Electronic Supplementary Material (ESI) for Chemical Communications.

Comparison of Design Strategies for α-Helix Backbone Modification in a Protein Tertiary Fold

Nathan A. Tavenor, Zachary E. Reinert, George A. Lengyel, Brian D. Griffith and W. Seth Horne*

Department of Chemistry, University of Pittsburgh, Pittsburgh, PA 15260.

SUPPORTING INFORMATION

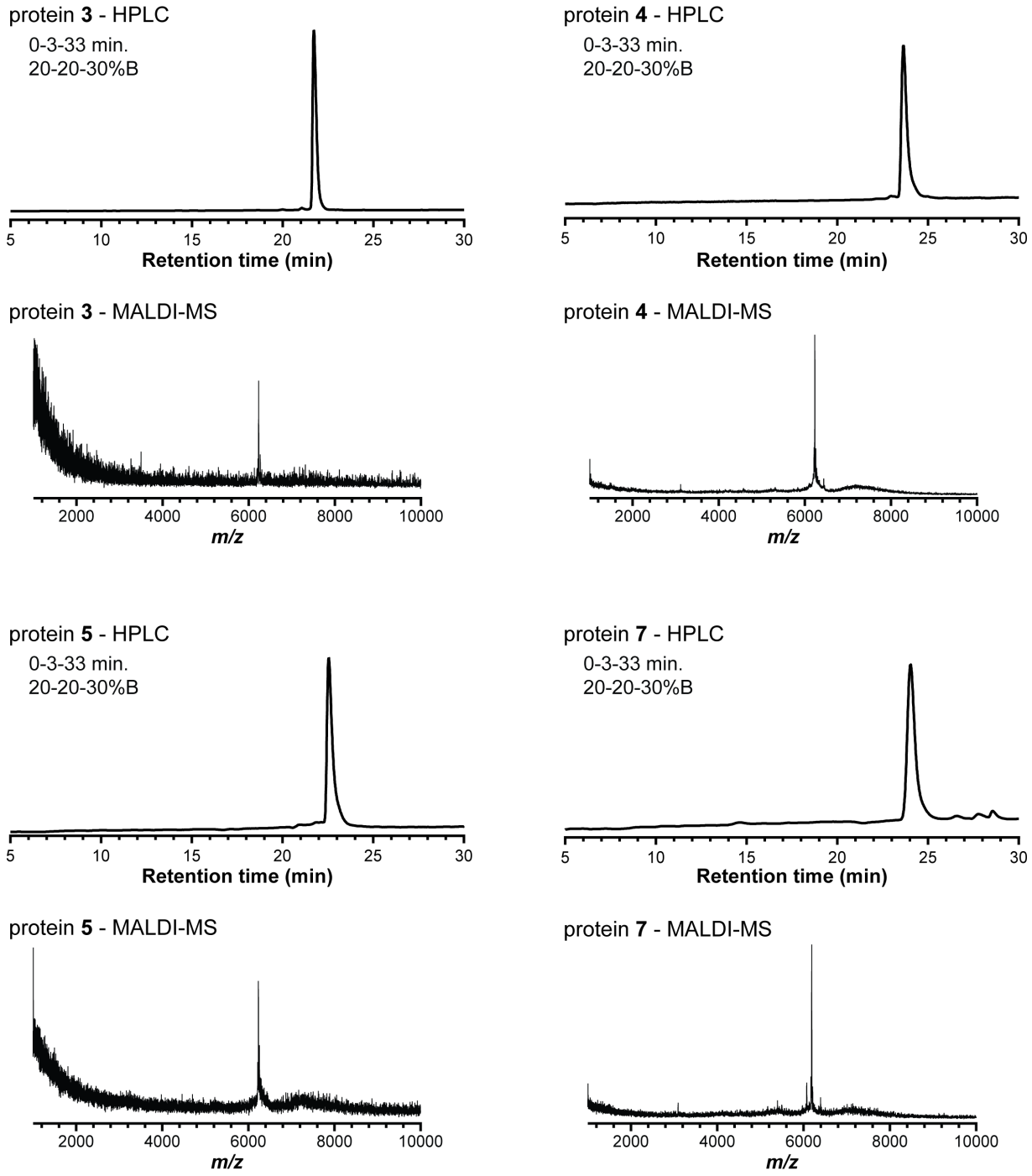


Figure S1. Analytical HPLC chromatograms and MALDI-TOF-MS spectra of purified synthetic proteins **3**, **4**, **5**, and **7**. HPLC experiments were performed on a C18 stationary phase (10 μm particle, 300 Å pore size) with indicated elution gradients (solvent A = 0.1 % TFA in water, solvent B = 0.1% TFA in acetonitrile).

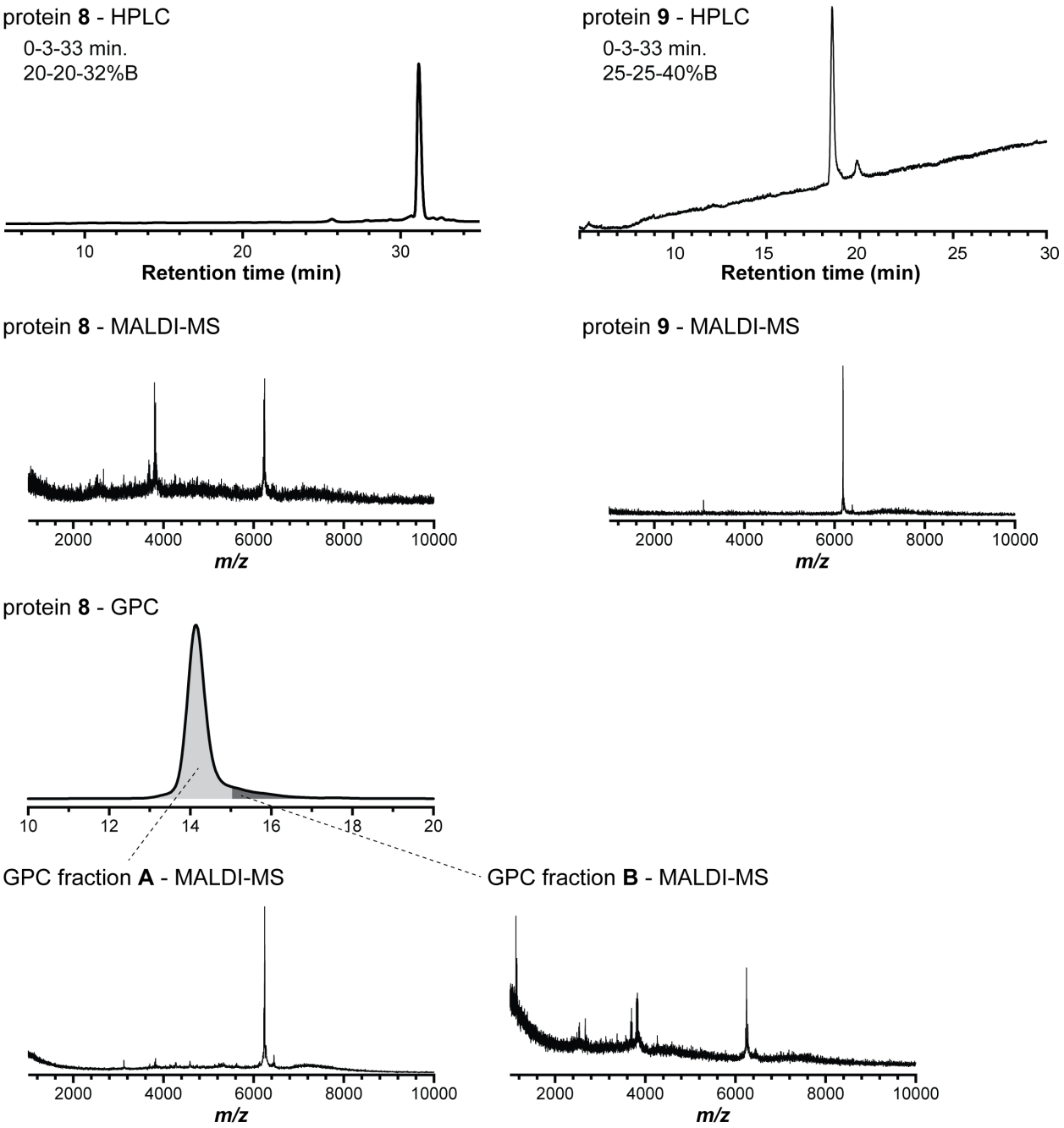


Figure S2. Analytical HPLC chromatograms and MALDI-TOF-MS spectra of purified synthetic proteins **8** and **9**. HPLC experiments were performed on a C18 stationary phase (10 μm particle, 300 Å pore size) with indicated elution gradients (solvent A = 0.1 % TFA in water, solvent B = 0.1% TFA in acetonitrile). Additional analysis of protein **8** by gel permeation chromatography (GPC, isocratic elution in PBS on a Superdex 75 10/100 column) indicates the truncation product visible in the MALDI spectrum is a minor component of the sample.

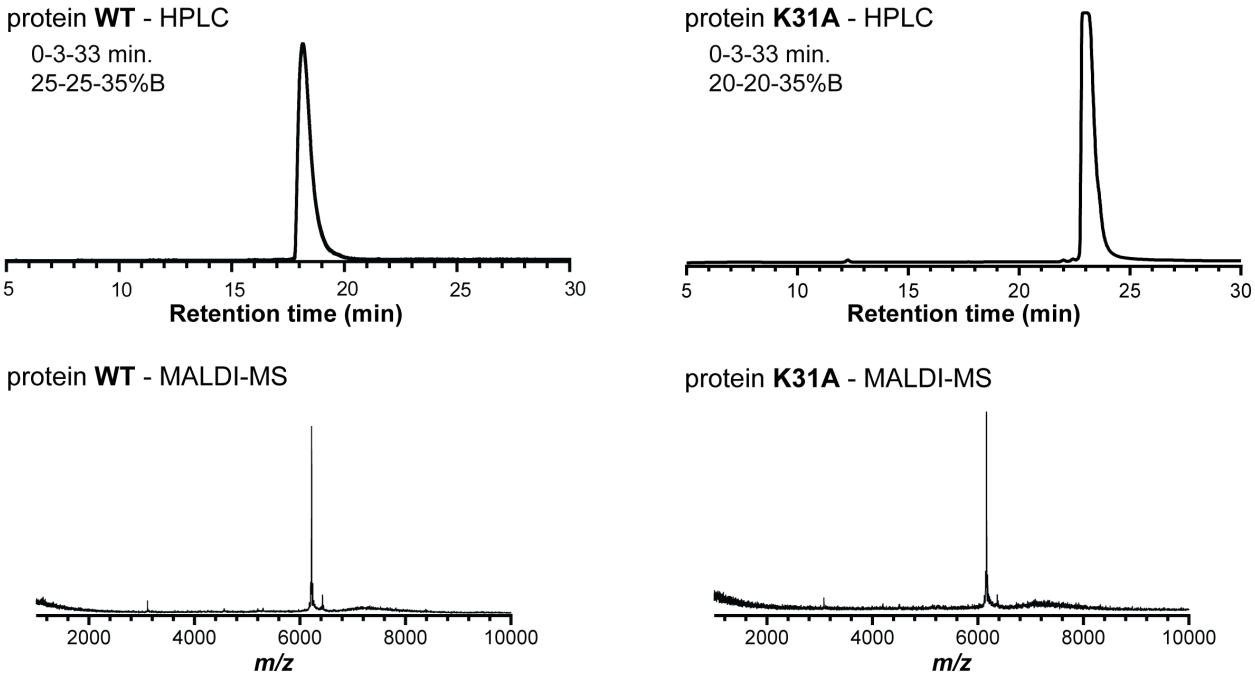


Figure S3. Analytical HPLC chromatograms and MALDI-TOF-MS spectra of purified expressed proteins **WT** and **K31A**. HPLC experiments were performed on a C18 stationary phase (10 μm particle, 300 Å pore size) with indicated elution gradients (solvent A = 0.1 % TFA in water, solvent B = 0.1% TFA in acetonitrile).

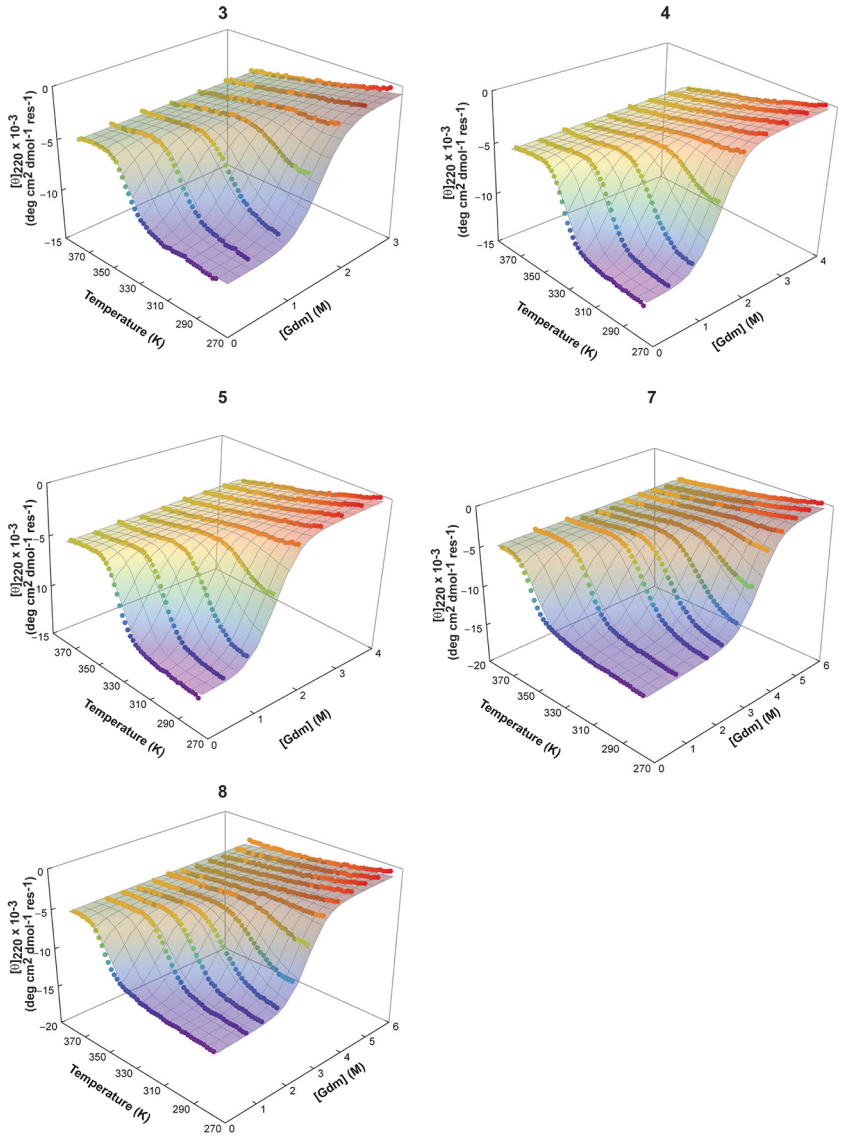


Figure S4. CD data at 220 nm for proteins **3**, **4**, **5**, **7**, and **8** as a function of temperature and chemical denaturant concentration. Raw data (points) are fit (surface) to extract thermodynamic parameters for the folding equilibrium. Data for proteins **1**, **2** and **6** were published previously.[1]

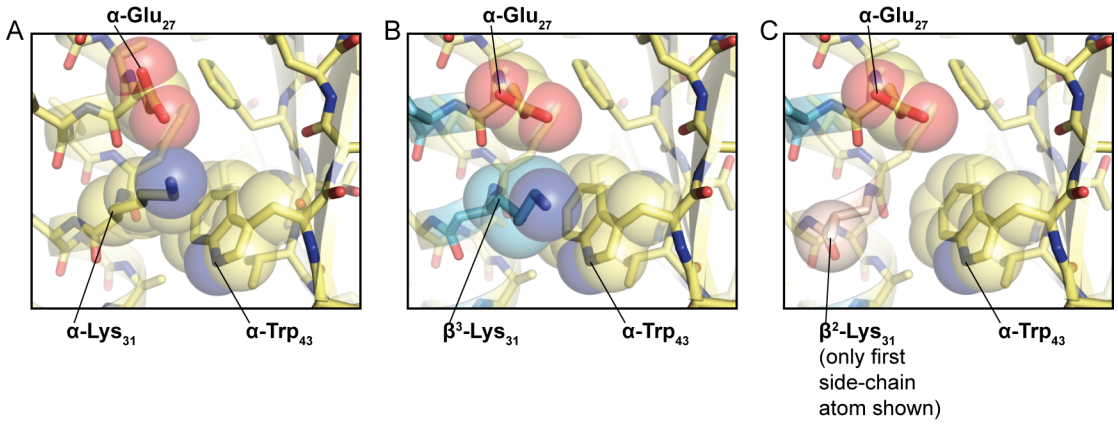


Figure S5. Packing interactions involving α-Lys_{31} from the crystal structure of wild-type GB1 (A) and β^3-Lys_{31} from the crystal structure of **2** (B) show a salt bridge to Glu_{27} and hydrophobic contact with Trp_{43}. A model incorporating a β^3-$Lys_{31}\rightarrow\beta^2$-Ala mutation in **2** (C) shows how the side chain would project away from the two interaction partners after the substitution.

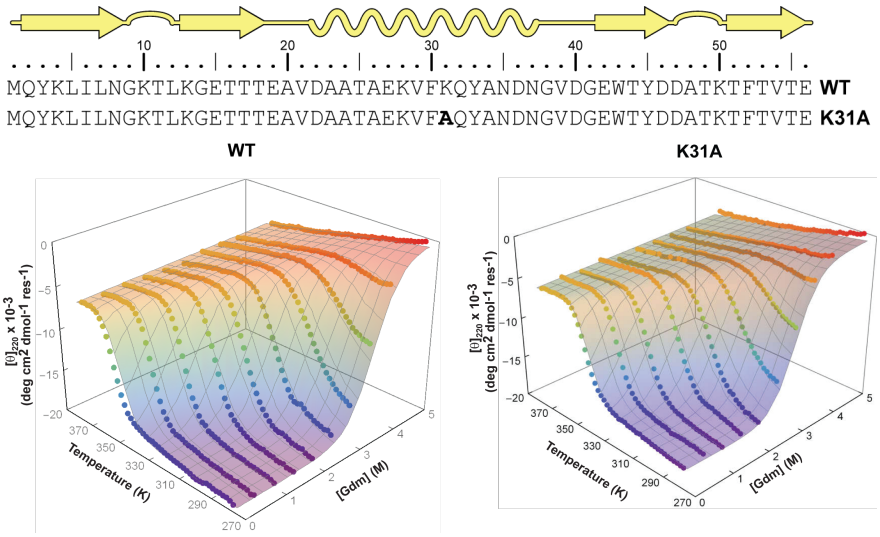


Figure S6. Sequences and coupled thermal / chemical denaturation CD data for wild-type GB1 (**WT**) and $Lys_{31}\rightarrow$Ala mutant (**K31A**). Analysis of folding thermodynamics for these two proteins (Table S3) supports the energetic importance of side-chain contacts involving Lys_{31}.

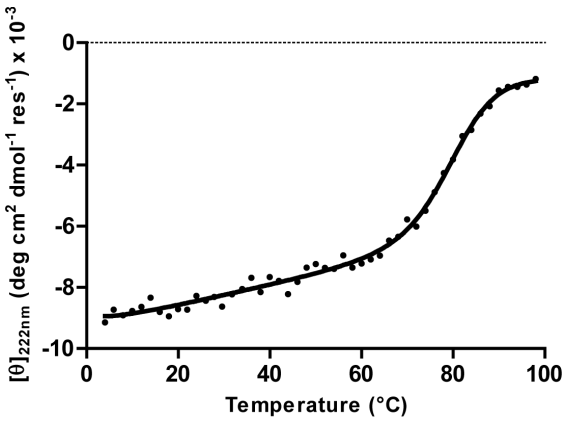


Figure S7. CD thermal melt of protein **9** at ~8 μM in 20 mM phosphate buffer pH 7. The concentration was difficult to measure accurately due to the low yield of purified material.

Table S1. MALDI-TOF MS data for proteins **3**-**5**, **7**-**9**, **K31A**, and **WT**.

#	$[M+H]^+$ *m/z* (average)	
	Calculated	**Observed**
3	6235.8	6233.4
4	6235.8	6234.8
5	6235.8	6234.6
7	6191.7	6191.9
8	6247.8	6247.5
9	6191.7	6188.2
K31A	6164.7	6164.7
WT	6223.8	6223.0

Table S2. X-ray diffraction data collection and refinement statistics for proteins **3**, **4**, and **7.**

	3	**4**	**7**
Data Collection			
Unit cell dimensions (Å, °)	a = 92.8, b = 22.4, c = 65.3 α = γ = 90, β = 134.1	a = b = 51.9, c = 96.4 α = β = γ = 90	a = 74.4, b = 73.4, c = 79.4 α = γ = 90, β=99.3
Space group	C2	$P4_1$	C2
Resolution (Å)	23.44-1.95 (2.02–1.95)	51.95-1.80 (1.86-1.80)	41.15-2.15 (2.23-2.15)
Total observations	34,719	297,770	260,925
Unique observations	7,188	22,477	22,880
Redundancy	4.8 (3.2)	13.25 (13.18)	11.40 (3.04)
Completeness (%)	97.8 (90.4)	95.0 (90.5)	99.2 (93.2)
I/σ	18.5 (3.6)	25.3 (4.9)	15.9 (4.2)
R_{merge} (%)	6.9 (15.2)	6.6 (40.5)	13.7 (23.9)
Refinement			
Resolution (Å)	23.44–1.95	51.95-1.80	41.15-2.15
R (%)	19.9	19.9	21.74
R_{free} (%)	23.0	21.7	25.18
Avg. B factor (Å^2)	22.1	27.3	24.33
RMSD			
Bonds (Å)	0.005	0.006	0.008
Angles (°)	1.04	1.13	1.07
Twinning			
Twin fraction	n/a	0.27	n/a
Twin Law	n/a	H, -K, -L	n/a

Table S3. Thermodynamic parameters for the unfolding of synthetic proteins **1-9** and expressed proteins **WT** and **K31A**.[a]

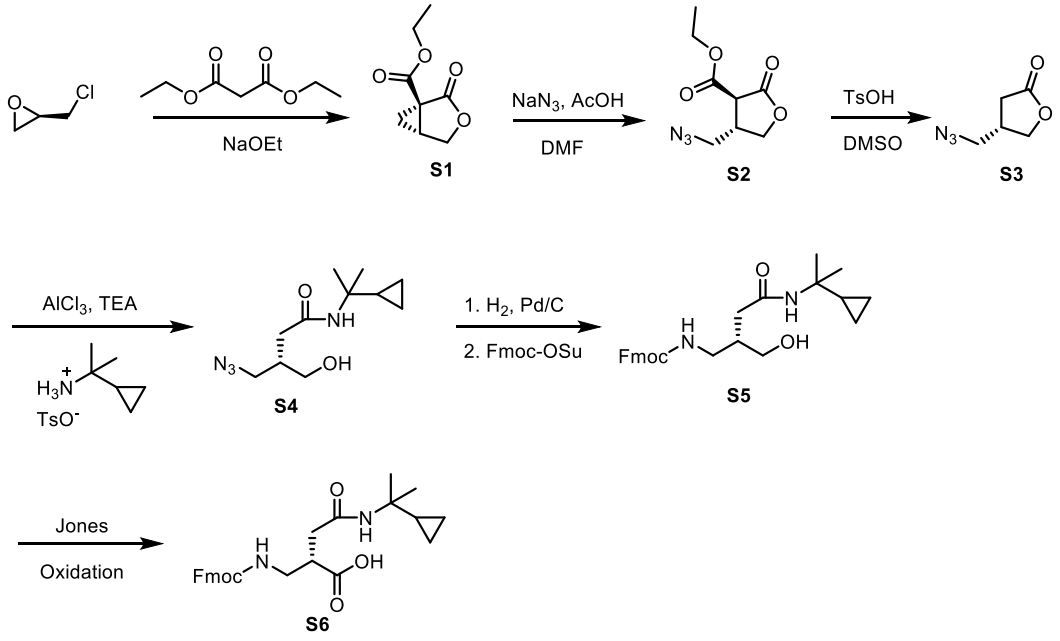
Protein	ΔH° (kcal mol^{-1})	TΔS° (kcal mol^{-1})	ΔG° (kcal mol^{-1})	ΔC_p (kcal mol^{-1} K^{-1})	*m* (kcal mol^{-1} M^{-1})	T_m (°C)[b]
1	22.0 ± 0.6	16.1 ± 0.3	5.9 ± 0.7	0.60 ± 0.02	1.80 ± 0.04	82.1 ± 0.1
2	18.3 ± 0.4	15.2 ± 0.3	3.1 ± 0.5	0.53 ± 0.02	2.48 ± 0.05	61.6 ± 0.1
3	18.8 ± 0.6	15.8 ± 0.3	3.0 ± 0.7	0.45 ± 0.03	2.25 ± 0.07	64.0 ± 0.2
4	18.7 ± 0.4	16.1 ± 0.3	2.6 ± 0.5	0.48 ± 0.02	2.36 ± 0.04	61.4 ± 0.4
5	17.0 ± 0.3	15.5 ± 0.3	1.5 ± 0.4	0.48 ± 0.02	2.45 ± 0.04	47.7 ± 0.2
6	18.4 ± 0.5	14.9 ± 0.3	3.5 ± 0.6	0.48 ± 0.02	2.00 ± 0.05	70.8 ± 0.2
7	22.7 ± 0.6	17.9 ± 0.5	4.8 ± 0.7	0.44 ± 0.02	1.57 ± 0.04	77.9 ± 0.1
8	19.9 ± 0.4	15.8 ± 0.3	4.1 ± 0.5	0.46 ± 0.02	1.73 ± 0.04	76.2 ± 0.1
9[c]	-	-	-	-	-	79.6 ± 0.5
WT	23.0 ± 0.5	17.9 ± 0.3	5.1 ± 0.6	0.57 ± 0.02	1.71 ± 0.04	78.5 ± 0.3
K31A	22.7 ± 0.4	18.5 ± 0.3	4.2 ± 0.5	0.67 ± 0.02	1.77 ± 0.03	67.6 ± 0.1

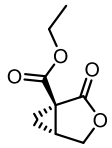
[a] Thermodynamic values are at 298 K and reported errors are from parameter uncertainties in the fit. Data for proteins **1**, **2**, and **6** were published previously.[1] [b] Midpoint of the CD thermal unfolding transition in the absence of chemical denaturant. [c] Protein **9** measured at 8 μM (0 M denaturant only) instead of 40 μM due to poor synthetic yield.

Materials and Methods

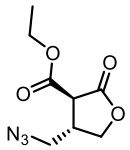
General Information. Solvents and all other reagents were purchased from Aldrich, Baker, EMD, or Fisher and used without further purification. HOBt was purchased from Anaspec Inc. HCTU, NovaPEG Rink Amide Resin, and Fmoc-protected α-amino acids were purchased from Novabiochem. Fmoc-protected β^3-amino acids were purchased from Aapptec. Column chromatography was performed using Silicycle SiliaFlash P60 (230-400 mesh) silica gel. Optical rotations were measured on a Perkin-Elmer 241 digital polarimeter with a sodium lamp at ambient temperature. NMR spectra were recorded on a Bruker Advance 400 spectrometer. Fmoc-β^2-Ala-OH and Fmoc-β^2-Lys(Boc)$_2$-OH were synthesized according to published routes.[2] Fmoc-C_α-Me-Val-OH was purchased from Aspira. Fmoc-C_α-Me-Lys(Boc)-OH was purchased from W & J Pharma Chem Inc.

Scheme S1. Synthesis of Fmoc-β^2-Asn(Dmcp)-OH (**S6**)



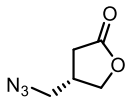

S1

Ethyl (1*S*, 5*R*)-2-oxo-3-oxabicyclo [3.1.0] hexane-1-carboxylate (**S1**): Compound **S1** was synthesized according to a published protocol.[3] Sodium (1.15 g, 50 mmol) was dissolved in ethanol (115 mL) under a N_2 atmosphere. The solution was cooled in an ice bath and diethyl malonate (9.2 mL, 60 mmol) was slowly added. After 10 minutes, *R*-epichlorohydrin (4.0 mL, 50 mmol) was slowly added over 20 minutes. The solution was refluxed overnight and concentrated. The residue was dissolved in water (100 mL) and the aqueous solution was extracted three times with 100 mL DCM. The organic layers were combined, dried with magnesium sulfate and concentrated. The concentrate was purified using column chromatography (20 % ethyl acetate in hexanes) to afford the product as a colorless oil (4.4 g, 26 mmol, 52 % yield). NMR data agreed with previously reported results.[3]


S2

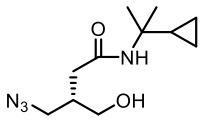
Ethyl (3*S*,4*S*)-4-(azidomethyl)-2-oxotetrahydrofuran-3-carboxylate (**S2**): Compound **S2** was synthesized according to a published protocol.[3] A solution of **S1** (4.4 g, 26 mmol), sodium azide (6.7 g, 104 mmol), glacial acetic acid (5.9 mL, 104 mmol), and triethylamine (72 µL, 0.5 mmol) in anhydrous DMF (100 mL) was heated at 70 °C for 4 h under a N_2 atmosphere. The DMF was removed under reduced pressure and 200 mL saturated ammonium chloride solution was added. The aqueous solution was extracted three times

with 200 mL DCM. The organic layers were washed three times with 100 mL saturated ammonium chloride, dried with magnesium sulfate, and concentrated to give the product as a colorless oil (3.9 g, 18.4 mmol, 71 % yield). NMR data agreed with previously published results.[3]



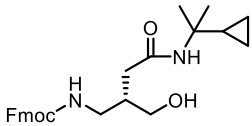
S3

(*S*)-4-(azidomethyl) dihydrofuran-2(3H)-one (**S3**): Compound **S3** was synthesized according to a published protocol.[4] A solution of **S2** (3.9 g, 18.4 mmol) and *p*-toluenesulfonic acid monohydrate (14.0 g, 74 mmol) in DMSO was heated at 120 °C overnight. The reaction was cooled, and 200 mL water was added. The solution was extracted four times with 200 mL ethyl acetate. The organic layers were washed three times with 100 mL saturated ammonium chloride, dried with magnesium sulfate, and concentrated to give the product as a colorless oil (1.5 g, 10.6 mmol, 57 % yield). NMR data agreed with previously published results.[4]



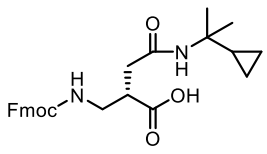
S4

(*S*)-4-azido-N-(2-cyclopropylpropan-2-yl)-3-(hydroxymethyl) butanamide (**S4**): To a solution of aluminum chloride (3.1 g, 23.3 mmol) suspended in anhydrous DCM (100 mL) cooled on ice under a N_2 atmosphere was added triethylamine (6.5 mL, 47 mmol). The solution was stirred for 15 minutes before **S3** (1.5 g, 10.6 mmol) and α,α-dimethyl-cyclopropylmethanamine *p*-toluenesulfonic acid (3.2 g, 11.7 mmol) were added. The solution was stirred overnight at ambient temperature and concentrated. The residue was dissolved in 200 mL ethyl acetate and washed twice with 100 mL 5% sodium bicarbonate solution, once with 100 mL saturated ammonium chloride, and once with 100 mL brine. The organic layers were dried with magnesium sulfate and concentrated to afford the product as a colorless oil (1.6 g, 6.7 mmol, 63 % yield). $[\alpha]^{20}_{=}$ -7.1 (c = 1.1 in $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.67 (s, 1 H), 3.71 (m, 2 H), 3.47 (m, 2 H), 2.25 (m, 3 H), 1.28 (m, 8 H), 0.43 (m, 4 H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.0, 62.6, 52.9, 51.5, 37.1, 36.7, 23.0, 20.4, 0.0. HRMS m/z calculated for $C_{11}H_{21}N_4O_2$ $(M+H)^+$ 241.1659; found 241.1660.



S5

(9*H*-fluoren-9-yl) methyl-(*S*)-(4-((2-cyclopropylpropan-2-yl) amino)-2-(hydroxymethyl)-4-oxobutyl) carbamate (**S5**): A solution of **S4** (1.6 g, 6.7 mmol), palladium hydroxide on carbon (0.3 g), and palladium on carbon (0.3 g) in MeOH (75 mL) was stirred for two days under a H_2 atmosphere. The reaction was filtered through Celite with MeOH and concentrated to afford the amino alcohol, which was carried forward without further purification. To a solution of the amino alcohol in DCM (50 mL) was added FmocOSu (2.5 g, 7.4 mmol) and DIEA (4.8 mL, 27 mmol). The reaction was stirred for 4 h and concentrated. The residue was dissolved in 100 mL ethyl acetate, washed with 30 mL 5% sodium bisulfate, dried with magnesium sulfate, and concentrated. Column chromatography (50 % ethyl acetate in hexanes) afforded the product as a white solid (1.5 g, 3.4 mmol, 50% yield). $[\alpha]^{20}_{=}$ +1.25 (c = 0.8 in $CHCl_3$). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.90 (d, *J* = 8 Hz, 2 H), 7.70 (d, *J* = 8 Hz, 2 H), 7.43 (t, *J* = 4 Hz, 2 H), 7.34 (t, *J* = 4 Hz, 2 H), 7.28 (s, 1 H), 7.23 (t, *J* = 4 Hz, 1 H), 4.46 (t, *J* = 4 Hz, 1 H), 4.30, (m, 3 H), 3.30 (m, 2 H), 3.00 (m, 2 H), 2.01 (m, 3 H), 1.27 (m, 1 H), 1.15 (s, 6 H), 0.28 (m, 4 H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 169.9, 155.3, 142.8, 139.6, 126.5, 125.9, 124.1, 119.0, 64.0, 60.5, 51.2, 45.6, 40.1, 35.2, 23.6, 19.4, 0.0. HRMS m/z calculated for $C_{26}H_{33}N_2O_4$ $(M+H)^+$ 437.2440; found 437.2427.



S6

Fmoc-(*S*)-β^2-Asn(Dmcp)-OH (**S6**): To a stirred solution of sodium dichromate in water (1 M) was added concentrated sulfuric acid to a final concentration of 4 M. The solution was then diluted with water to a final concentration of 0.5 M sodium dichromate and 2 M sulfuric acid. To a solution of **S5** (2.2 g, 5.0 mmol) in acetone (78 mL) was added the above prepared Jones reagent (12.5 mL). After 2.5 h, 20 mL isopropanol was added, and the solution was stirred for 30 minutes.

The reaction was diluted with 400 mL ethyl acetate, washed once with 200 mL 5 % sodium bisulfate, twice with 200 mL brine, dried with magnesium sulfate, and concentrated. The residue was purified by column chromatography (dry-loaded in 1 % TEA 50 % ethyl acetate in hexanes, eluted with 1 % acetic acid in ethyl acetate) to afford the product as a white solid (0.58 g, 1.3 mmol, 26 % yield). $[\alpha]^{20}_{=}$ -28.8 (c = 1.0 in $CHCl_3$). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.87 (d, *J* = 8 Hz, 2 H), 7.68 (d, *J* = 8 Hz, 2 H), 7.41 (t, *J* = 4 Hz, 2 H), 7.34 (t, *J* = 4 Hz, 2 H), 4.23 (m, 3 H), 3.19 (m, 2 H), 2.80 (m, 1 H), 2.22 (m, 2 H), 1.15 (m, 7 H), 0.26 (m, 4 H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 175.2, 170.3, 156.6, 144.4, 144.3, 141.2, 128.1, 127.5, 125.7, 120.6, 65.9, 52.8, 47.2, 42.4, 42.3, 36.1, 25.3, 25.2, 21.1, 1.64, 1.56. HRMS m/z calculated for $C_{26}H_{29}N_2O_5$ $(M-H)^-$ 449.2071; found 449.2081.

Protein Synthesis. Synthesis and purification of proteins **1**, **2**, and **6** has been described previously.[1, 5] Proteins **3-5** were synthesized at room temperature (*Method 1*), while **7-9** were prepared by a combination of room temperature and microwave-assisted reactions (*Method 2*). All syntheses were carried out on a 70 μmol scale using NovaPEG Rink Amide resin.

Method 1. Room temperature reactions were performed on a PTI Tribute automated synthesizer. In a standard coupling reaction, 2.5 mL of a solution composed of 0.2 M HCTU, 0.4 M N-methylmorpholine in DMF was added to 7 equivalents of Fmoc-amino acid relative to resin. After a 2 minute pre-activation, the solution was added to the resin and vortexed for 45 minutes. Deprotections were performed by two treatments with 3 mL of 20% v/v 4-methylpiperidine in DMF for 4 minutes each. The resin was washed four times with 3 mL of DMF after each coupling and deprotection step. After the final Fmoc deprotection, the resin was rinsed three times with 3 mL each DMF, DCM, and MeOH. The resin was dried in a vacuum desiccator for 20 minutes prior to TFA cleavage.

Method 2. Microwave reactions were performed using a CEM Microwave-Assisted Reaction System (MARS). Coupling cycles consisted of a 1.5 minute ramp to 90°C followed by a 2 minute hold, while deprotection cycles consisted of a 1.5 minute ramp to 90°C followed by a 1 minute hold.[6] Coupling solutions included protected amino acid (7 equiv), HATU (6.9 equiv), and DIEA (10.5 equiv) in DMF, and were preactivated for 2 minutes prior to addition to resin. Fmoc deprotections made use of 20% v/v 4-methylpiperidine in DMF.

Unless otherwise indicated, synthesis was carried out by *Method 1*. Following are the modifications to the standard automated method made for specific proteins. For proteins **3-5** and **7-9**, Glu_{56} was double-coupled, and pseudoproline dipeptides were used for residues $Ala_{48}Thr_{49}$ and $Glu_{15}Thr_{16}$. Pseudoproline dipeptides were coupled with PyAOP or HATU for 90 minutes at room temperature. For protein **7**, Aib residues were coupled with PyAOP for 90 minutes and the Ala residues immediately following each were double-coupled with PyAOP for 90 minutes each. For protein **8**, αMeVal residues were coupled with HATU for 90 minutes, and the resin was capped with DMF/DIEA/Ac_2O (8/2/1, v/v/v) for 10 minutes prior to deprotection. The Ala residues following each were double-coupled in the microwave (*Method 2*), with capping. For protein **9**, Aib_{35} was coupled with HATU for 90 minutes. Ala_{34} was double-coupled with HATU for 90 minutes each, followed by capping. For residues 23-31, synthesis proceeded in the microwave (*Method 2*). Residues Phe_{30}, Glu_{27} and Ala_{23} were double-coupled. Capping was done after $\alpha MeLys_{31}$, Phe_{30}, $\alpha MeLys_{28}$, Glu_{27}, and Ala_{23}.

All proteins were cleaved from the resin in a cocktail of TFA/H_2O/EDT/TIS (92.5/3/3/1.5 by volume) for approximately 3.5 hours on a rocker. Crude protein was precipitated from the TFA solution by addition of cold Et_2O, and the mixture was centrifuged and decanted. The crude pellet was suspended in ~7 mL of 6M guanidinium, 0.025 M phosphate pH 7. The organic and aqueous layers of the resulting suspension were separated by centrifuge prior to purification.

Protein Expression. Proteins (**WT** and **K31A**) were expressed in *E. coli* following published protocols[7] using plasmids graciously provided by Timothy F. Cunningham and Sunil Saxena (University of Pittsburgh).

Protein Purification. All proteins were purified by preparative (300 Å pores, 10 μm beads) C18 reverse-phase HPLC using gradients composed of 0.1% TFA in water/acetonitrile. Protein identities were confirmed using mass spectrometry on a Voyager DE Pro MALDI-TOF instrument (Table S1). Following HPLC, proteins were further purified by anion-exchange chromatography on a MonoQ 5/50GL column (GE Healthcare) using 0.02 M Tris pH 8 buffer eluted with increasing concentrations of NaCl. Following ion-exchange, protein **9** was further purified using semi-preparative (300 Å pores, 5 μm beads) C18 reverse-phase HPLC using gradients as described above. Final purity of each protein was ≥95% by analytical RP-HPLC (Figures S1-S3).

Circular Dichroism Spectroscopy. Circular dichroism measurements and data analysis were performed as described previously.[1] Concentration for all proteins was 40 μM except for protein **9** which was 8 μM due to poor synthetic yield.

Crystallography. Crystals were grown using the hanging drop vapor diffusion method. Stock solutions (18 mg/mL for **3**, 20 mg/mL for **4** and **7**) were mixed (0.7 μL + 0.7 μL) with crystallization buffer (Table S2) and allowed to equilibrate at room temperature over a well of that buffer. Harvested crystals were cryoprotected with 30% v/v glycerol in the mother liquor then flash frozen in liquid nitrogen. X-ray diffraction data were collected using Cu/K_α radiation on a Rigaku/MSC diffractometer (FR-E generator, VariMax optics) with a Saturn 944 CCD detector for **3** and **7** or a RAXIS HTC image plate detector for **4**. Crystals were maintained at 100 K during diffraction experiments with an X-Stream 2000 low-temperature system.

Crystallization buffer conditions.

Protein	Buffer
3	0.2 M NaOAc pH 4.6, 20% PEG 4000
4	0.1 M Na Cacodylate pH 6.5, 0.1 M $Mg(OAc)_2$, 20% PEG 4000
7	0.1 M NaOAc pH 4.5, 0.2 M $(NH_4)_2SO_4$, 20% PEG 4000

Raw diffraction data were processed with d*TREK. Structures were solved by molecular replacement with a published structure of the expressed wild-type GB1 (PDB 4QMT) as a search model. Model refinement was performed with Phenix,[8] and manual real-space model building was accomplished using Coot.[9] Final data collection and refinement statistics may be found in Table S2. Protein **4** exhibited signs of twinning as determined by Xtriage in Phenix, and the final structure was refined with a twin fraction of 0.27 and twin law of (H, -L, -K). Final coordinates and reflection files have been deposited in the PDB under accession codes 5HFY (protein **3**), 5HG2 (protein **4**), and 5HI1 (protein **7**).

References

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