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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.

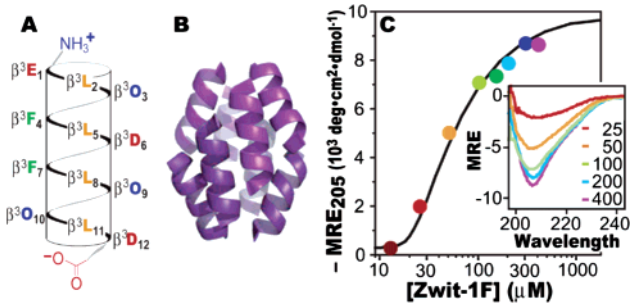

Figure 1. (A) Helical net representation of the Zwit-1F monomer. β^3-Amino acids are designated by the single letter corresponding to the equivalent α-amino acid. O signifies ornithine. (B) Zwit-1F octamer structure determined by X-ray crystallography.[1] (C) Plot of MRE_{205} as a function of [Zwit-1F] fit to a monomer−octamer equilibrium. Inset: CD spectra (MRE in units of 10^3 deg·cm^2·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, MRE_{205})[2] but undergoes a large increase in helical structure between 20 and 200 μM (Figure 1C). The concentration dependence of MRE_{205} fits a monomer−octamer equilibrium with an association constant of 4.0×10^{30} 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 histones[5] (hetero-octamer), TATA binding protein[6] (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 Å^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 Å^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 natural helical bundle proteins GCN4 and ROP (Table 1). In fact, ΔG_{area} for Zwit-1F is close to the average value (7.0 ± 2.8 cal·mol^{-1}·A^{-2}) observed for protein complexes burying at least 1000 Å^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.

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 cal·mol^{-1}·Å^{-2}. Interaction surface areas and ΔG_{area} calculated as described in Supporting Information.

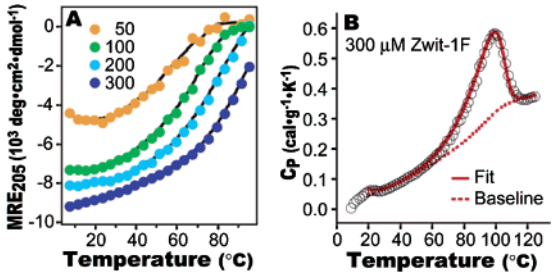

Figure 2. (A) Temperature-dependent CD analysis of Zwit-1F. Plot of MRE_{205} as a function of temperature at the indicated Zwit-1F concentration (μM). (B) DSC analysis of Zwit-1F unfolding fit to a subunit dissociation model. Raw data are shown as black circles.[3]

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 ($\partial C_p/\partial T = 5.1$ cal·mol^{-1}·K^{-2} = 3.1 mcal·g^{-1}·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 mcal·g^{-1}·K^{-2} in the folded state,[15] but GCN4 ($\partial C_p/\partial T = 3.6$ mcal·g^{-1}·K^{-2})[16] and some

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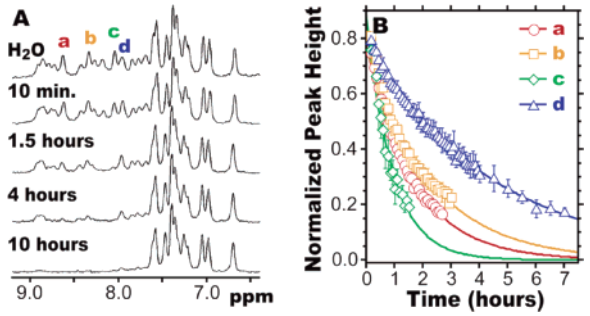

Figure 3. (A) 500 MHz ^{1}H NMR spectra of 1.5 mM Zwit-1F, acquired in phosphate-buffered "H_2O" (9:1 H_2O/D_2O) or at the indicated times after reconstitution of a lyophilized Zwit-1F sample in phosphate-buffered D_2O. (B) Peak heights of the indicated resonances (normalized to the peak at 6.70 ppm)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

ROP mutants ($\partial C_p/\partial T = 4-5$ mcal·g^{-1}·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 ± 0.3 kcal·mol^{-1} and 1.4 ± 0.1 kcal·mol^{-1}·K^{-1}, respectively. Substituting these values into the Gibbs−Helmholz equation[3] 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 cal·g^{-1}, within the range observed for natural globular proteins (5.2−11.8 cal·g^{-1}),[19,20] but somewhat lower than GCN4 (7.7 cal·g^{-1})[21] and ROP (9.5 cal·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 ^{1}H 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_{rc}/k_{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 2.9×10^{-4} 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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