

# Biophysical and Structural Characterization of a Robust Octameric $\beta$ -Peptide Bundle

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**General Methods.** Fmoc-protected amino acids, *O*-Benzotriazole-*N,N,N'*-tetramethyluronium-hexafluoro-phosphate (HBTU), *N*-hydroxybenzotriazole (HOBt), and Wang resin were purchased from Novabiochem (San Diego, CA). Dimethylformamide (DMF), *N*-methyl-2-pyrrolidone (NMP), *N*-methyl morpholine (NMM), trifluoroacetic acid (TFA) and piperidine (Pip) were purchased from American Bioanalytical (Natick, MA). *N*-Fmoc- $\beta^3$ -p-iodohomophenylalanine was purchased from Peptech (Burlington, MA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO). Mass spectra were acquired with an Applied Biosystems Voyager-DE Pro MALDI-TOF mass spectrometer (Foster City, CA). Reverse-phase HPLC was performed using a Varian Prostar HPLC and Vydac analytical (C8, 300 Å, 5  $\mu$ m, 4.6 mm X 150 mm) or preparative (C8, 300 Å, 5  $\mu$ m, 25 mm X 250 mm) columns, using water/acetonitrile gradients containing 0.1% TFA. Circular dichroism (CD) spectra were acquired with a Jasco J-810 Spectropolarimeter (Jasco, Tokyo, Japan) equipped with a Peltier temperature control. Analytical ultracentrifugation (AU) was performed using a Beckman XL-I

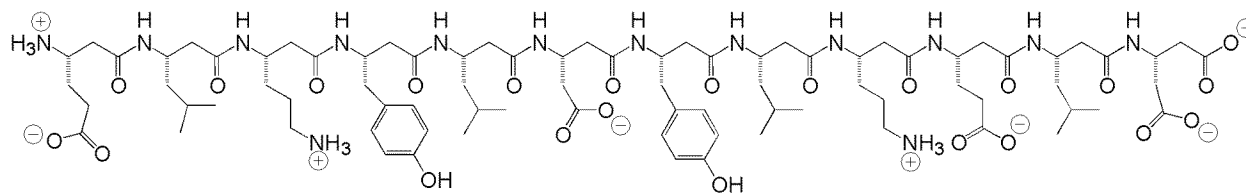
instrument. Differential scanning calorimetry experiments were performed on a Microcal VP-DSC instrument (Northampton, MA) in the labs of Dr. Jeff Hoch at the University of Connecticut Health Center (Farmington, CT).  $^1\text{H}$  NMR experiments were performed on a Bruker 500 MHz instrument. All experiments were performed in phosphate buffer (10 mM  $\text{NaH}_2\text{PO}_4$ , 200 mM NaCl, pH adjusted to 7.1 with NaOH), except as noted.

**$\beta^3$ -peptide synthesis and purification.**  $\beta^3$ -peptides were synthesized on a 25  $\mu\text{mole}$  scale using standard Fmoc chemistry and Wang resin loaded with  $\beta^3$ -homoaspartic acid as previously described.<sup>1</sup> One cycle of peptide elongation consisted of the following steps: First, the loaded resin was washed with DMF (6 X 30 sec) and the terminal Fmoc protecting group removed with 20% Pip in DMF (1 X 2 min, 2 X 8 min). This deprotection step was supplemented with a second deprotection step using diazabicyclo[5.4.0]undec-7-ene (DBU) after five residues have been attached to the resin (1 X 2 min 20% Pip/DMF, 2 X 8 min 20% Pip/DMF, 1 X 8 min 2:2:96 Pip/DBU/DMF, and 1 X 8 min 20% Pip/DMF). After deprotection, the resin was washed with DMF (6 X 30 sec) and the next  $\beta^3$ -amino acid coupling reaction was carried out for 30 min using 2 equiv. of the appropriate  $\beta^3$ -amino acid, 1.8 equiv. HBTU, 2 equiv. HOBt, and 5 equiv. diisopropylethylamine (DIEA). This coupling was the repeated with fresh reagents for another 30 min. The resin was then washed with DMF (6 X 30 sec) before the next cycle of elongation. This elongation cycle was repeated until the  $\beta$ -peptide sequence was complete. Once the final Fmoc protecting group had been removed, the resin was washed with DMF (8 X 30 sec) and methylene chloride (8 X 30 sec), dried for 20 min under nitrogen, and treated with a cleavage cocktail composed of 1% v/v each of water, triisopropylsilane and phenol in TFA (2 X 60 min). The cleaved peptides were precipitated with 50% v/v pentane/ether. After decanting the ether

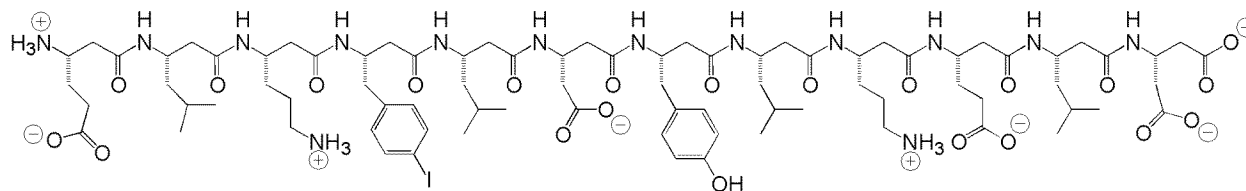
and drying with nitrogen, the precipitated peptides were dissolved in 30% v/v acetonitrile in water and purified by HPLC.

The success of each synthesis was assessed by both HPLC and MALDI-TOF analysis of the crude reaction mixture.  $\beta$ -peptides were then purified to homogeneity by reverse-phase HPLC. The identities and purities of the purified  $\beta$ -peptides were assessed by analytical HPLC and mass spectrometry. MALDI mass spectra were obtained using peptide samples in  $\alpha$ -cyano-4-hydroxycinnamic acid (CHCA) matrix. The masses found were: Acid-1Y (m/z observed, calculated):  $[M+H]^+$  1685, 1683; Acid-1Y\* (m/z observed, calculated):  $[M+H]^+$  1794, 1793 (Figure S1). Following purification,  $\beta$ -peptides were lyophilized and reconstituted in buffer (previously described) for characterization.

### Acid-1Y



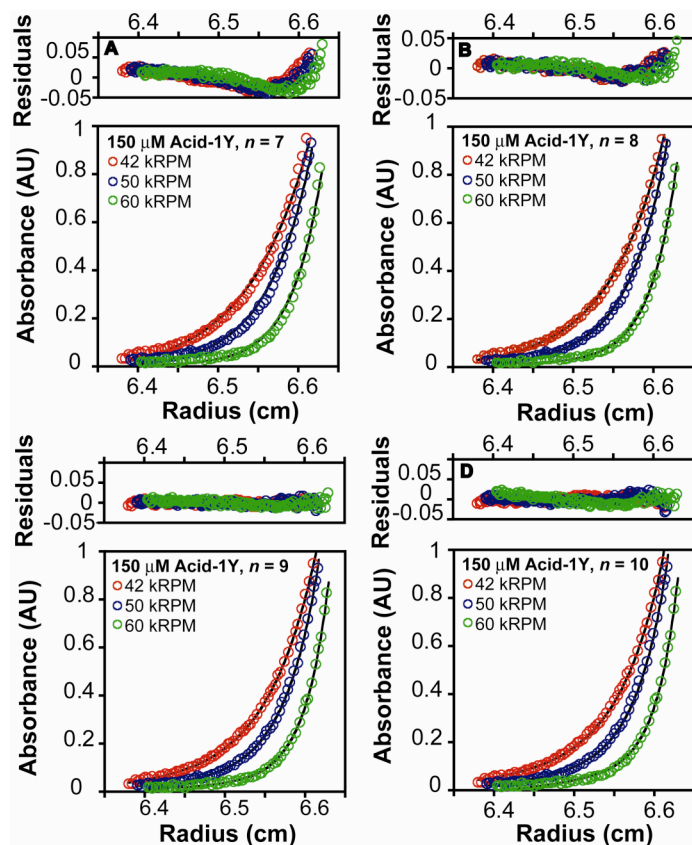
### Acid-1Y\*



**Figure S1.** Chemical structures of Acid-1Y and Acid-1Y\*.

**Sedimentation Equilibrium Analytical Ultracentrifugation.** HPLC-purified, lyophilized samples of Acid-1Y (300  $\mu$ M, 150  $\mu$ M and 75  $\mu$ M) and Acid-1Y\* (280  $\mu$ M, 140  $\mu$ M and 70  $\mu$ M) were resuspended and centrifuged at 25  $^{\circ}$ C to equilibrium at three different speeds (42,000,

50,000 and 60,000 rpm). Centrifugation was performed in an AN 60-Ti 4-hole rotor equipped with six-channel, carbon-epoxy composite centerpieces (Beckman). Absorbance was monitored at 280 nm. Data were collected with a step size of 0.001 cm with scans occurring at 3 h intervals. Samples were determined to have reached equilibrium when no significant changes in radial concentration were observed in three successive scans using the program Match within the Heteroanalysis software suite (available from the National Analytical Ultracentrifugation Facility website, <http://vm.uconn.edu/~wwwbiotc/uaf.html>). The partial specific volume of each  $\beta$ -peptide was calculated from functional group composition according to Durchschlag and Zipper.<sup>2</sup> The data was fit to a monomer-*n*-mer equilibrium model using the Heteroanalysis software. Fixed parameters: Acid-1Y monomer MW = 1682 Da,  $\bar{v}$  = 0.7846 cm<sup>3</sup>/g,  $d$  = 1.00674 g/mL,  $\epsilon_{280}$  = 1360 M<sup>-1</sup>•cm<sup>-1</sup>, and  $n$  = 8; Acid-1Y\* monomer MW = 1792 Da,  $\bar{v}$  = 0.7513 cm<sup>3</sup>/g,  $d$  = 1.00674 g/mL,  $\epsilon_{280}$  = 1360 M<sup>-1</sup>•cm<sup>-1</sup>, and  $n$  = 8. Fitted parameters: Acid-1Y  $\ln K_a$  = 83.3  $\pm$  1.8, RMSD = 0.01002 and baseline deviation <0.02; Acid-1Y\*  $\ln K_a$  = 65.6  $\pm$  0.1, RMSD = 0.00708 and baseline deviation <0.02. The data shown was fit with these parameters and the resulting residuals.



**Figure S2.** Acid-1Y self-association monitored by analytical ultracentrifugation (AU) (150  $\mu$ M Acid-1Y) and fit to monomer- $n$ -mer equilibria where (A)  $n = 7$ ; (B)  $n = 8$ ; (C)  $n = 9$ ; and (D)  $n = 10$ . Samples were prepared in 10 mM  $\text{NaH}_2\text{PO}_4$ , 200 mM NaCl (pH 7.1) and centrifuged to equilibrium at 25  $^\circ\text{C}$  at speeds of 42 (red), 50 (green), or 60 (blue) kRPM. The experimental data points are shown as open circles; lines indicate a fit to a monomer- $n$ -mer

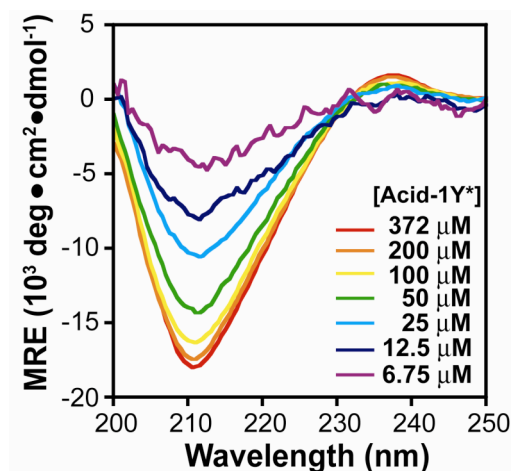
model as described in the experimental section. We note that the data fit poorly to a monomer-hexamer model ( $n = 6$ ), with a RMSD significantly higher (0.2159) than the RMSD values for other fits including  $n = 7$  (0.01367),  $n = 8$  (0.00706) and  $n = 9$  (0.01370).

**Circular Dichroism (CD).** Wavelength dependent CD spectra were acquired for various concentrations of Acid-1Y and Acid-1Y\* at 25  $^\circ\text{C}$  in continuous scan mode with 0.5 nm data pitch, 50 nm/min scanning speed, 4 sec response, 0.5 nm band width and 3 accumulations. The concentration dependence of the molar residue ellipticity (MRE) at 205 nm was determined by least-squares fitting of the total peptide monomer concentration as a function of experimental MRE with the following equation in Kaleidagraph (Synergy Software; Reading, PA)<sup>3</sup>:

$$[\text{Peptide}]_{\text{Total}} = \left\{ \frac{(\text{MRE}_{\text{Exp}} - \text{MRE}_{\text{Mon}})(1/K_a)}{n(\text{MRE}_{\text{Nmer}} - \text{MRE}_{\text{Mon}}) \left[ 1 - \left( \frac{\text{MRE}_{\text{Exp}} - \text{MRE}_{\text{Mon}}}{\text{MRE}_{\text{Nmer}} - \text{MRE}_{\text{Mon}}} \right)^n \right]} \right\}^{1/(n-1)}$$

The values obtained for the Acid-1Y fitted parameters were  $\text{MRE}_{\text{Mon}} = 11160 \text{ deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1}$  (constant, not fit in equation),  $\text{MRE}_{\text{Nmer}} = 24900 \pm 180 \text{ deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1}$ , and  $\ln K_a = 82.5 \pm 1.8$ . The curve in Figure 2B was fit using these parameters. Wavelength-dependent CD spectra for Acid-1Y\* also shows a concentration dependent increase in helical structure (Figure S3), analogous to that observed for Acid-1Y. The values obtained for the Acid-1Y\* fitted parameters for a monomer-octamer model were  $\text{MRE}_{\text{Mon}} = 0 \text{ deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1}$  (constant, not fit in equation),  $\text{MRE}_{\text{Nmer}} = 16755 \pm 58 \text{ deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1}$ , and  $\ln K_a = 81.1 \pm 0.8$ . However, analyses of  $\text{MRE}_{208}$  as a function of  $[\text{Acid-1Y}^*]$  were ambiguous in discriminating between monomer-hexamer and monomer-octamer models. To resolve this ambiguity in favor of the monomer-octamer model

we used the results of the AU data and the crystal structure.



**Figure S3.** Acid-1Y\* self-association measured by circular dichroism spectroscopy (CD). Wavelength-dependent CD spectra of Acid-1Y\* at the indicated  $[\text{Acid-1Y}^*]$ .

Temperature-dependent CD spectra were obtained at 205 nm between 5 and 95 °C, using the variable temperature module provided with the instrument. Data were collected with a 1 °C data pitch, 5 sec delay time, 1 °C/min temperature slope, 4 sec response time, and 1 nm band width. The first derivatives of the temperature-dependent CD spectra for the various concentrations of Acid-1Y were calculated and the  $T_m$  values reported correspond to the maximum of the  $\delta MRE_{205}/\delta T$  plots. Refer to Figure 3A for a plot of the  $\delta MRE_{208}/\delta T$  as a function of temperature for Acid-1Y.

**1-anilino-8-naphthalenesulfonate (ANS) Binding.** Stock solutions of 400  $\mu$ M Acid-1Y were prepared in 10 X PBC (100 mM phosphate, borate, and citrate) supplemented with 200 mM NaCl, pH 7.0; a 10  $\mu$ M stock of ANS was prepared in the same buffer (from an initial stock of 20  $\mu$ M ANS in 10 X PBC). Binding reactions were performed by mixing 80  $\mu$ L of the ANS solution with an appropriate volume of the Acid-1Y stock solution and diluting to a final volume of 160  $\mu$ L with 10 X PBC, 200 mM NaCl in order to produce 400, 200, 100, 50 and 25  $\mu$ M solutions of Acid-1Y. Fluorescence intensity (counts/s) measurements were made using a Photon Technology International (Lawrenceville, NJ) Quantamaster C-60 spectrofluorimeter at 25 °C in a 1 cm path length Hellma (Mullheim, Germany) cuvette. The sample was excited with 350 nm light (4 nm slit width) and fluorescence emission was measured at 1 nm intervals between 400 and 600 nm. ANS binding was not performed on Acid-1Y\*.

**Differential Scanning Calorimetry.** Temperature scans were performed from 5 to 125 °C at a scan rate of 1 °C/min. with a 10 min. equilibration period at 5 and 125 °C. All scans were performed with an excess pressure of 40 PSI. Samples of 300  $\mu$ L (142  $\mu$ L sample cell) were injected from the autosampling tray after being held at 10 °C. The raw data of temperature, power and time were converted to plots of heat capacity versus temperature. This data was fit to

a two-state model with dissociation of eight subunits using the program EXAM.<sup>4</sup> This process involves fitting  $\Delta H_{\text{Cal}}(T_{1/2})$ ,  $\Delta C_P(T_{1/2})$ ,  $\Delta C_P'(T_{1/2})$ ,  $T_{1/2}$ ,  $B_A$ ,  $B_A'$ ,  $B_B$ ,  $B_B'$  in the following equations where  $N$  and  $n$  are fixed:

For the transition  $A \rightleftharpoons nB$ , the heat required to melt  $N$  moles is

$$\frac{dQ}{dT} = (1 - \alpha)[B_A + B_A'(T - T_{1/2})] + N \left[ \frac{\Delta H_{\text{Cal}}(T)}{RT^2} \right] f(\alpha) + \alpha[B_B + B_B'(T - T_{1/2})]$$

where  $N_A$  is the number of moles of A,  $N_B$  is the number of moles of B and  $f(\alpha) = \frac{\alpha(1 - \alpha)}{n(1 - \alpha) + \alpha}$

and  $\alpha = 1 - \frac{N_{A,T}}{N_{A,T=0}} = \frac{N_{B,T}}{N_{B,T=\infty}}$ . The free energy is determined relative to  $T_{1/2}$  using

$$\frac{K}{K_{T_{1/2}}} = \frac{\frac{(\alpha N_{B,T=\infty})^n}{(1 - \alpha)N_{A,T=0}}}{\frac{(1/2 N_{B,T=T_{1/2}})^n}{(1 - 1/2)N_{A,T=T_{1/2}}}} = \frac{(2\alpha)^n}{2(1 - \alpha)} = e^{-\frac{\Delta\Delta G}{RT}} \text{ where } \alpha = 1/2 \text{ at } T_{1/2}.$$

$\Delta\Delta G$  is determined by the following thermodynamic equations:

$$\Delta H_{\text{Cal}}(T) = \Delta H_{\text{Cal}}(T_{1/2}) + \Delta C_P(T_{1/2})(T - T_{1/2}) + 1/2\Delta C_P'(T - T_{1/2})^2 \quad \Delta C_P(T) = \Delta C_P(T_{1/2}) + \Delta C_P'(T - T_{1/2})$$

$$\frac{-\Delta\Delta G}{T} = \left[ \frac{\Delta H_{\text{Cal}}(T_{1/2})}{T_{1/2}} \right] \left[ \frac{T - T_{1/2}}{T} \right] + \Delta C_P(T_{1/2}) \left[ \ln \frac{T}{T_{1/2}} - \frac{T - T_{1/2}}{T} \right] + 1/2\Delta C_P' \left[ \frac{(T + T_{1/2})(T - T_{1/2})}{T} - 2T_{1/2} \ln \frac{T}{T_{1/2}} \right]$$

The fitting results are shown in Figure 3B for 300  $\mu\text{M}$  Acid-1Y. The values of the fitted parameters are as follows:  $\Delta H(T_{1/2}) = 132.0 \pm 0.4 \text{ kcal}\cdot\text{mol}^{-1}$ ,  $\Delta C_P(T_{1/2}) = 1.8 \text{ kcal}\cdot\text{mol}^{-1}\cdot\text{K}^{-1}$ ,  $\Delta C_P'(T_{1/2}) =$ ,  $T_{1/2} = 356.3$ . The equilibrium constant at 25 °C ( $K = 6.1 \times 10^{-36}$ ) was calculated using  $K(T) = 1/(K(T_{1/2}) \cdot e^{-\Delta\Delta G/RT})$ , where  $K(T_{1/2}) = (C_T/2)^n / (C_T/2n)$  and  $C_T$  is the total peptide concentration.

The van't Hoff enthalpy was calculated from the integrated calorimetric enthalpy ( $\Delta H_{\text{Cal}}^*$ ), obtained by integrating the area between the thermogram and the baseline in Figure 5 for Zwit-



1F and Acid-1Y. This area was used in the following equation to calculate the van't Hoff enthalpy according to Sturtevant<sup>5</sup> and Marky and Breslauer<sup>6</sup>:

$$\Delta H_{\text{vH}} = 2(n + 1)RT_{1/2}^2 \frac{C_{\text{P}}^{\text{Max}}}{\Delta H_{\text{Cal}}^*}$$

where  $C_{\text{P}}^{\text{Max}}$  is the maximum difference between the thermogram and the baseline heat capacity and  $n$  is the number of subunits. The  $\Delta H_{\text{vH}}/\Delta H_{\text{Cal}}$  ratio was calculated using the fitted  $\Delta H_{\text{Cal}}(T_{1/2})$  from above.

**Crystallization and Structure Determination.** Acid-1Y\* (2.00 mM) was crystallized by hanging drop vapor diffusion over a buffer composed of 1.35 M sodium citrate and 0.1 M HEPES (pH 7.5). Crystals were cryoprotected using a step-wise transfer from mother liquor containing 0% glycerol to mother liquor supplemented with 7.5% glycerol and then 12% glycerol. X-ray diffraction data to 1.54 Å resolution was collected at 100K at the Yale Center for Structural Biology. Data was processed to 2.3 Å resolution using the HKL2000 software suite.<sup>7</sup> Molecular replacement using a tetramer of the previously solved Zwit-1F octamer<sup>8</sup> was used to obtain phases calculated to 2.3 Å using CNS.<sup>9</sup> The data was determined to have partial merohedral twinning by the Yeate's twinning test with a twinning fraction ( $\alpha$ ) equal to  $0.307932 \pm 0.004175$  following a twin operation of 2 along  $a^*$ ,  $b^*$ .<sup>10</sup> Detwinning, density modification and NCS averaging were performed using CNS.<sup>9</sup> The program XFIT was used to build and refine eight  $3_{14}$ -helices into the electron density maps.<sup>11</sup> Refinement was performed using CNS with an  $R_{\text{free}}$  set to 10% of the data.<sup>9</sup> Pymol was used for Figure preparation.<sup>12</sup>

**Table S1.** Data collection, phasing and refinement statistics

<b>Acid-1Y*</b>	
<b>Data Collection</b>	
Space group	P3 <sub>2</sub>
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	41.345, 41.345, 66.31
$\alpha$ , $\beta$ , $\gamma$ (°)	90, 90, 120
Resolution (Å)	50 - 2.30 (2.38-2.30) <sup>a</sup>
<i>R</i> <sub>sym</sub> or <i>R</i> <sub>merge</sub>	10.2 (33.8)
<i>I</i> / $\sigma$ <i>I</i>	17.10 (3.48)
Completeness (%)	99.6 (96.9)
Redundancy	5.1 (3.6)
<b>Refinement</b>	
Resolution (Å)	50 – 2.30
No. reflections	10,294
<i>R</i> <sub>work</sub> / <i>R</i> <sub>free</sub> (%)	21.52 / 24.95
No. atoms	
Protein	952
Water	38
B-factors	
Protein	39.758
Water	32.6549
r.m.s deviations	
Bond lengths (Å)	0.005810
Bond angles (°)	0.78714

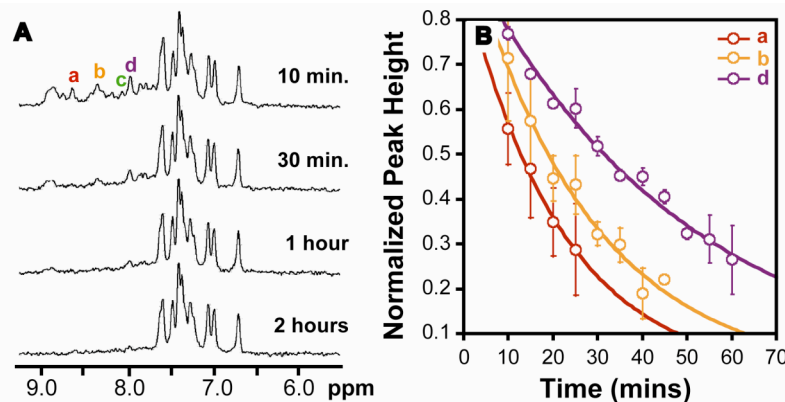
<sup>a</sup>Highest resolution shell is shown in parenthesis

**Deuterium Exchange NMR Studies.** All spectra were acquired at 25 °C using the WATERGATE solvent suppression pulse sequence ZZPWG (see [www.bruker.com](http://www.bruker.com) for pulse sequence information). Before acquisition under the WATERGATE pulse sequence, a standard proton spectrum was acquired using a 1.5 mM Zwit-1F sample in a 9:1 mixture of H<sub>2</sub>O and D<sub>2</sub>O and the position of the water peak was determined.<sup>13</sup> The transmitter offset (<sup>01</sup>P) was set to this frequency for ZZPWG acquisitions. Post processing was performed with a *sfil* type baseline correction set to 1.5 ppm and a line broadening factor of 5.0 Hz. In order to acquire deuterium exchange data, the Acid-1Y sample was dissolved in phosphate buffer spiked with TMSP and lyophilized. The lyophilized sample was resuspended in D<sub>2</sub>O at a concentration of 750 μM just prior to insertion into the instrument. Prior to loading the Acid-1Y sample, the spectrometer was locked and shimmed on a sample of Zwit-1F in 9:1 H<sub>2</sub>O/D<sub>2</sub>O in phosphate buffer. Amide exchange rates vary logarithmically with pD in the EX2 regime (pH < 9.5 for most α-amino acid proteins) and therefore the pD of the Acid-1Y samples used in the NMR studies were carefully monitored.<sup>14</sup> Our experiments were confirmed to belong to the EX2 regime as previously determined.<sup>13</sup> We measured the pD of our Acid-1Y NMR samples after exchange was complete using a pH meter (Thermo Orion Model 410, Beverly, MA) in the laboratory of Prof. Ann Valentine (Yale University). The samples were determined to have values of 6.80 ± 0.5 and 6.87 ± 0.5 pH units, which corresponds to corrected values of 7.20 and 7.27 pD units for Acid-1Y samples 1 and 2 respectively.<sup>15</sup> The 750 μM Zwit-1F samples were also determined to have a corrected pD of ~ 7.2. Protection factors were calculated using  $k_{rc}$  values derived from poly-β-homoglycine or using α-amino acid dipeptides.<sup>13, 16, 17</sup> The former method has been described previously;<sup>13</sup> for the latter, we calculated P using  $k_{rc}$  values derived from all α-amino acid dipeptides represented by the sequence of Acid-1Y (NH<sub>3</sub><sup>+</sup>-E-L-O-Y-L-D-Y-L-O-E-L-D-COO<sup>-</sup>).

The most conservative calculation of P for Acid-1Y makes reference to the  $k_{rc}$  value for Glu-Leu; the P values calculated in this way vary by a factor of four from the P values calculated with reference to the  $k_{rc}$  value for poly- $\beta$ -homoglycine (Table S1).

**Table S1.** Protection factors calculated from two  $k_{ex}$  sources, poly- $\beta$ -homoglycine and  $\alpha$ -amino acid dipeptide (Glu-Leu)

$k_{ex}$ source	Protection Factors			
	Peak A	Peak B	Peak C	Peak D
poly- $\beta$ -homoglycine <sup>18</sup>	59000	52300	69300	20500
$\alpha$ -amino acid dipeptide (Glu-Leu) <sup>16</sup>	14000	13000	17000	5000



**Figure S4.** NMR Studies of Zwit-1F at 750  $\mu$ M concentration. (A) 500 MHz <sup>1</sup>H NMR spectra of Zwit-1F acquired at the indicated times after the lyophilized Zwit-1F

sample was reconstituted in phosphate-buffered D<sub>2</sub>O. (B) Peak heights of the indicated resonances (plotted as normalized values to a peak at 6.39 ppm) are fit to exponential decays for one of the Acid-1Y deuterium exchange NMR experiments.



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