**) (2,2'-(*E*)-ethene-1,2-diylbis(5-nitrobenzenesulfonic acid; dinitrostilbene disulfonic acid) was purchased from City Chemical (New York) and used without purification.

*Synthesis of 2-[bromo(6-nitro-1,1-dioxido-3H-2,1-benzoxathiol-3-yl)methyl]-5-nitrobenzenesulfonic acid (**4**).*

16 g (0.034 mol) of the sodium salt of (**3**) were dissolved in 75 mL of water at 55-58^{o}C. Then 3.46 mL (0.065 mol Br_2) of bromine were added with vigorous stirring. After 5 min the reaction mixture was cooled to 0-5^{o}C. The precipitate was filtered then recrystallized from water to give 4.7 g of (**4**) (yield 25%).

^{1}H-NMR: (400 MHz, DMSO-d6) δ ppm 6.60 (d, J=9.4 Hz, 1H) 7.73 (d, J=9.4 Hz, 1H) 8.01 (d, J=8.7 Hz, 1H), 8.15 (d, J=8.5 Hz, 1H) 8.33 (dd, J=8.7, 2.4 Hz, 1H) 8.58 (dd, J=8.5 Hz, 2.4 Hz) 8.62 (d, J=2.4 Hz, 1H) 8.63 (d, J=2.4 Hz, 1H).

^{13}C-NMR: (101 MHz, DMSO-d6) δ ppm 44.2 (s, 1C) 81.6 (s, 1C) 120.4 (s, 1C) 122.7 (s, 1C) 124.9 (s, 1C) 129.1 (s, 1C) 130.5 (s, 1C) 134.3 (s, 1C) 135.3 (s, 1C) 138.0 (s, 1C) 141.6 (s, 1C) 142.6 (s, 1C) 148.3 (s, 1C) 149.8 (s, 1C)

HR-ESI-MS: (negative mode) $C_{14}H_8N_2O_{10}S_2Br$ calc'd: 506.8809, obs'd 506.8828.

*Synthesis of 2,2'-ethyne-1,2-diylbis(5-nitrobenzenesulfonic acid) (**5**).*

To a stirred solution of (**4**) (9 g, 0.017 mol) in water (150 mL) at 60-70^{o}C was added 30 mL of a 50% solution of potassium hydroxide. The precipitate was collected and recrystallized from water to yield 4.9 g of (**5**) (yield 65%).

^{1}H-NMR: (400 MHz, D_2O) δ ppm 8.07 (d, J=8.6 Hz, 2H) 8.44 (dd, J=8.6, 2.3 Hz, 2H) 8.75 (d, J=2.3 Hz, 2H)

^{13}C-NMR: (101 MHz, DMSO-d6) δ ppm 97.5 (s, 2C) 122.4 (s, 2C) 124.1 (s, 2C) 127.1 (s, 2C) 135.9 (s, 2C) 146.8 (s, 2C) 151.1 (s, 2C).

HR-ESI-MS: (negative mode) $C_{14}H_7N_2O_{10}S_2$ calc'd: 426.9547, obs'd 426.9570.

*Synthesis of 2,2'-ethyne-1,2-diylbis(5-aminobenzenesulfonic acid) (**7**).*

To a vigorously stirred solution of (**5**) (1 g, 2.7 mmol) in 35% ammonium hydroxide/water (150 mL) at room temperature was added in one portion 2 g (0.030 mol) of zinc powder (Aldrich). After 24h the solid was filtered off and the solvent was reduced to 50 mL by rotary evaporation. The precipitate was filtered again and the remaining liquid was mixed with silica gel, evaporated to dryness and introduced onto a silica gel column. The product was chromatographed using a mobile phase composed ethyl acetate/methanol 7:3. (yield 67%).

^{1}H-NMR: (400 MHz, D_2O) δ ppm 6.9 (dd, J=8.2, 2.3 Hz, 2H) 7.3 (d, J=2.3 Hz, 2H) 7.5 (d, J=8 Hz, 2H)

^{13}C-NMR: (101 MHz, D_2O) δ ppm 91.2 (s, 2C) 109.9 (s, 2C) 114.1 (s, 2C) 118.2 (s, 2C) 135.7 (s, 2C) 143.8 (s, 2C) 147.1 (s, 2C).

HR-ESI-MS: (negative mode) $C_{14}H_{11}N_2O_6S_2$ calc'd: 367.0064, obs'd 367.0059.

*Synthesis of 2,2'-ethyne-1,2-diylbis{5-[(chloroacetyl)amino]benzenesulfonic acid} (**1**).*

To a solution of (**7**) (0.14 g, 0.4 mmol) in water (10 mL) at 0^{o}C was added 0.15 g (3.8 mmol) of sodium hydroxide followed by 0.15 mL (0.21g, 1.9 mmol) chloroacetyl chloride. The mixture was stirred for 1h at 0^{o}C. The precipitate was filtered, washed with cold water and several times with THF. (yield 75%).

^{1}H-NMR: (400 MHz, DMSO-d6) δ ppm 4.25 (s, 4H) 7.52 (d, J=8.4 Hz, 2H) 7.72 (dd, J=8.4, 2.4 Hz, 2H) 7.97 (d, J=2.4 Hz, 2H) 10.5 (s, 2H)

^{13}C-NMR: (101 MHz, DMSO-d6) δ ppm 44.3 (s, 2C) 93.5 (s, 2C) 116.4 (s, 2C) 118.2 (s, 2C) 119.5 (s, 2C) 135.6 (s, 2C) 138.2 (s, 2C) 149.0 (s, 2C) 165.3 (s, 2C).

HR-ESI-MS: (negative mode) $C_{18}H_{13}N_2O_8S_2Cl_2$ calc'd: 518.9495, obs'd 518.9468.

*Synthesis of 2,2'-ethane-1,2-diylbis(5-aminobenzenesulfonic acid) (**6**).*

Reduction of (**3**) was performed essentially as described by Huang-Minlon.[3] A mixture of 2 g (x mol) of (**3**), 40 mL diethylene glycol and 8 mL of 85% hydrazine hydrate was refluxed for 30 min. The condenser was then removed to allow the aqueous liquor to evaporate and the temperature of the reaction mixture to rise to about 200^{o}C. Refluxing at this temperature was continued until the dark colored solution became light brown (1-3 h). The reaction mixture was cooled, diluted with water and acidified with concentrated hydrochloric acid to precipitate (**6**)(yield 46%).

^{1}H-NMR: (400 MHz, D_2O) δ ppm 3.16 (s, 4H) 6.86 (dd, J=8.0, 2.4 Hz, 2H) 7.15 (d, J=8.0 Hz, 2H) 7.29 (d, J=2.4 Hz, 2H)

^{13}C-NMR: (101 MHz, D_2O) δ ppm 34.0 (s, 2C) 115.0 (s, 2C) 119.2 (s, 2C) 130.4 (s, 2C) 132.8 (s, 2C) 141.5 (s, 2C) 144.4 (s, 2C).

HR-ESI-MS: (negative mode) $C_{14}H_{15}N_2O_6S_2$ calc'd: 371.0377, obs'd 371.0355.

*Synthesis of 2,2'-ethane-1,2-diylbis{5-[(chloroacetyl)amino]benzenesulfonic acid} (**2**).*

To a solution of (**6**) (0.15 g, 0.4 mmol) in water (10 mL) at 0^{o}C was added 0.13 g (3 mmol) of sodium hydroxide followed by 0.2 mL (0.27g, 2.1 mmol) chloroacetyl chloride. The mixture was stirred for 1h at 0^{o}C. The precipitate was filtered, washed with cold water and several times with THF. (yield 76%).

^{1}H-NMR: (400 MHz, DMSO-d6) δ ppm 3.21 (s, 4H) 4.25 (s, 4H) 7.22 (d, J=8.4 Hz, 2H) 7.56 (dd, J=8.4, 2.3 Hz, 2H) 7.89 (d, J=2.3 Hz, 2H) 10.3 (s, 2H)

^{13}C-NMR: (101 MHz, DMSO-d6) δ ppm 34.3 (s, 2C) 44.3 (s, 2C) 119.0 (s, 2C) 120.2 (s, 2C) 131.3 (s, 2C) 135.7 (s, 2C) 136.7 (s, 2C) 147.1 (s, 2C) 165.0 (s, 2C).

HR-ESI-MS: (negative mode) $C_{18}H_{17}N_2O_8S_2Cl_2$ calc'd: 522.9808, obs'd 522.9785.

Peptides and proteins.

Peptides FK11W(acetyl-WGEACAREAAAREAACRQ-amide) and FK22C (acetyl-WEAAAREAAAREAAAREACAREAAAREAACRQ-amide) were prepared using standard Fmoc-based solid-phase peptide synthesis as described previously.[4] All peptides were HPLC purified (using a Zorbax SB-C18 column with a gradient 5-60% acetonitrile/water containing 0.1% trifluoroacetic acid). The compositions of the peptides were confirmed by MALDI-MS (FK11W, calculated 1988.9 Da, observed 1989.2 Da; FK22C, calculated 3427.6Da, observed 3427.7Da.)

Cross-linking of peptides was preformed in 50 mM sodium phosphate at pH 8.5 containing 10 mM TCEP. To the reaction mixture, a final concentration of 0.5 mM peptide, 2 mM of cross-linker was added and stirred at 37 degree for 2 hours. The reaction was quenched by neutralization with 1% trifluoroacetic acid. For cross-linking with DPDPB, FK11W was first incubated with immobilized TCEP (Pierce, Inc.) at 25ºC for 1 hour to reduce any disulfide bonds. After centrifugation, the TCEP-free FK11W solution was added to 2 mM of DPDPB in methanol. The mixture was stirred at 25ºC for 30 min. The cross-linked peptides were purified using HPLC (Zorbax SB-C18 column with a gradient 5-60% acetonitrile/water containing 0.1% trifluoroacetic acid). The compositions of the cross-linked peptides were checked by MALDI-MS (EY-CBS-FK11W, calculated 2437.2 Da, observed 2437.4 Da; EA-CBS-FK11W, calculated 2441.2 Da, observed 2440.8 Da; DPDPB-FK11W, calculated 2251.0 Da, observed 2250.9 Da; EY-CBS-FK22C, calculated 3875.6 Da, observed 3877.3 Da.)

The double-cysteine mutant of the FynSH3 domain (T14CE33C-SH3) plasmid was created by site-directed mutagenesis using primers:
CGGGGCGACTTAAGTTTTCACAAAGGAGAAAAATTTCAAATTCTTAACAGCTCGTGC GGAGACTGGTGGGAG (forward) and CGGGGCACTTAAGTCATCTTCGCATCTTGCTTCATAGTCATAAAGCGC (reverse). The proteins were over-expressed in BL21*(DE3) cells and purified with NTA-Ni resin (Qiagen) as described previously.[5] The double-cysteine mutant proteins were then reacted with EY-CBS the same way as described above except incubation was performed at 37ºC for 6 hours. The reaction mixture was then loaded onto NTA-Ni resin. After washing with Buffer A (20 mM imidazole, 10 mM sodium phosphate pH 7.0) for 5 column volumes to remove the free cross-linker, the cross-linked T14CE33C-FynSH3 (EY-CBS-T14CE33C-FynSH3) was eluted with 2 column volumes of Buffer B (500 mM imidazole, 10 mM sodium phosphate pH 7.0). EY-CBS-T14CE33C-FynSH3 in Buffer B was then transferred to Buffer C (10 mM phosphate pH 7.0, 0.1 mM TCEP) using a G25 desalting column. The proteins were characterized by ESI-MS. (Uncross-linked T14CE33C-FynSH3, calculated 9286.1Da, observed 9284.0Da; cross-linked EY-CBS-T14CE33C-FynSH3,

calculated 9734.1Da, observed 9736.0Da.)

Langevin dynamics.

Linker end-to-end distance distributions were calculated using Langevin dynamics methods running under Hyperchem 8.0 (Hypercube Inc.) essentially as described previously.[6] Models of linkers were built and minimized using the Amber molecular mechanics force field (Amber99) with methyl groups linked to sulfur atoms to represent the Cys beta carbons. Multiple 10 ns runs were performed at 300K using a variety of starting geometries. Cross-linker distributions were built from sulfur-to sulfur distances measured at 1 ps time steps.

CD spectra and thermal melting curves.

CD spectra and thermal melting curves were recorded on an Olis RSM 1000 circular dichroism spectrophotometer with a Quantum Northwest Peltier accessory. CD spectra were obtained at a series of temperatures from 2ºC to 90ºC with a 4ºC increments. Each spectrum was scanned from 260 nm to 190 nm with an integration time of 5 s at each wavelength. All the spectra were baseline-corrected using a blank consisting of 0.5 mM TCEP, 10 mM phosphate buffer at pH 7.0. All CD measurements were carried out in a 1 mm pathlength cuvette with a protein concentration of 50 μM in 0.5 mM TCEP, 10 mM phosphate buffer at pH 7.0. Uncross-linked peptide concentration was determined by UV absorbance at 280 nm based on tryptophan absorbance. The cross-linked peptide concentration was determined by bicinchoninic acid (BCA) (Pierce, Inc) assay taking each corresponding uncross-linked peptide as reference. Helix percentage was calculated at from MRE at 222 nm as described.[4]

EY-CBS-T14CE33C-FynSH3 modeling.

To build a model of EY-CBS-T14CE33C-FynSH3, the wild-type FynSH3 X-ray structure (PDB: 1A0N) was used as a starting point. Two residues T14 and E33 were mutated to Cys using the program HyperChem (Hypercube, Inc). The cross-linker EY-CBS was also built in HyperChem and optimized using the BIO force field. EY-CBS was then covalently linked to T14CE33C-FynSH3 through Cys residues and the overall structure was further optimized (Fig S1).

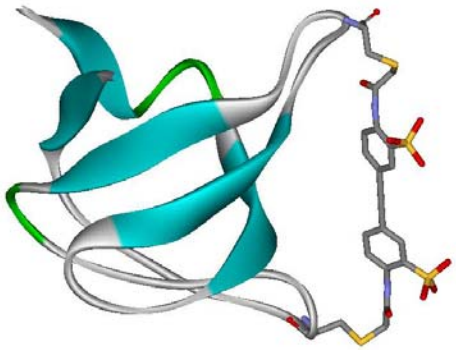


Fig S1. Model of EY-CBS-T14CE33C-FynSH3

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