

Biophysical Characterization of a β -Peptide Bundle: Comparison to Natural Proteins

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We recently described the high-resolution X-ray structure of a helical bundle composed of eight copies of the β -peptide Zwit-1F (Figure 1A,B).¹ Like many proteins in nature, the Zwit-1F octamer contains parallel and antiparallel helices, extensive inter-helical electrostatic interactions, and a solvent-excluded hydrophobic core. Here we explore the stability of the Zwit-1F octamer in solution using circular dichroism (CD) spectroscopy, analytical ultracentrifugation (AU), differential scanning calorimetry (DSC), and NMR. These studies demonstrate that the thermodynamic and kinetic properties of Zwit-1F closely resemble those of natural α -helical bundle proteins.

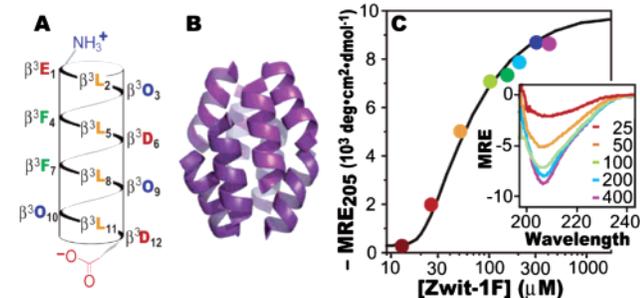


Figure 1. (A) Helical net representation of the Zwit-1F monomer. β^3 -Amino acids are designated by the single letter corresponding to the equivalent α -amino acid. O signifies ornithine. (B) Zwit-1F octamer structure determined by X-ray crystallography.¹ (C) Plot of MRE₂₀₅ as a function of [Zwit-1F] fit to a monomer–octamer equilibrium. Inset: CD spectra (MRE in units of $10^3 \text{ deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1}$) at the indicated [Zwit-1F] (μM).

CD spectroscopy indicates that Zwit-1F is minimally 3_{14} -helical in dilute solution (as judged by the molar residue ellipticity at 205 nm, MRE₂₀₅)² but undergoes a large increase in helical structure between 20 and 200 μM (Figure 1C). The concentration dependence of MRE₂₀₅ fits a monomer–octamer equilibrium with an association constant of $4.0 \times 10^{30} \text{ M}^{-7}$ ($\ln K_a = 70.5 \pm 1.9$).³ This value matches the result of AU analysis, which fits a monomer–octamer equilibrium with $\ln K_a = 71.0 \pm 0.9$.³ Taken together, the AU and CD data support a model in which unfolded Zwit-1F monomer is in equilibrium with folded octamer.⁴

Examples of natural octameric proteins include the histones⁵ (hetero-octamer), TATA binding protein⁶ (octamer in 1 M KCl), and the thermodynamically and structurally characterized hemerythrin ($\ln K_a = 84$).⁷ Although Zwit-1F is less stable than hemerythrin, it is smaller in mass (13.1 vs 110 kDa) and interaction surface area (7000 vs 15 000 \AA^2).^{1,8} To compare the stability of Zwit-1F to that of proteins of diverse size and stoichiometry, we calculated the free energy of association per \AA^2 of buried surface area (ΔG_{area}). Issues of molecularity aside, the ΔG_{area} of Zwit-1F is higher than that of hemerythrin, the tetrameric aldolase, and

Table 1. Comparison of Protein Association Parameters^a

protein (stoichiometry)	MW _{monomer}	ΔG_{area}
Zwit-1F (8)	1.6 kDa	5.9
hemerythrin (8)	13.8 kDa	3.3 ⁷
aldolase (4)	39.2 kDa	3.9 ¹¹
GCN4 (2)	4.0 kDa	4.8 ¹²
ROP (2)	7.2 kDa	≥ 3.0 ¹³

^a ΔG_{area} values in units of $\text{cal}\cdot\text{mol}^{-1}\cdot\text{\AA}^{-2}$. Interaction surface areas and ΔG_{area} calculated as described in Supporting Information.

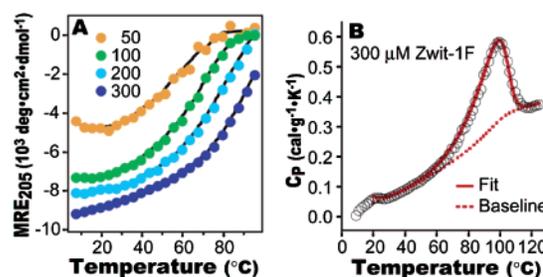


Figure 2. (A) Temperature-dependent CD analysis of Zwit-1F. Plot of MRE₂₀₅ as a function of temperature at the indicated Zwit-1F concentration (μM). (B) DSC analysis of Zwit-1F unfolding fit to a subunit dissociation model. Raw data are shown as black circles.³

natural helical bundle proteins GCN4 and ROP (Table 1). In fact, ΔG_{area} for Zwit-1F is close to the average value ($7.0 \pm 2.8 \text{ cal}\cdot\text{mol}^{-1}\cdot\text{\AA}^{-2}$) observed for protein complexes burying at least 1000 \AA^2 of surface area upon association.^{9,10} The comparison between Zwit-1F and hemerythrin implies that the lower affinity of Zwit-1F is due to its small size and not an inherent instability of β^3 -peptide complexes.

Temperature-dependent CD studies (Figure 2A) show Zwit-1F to exhibit a concentration-dependent T_m , an inherent property of protein quaternary structure.¹⁴ The Zwit-1F T_m , which increases from 57 °C at 50 μM to 95 °C at 300 μM , is comparable to T_m values of thermostable proteins such as ubiquitin ($T_m = 90$ °C) and bovine pancreatic trypsin inhibitor ($T_m = 101$ °C).¹⁵ The Zwit-1F T_m is significantly higher than the T_m of GCN4 (41–78 °C at 1–880 μM)¹⁶ and ROP (58–71 °C at 0.5–160 μM).¹⁷ We note, however, that the unfolding of Zwit-1F is less cooperative: the width of the temperature derivative of the CD signal at half-maximum is 40 versus 20 °C for GCN4 or 15 °C for ROP.^{16,17}

A high T_m is not a definitive measurement of thermodynamic stability, so DSC was used to further characterize Zwit-1F unfolding (Figure 2B). At 300 μM concentration (where Zwit-1F is 87% octameric), the temperature-dependent heat capacity (C_p) peaks near the T_m identified by CD. This peak is embedded in a sloping baseline ($\partial C_p/\partial T = 5.1 \text{ cal}\cdot\text{mol}^{-1}\cdot\text{K}^{-2} = 3.1 \text{ mcal}\cdot\text{g}^{-1}\cdot\text{K}^{-2}$) that is similar to the C_p versus temperature plot of monomeric β^3 -peptides, for which no cooperative unfolding peak has yet been observed.² For most natural proteins, $(\partial C_p/\partial T)$ is about $1 \text{ mcal}\cdot\text{g}^{-1}\cdot\text{K}^{-2}$ in the folded state,¹⁵ but GCN4 ($\partial C_p/\partial T = 3.6 \text{ mcal}\cdot\text{g}^{-1}\cdot\text{K}^{-2}$)¹⁶ and some

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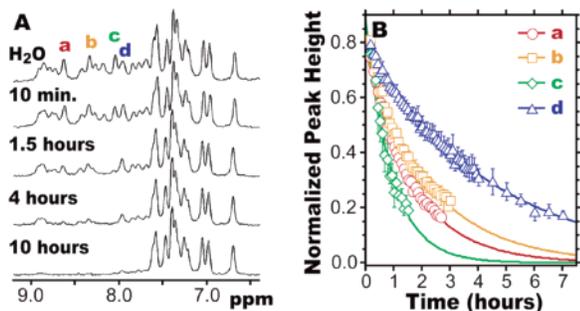


Figure 3. (A) 500 MHz ^1H NMR spectra of 1.5 mM Zwit-1F, acquired in phosphate-buffered “ H_2O ” (9:1 $\text{H}_2\text{O}/\text{D}_2\text{O}$) or at the indicated times after reconstitution of a lyophilized Zwit-1F sample in phosphate-buffered D_2O . (B) Peak heights of the indicated resonances (normalized to the peak at 6.70 ppm) fit to exponential decays.³ Bars indicate standard error.

ROP mutants ($\partial C_p/\partial T = 4\text{--}5 \text{ kcal}\cdot\text{g}^{-1}\cdot\text{K}^{-2}$)¹³ have sharply sloped pretransition baselines like Zwit-1F.

The DSC data fit well to a process defined by a two-state transition with dissociation of eight subunits using the program EXAM.^{3,18} The fitted enthalpy and heat capacity change per mole octamer are $107.4 \pm 0.3 \text{ kcal}\cdot\text{mol}^{-1}$ and $1.4 \pm 0.1 \text{ kcal}\cdot\text{mol}^{-1}\cdot\text{K}^{-1}$, respectively. Substituting these values into the Gibbs–Helmholtz equation³ yields an equilibrium constant of 5.3×10^{31} ($\ln K = 73.3 \pm 1.4$) at 25 °C, in excellent agreement with values derived from CD and AU data. The integrated calorimetric unfolding enthalpy (ΔH_{Cal}) for Zwit-1F is $7.2 \text{ cal}\cdot\text{g}^{-1}$, within the range observed for natural globular proteins ($5.2\text{--}11.8 \text{ cal}\cdot\text{g}^{-1}$),^{19,20} but somewhat lower than GCN4 ($7.7 \text{ cal}\cdot\text{g}^{-1}$)²¹ and ROP ($9.5 \text{ cal}\cdot\text{g}^{-1}$).¹⁷

The NMR spectra of many well-folded natural and designed proteins are characterized by differentiated amide resonances and slow hydrogen/deuterium exchange.²² The amide N–H resonances in the ^1H spectrum of Zwit-1F, under conditions where the sample is 97% octameric, span 1.4 ppm (Figure 3A). While this span is narrower than that observed in the NMR spectra of large proteins such as α -lactalbumin (3 ppm), it is comparable to that seen for coiled-coil proteins GCN4 and ROP (1.3 and 2.2 ppm, respectively).^{13,23,24} In contrast to Zwit-1F, the amide resonances of the poorly folded, monomeric β -peptide Acid-1Y^{A2,11} span only 0.5 ppm.³ These results indicate that the Zwit-1F fold in solution creates distinct electronic environments for the amide backbone protons.

Participation in a hydrogen bond can protect an amide N–H from exchange with bulk solvent; since exchange occurs from the unfolded state, a slow amide exchange rate constant (k_{ex}) correlates with protein stability in solution.²² Exchange is often characterized by a protection factor (P) equal to $k_{\text{rc}}/k_{\text{ex}}$, where k_{rc} is the rate constant for exchange of a random coil amide N–H under similar conditions. When a lyophilized sample of Zwit-1F is redissolved at 1.5 mM concentration in D_2O , 9 of 14 resolvable peaks require more than 4 h to become indistinguishable from baseline. The time dependence of exchange corresponds to exchange rate constants between 0.6×10^{-4} and $2.9 \times 10^{-4} \text{ s}^{-1}$. Using β -alanine (βG in our nomenclature) as a random coil model,^{3,25} a k_{ex} value of 0.6×10^{-4} corresponds to a protection factor of 2×10^4 for Zwit-1F. Thus, amide protons in Zwit-1F are less protected than those in large protein cores, where $P \geq 10^5$.^{22,26} However, the protection factor for Zwit-1F, like the span of amide resonances, is comparable to ROP (10^5 at 250 μM)¹³ and GCN4 (10^4 at 1.0 mM).^{23,24} Acid-1Y^{A2,11} undergoes amide N–H exchange in less than 10 min, showing that slow exchange requires a stable β -peptide fold.³

The biophysical experiments presented here describe the thermodynamic and kinetic stability of the Zwit-1F octamer in solution. The data allow us to quantify the similarity of Zwit-1F to GCN4 and ROP, two small, well-folded α -amino acid helix bundle

proteins. In fact, the T_m , ΔG_{area} , and ΔH_{Cal} for Zwit-1F are even comparable to much larger natural proteins. Taken together with the recent high-resolution structure of Zwit-1F,¹ these studies show that β -amino acid heteropolymers can assemble into quaternary complexes that resemble natural proteins in both solid-state structure and solution-phase stability. We note that our characterizations do not preclude some molten globule character of the Zwit-1F core in solution.²⁷ Nonetheless, these studies establish Zwit-1F as a remarkably protein-like stepping stone in the path toward fully synthetic mimics of biological molecules.

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Supporting Information Available: Experimental procedures, Table 1 calculations, and data fits (PDF). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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