Published in final edited form as:
J Am Chem Soc. 2007 May 2; 129(17): 5344–5345. doi:10.1021/ja070567g.

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

E. James Petersson[†], **Cody J. Craig**[†], **Douglas S. Daniels**[†], **Jade X. Qiu**[†], and **Alanna Schepartz**[*,†,‡]
Departments of Chemistry and Molecular, Cellular and Developmental Biology, Yale University, New Haven, Connecticut 06520-8107

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.

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 the unfolded Zwit-1F monomer is in equilibrium with the folded octamer.[4]

Few known natural proteins assemble as octamers. Examples include the histones[5] (hetero-octamer), TATA binding protein[6] (octamer in 1 M KCl), and the well-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}). 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] This comparison 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).

alanna.schepartz@yale.edu.
[†]Department of Chemistry.
[‡]Department of Molecular, Cellular and Developmental Biology.

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.

[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 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 ± 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] these values of k_{ex} correspond 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH and NFCR. We thank Dr. J. Hoch and Z. Sutter (UConn Health Center) for access to a Microcal VP-DSC microcalorimeter, and Dr. F. Schwarz (NIST) for the program EXAM.

References

1. Daniels DS, Petersson EJ, Qiu XJ, Schepartz A. J Am Chem Soc 2007;129:1532–1533. [PubMed: 17283998]
2. CD extrema between 205 and 215 nm have been attributed previously to the 3_{14}-helix. For details, see: Seebach D, Beck AK, Bierbaum DJ. Chem Biodiversity 2004;1:1111–1239.
3. See Supporting Information.
4. The MRE_{205} concentration dependence of Zwit-1F also fits a monomer–hexamer equilibrium, as did previous AU data. Although the Zwit-1F X-ray structure shows an octamer, we cannot currently exclude the presence of hexameric forms in solution.
5. Baxevanis AD, Godfrey JE, Moudrianakis EN. Biochemistry 1991;30:8817–8823. [PubMed: 1888742]
6. Daugherty MA, Brenowitz M, Fried MG. J Mol Biol 1999;285:1389–1399. [PubMed: 9917384]
7. Langerman NR, Klotz IM. Biochemistry 1969;8:4746–4757. [PubMed: 4312452]
8. Holmes MA, Stenkamp RE. J Mol Biol 1991;220:723–737. [PubMed: 1870128]
9. Nooren IMA, Thornton JM. J Mol Biol 2003;325:991–1018. [PubMed: 12527304]
10. Brooijmans N, Sharp KA, Kuntz ID. Proteins: Struct, Funct, Genet 2002;48:645–653. [PubMed: 12211032]
11. Tolan DR, Schuler B, Beernink PT, Jaenicke R. Biol Chem 2003;384:1463–1471. [PubMed: 14669989]
12. Wendt H, Baici A, Bosshard HR. J Am Chem Soc 1994;116:6973–6974.
13. Munson M, Balasubramanian S, Fleming KG, Nagi AD, Obrien R, Sturtevant JM, Regan L. Protein Sci 1996;5:1584–1593. [PubMed: 8844848]
14. Sturtevant JM. Annu Rev Phys Chem 1987;38:463–488.
15. Makhatadze GI, Privalov PL. Adv Protein Chem 1995;47:307–425. [PubMed: 8561051]
16. Dragan AI, Privalov PL. J Mol Biol 2002;321:891–908. [PubMed: 12206769]
17. Steif C, Weber P, Hinz HJ, Flossdorf J, Cesareni G, Kokkinidis M. Biochemistry 1993;32:3867–3876. [PubMed: 8471599]
18. Kirchhoff WM. NIST Technical Note 1993:1401.
19. Dragan AI, Li ZL, Makeyeva EN, Milgotina EI, Liu YY, Crane-Robinson C, Privalov PL. Biochemistry 2006;45:141–151. [PubMed: 16388589]
20. Makhatadze GI, Kim KS, Woodward C, Privalov PL. Protein Sci 1993;2:2028–2036. [PubMed: 7507751]
21. (a) Thompson KS, Vinson CR, Freire E. Biochemistry 1993;32:5491–5496. [PubMed: 8504069] See Supporting Information for comparison to GCN4 ΔH_{Cal} from (b) Dragan AI, Privalov PL. J Mol Biol 2002;321:891–908. [PubMed: 12206769]
22. Krishna MMG, Hoang L, Lin Y, Englander SW. Methods 2004;34:51–64. [PubMed: 15283915]

23. O'Shea EK, Lumb KJ, Kim PS. Curr Biol 1993;3:658–667. [PubMed: 15335856]

24. Lumb KJ, Kim PS. Biochemistry 1995;34:8642–8648. [PubMed: 7612604]

25. Glickson JD, Applequist J. J Am Chem Soc 1971;93:3276–3281. [PubMed: 5571295]

26. Schulman BA, Redfield C, Peng ZY, Dobson CM, Kim PS. J Mol Biol 1995;253:651–657. [PubMed: 7473740]

27. Although our DSC measurements support a two-state model, broad transitions of this type have been observed for proteins in molten globule states (Moosavi-Movahedi AA, Chamani J, Gharanfoli M, Hakimelahi GH. Thermochim Acta 2004;409:137–144. and designed proteins can become more structured in the solid state due to intermolecular crystal contacts Hill CP, Anderson DH, Wesson L, DeGrado WF, Eisenberg D. Science 1990;249:543. [PubMed: 2382133] A standard test for molten globule states is binding of the fluorophore ANS Goto Y, Fink AL. Biochemistry 1989;28:945–952. [PubMed: 2496758]), but this test is not feasible for Zwit-1F.[3] Experiments to examine the Zwit-1F structure using NMR relaxation techniques are in progress.

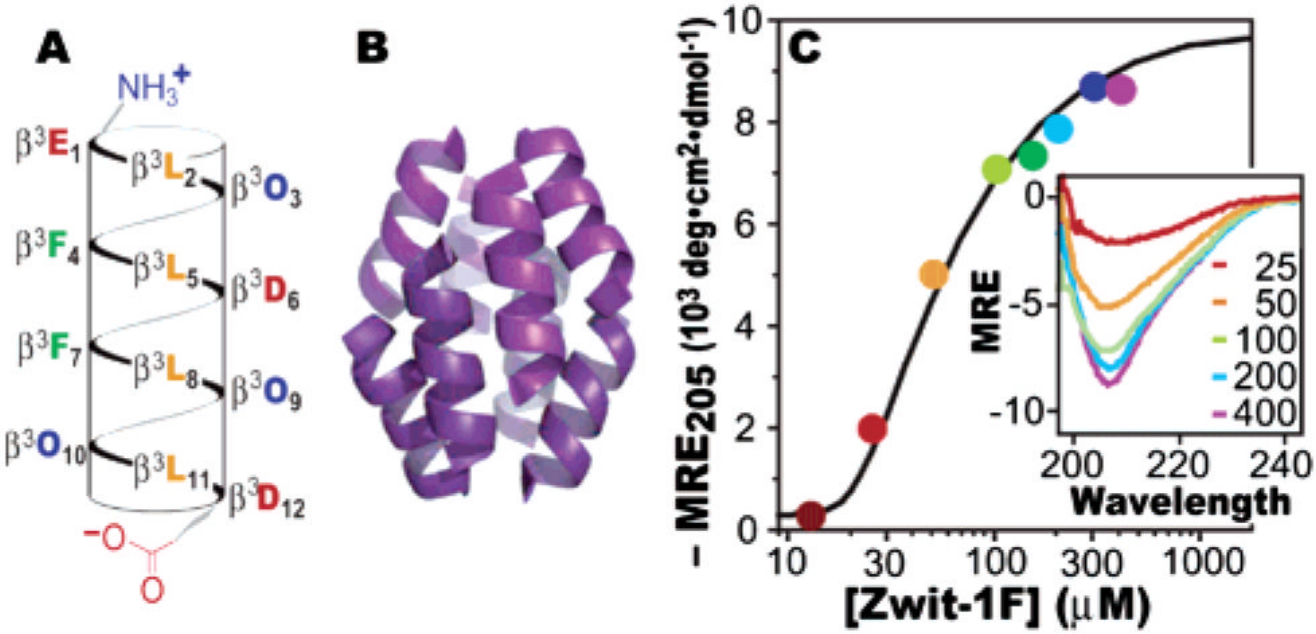

Figure 1.

(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).

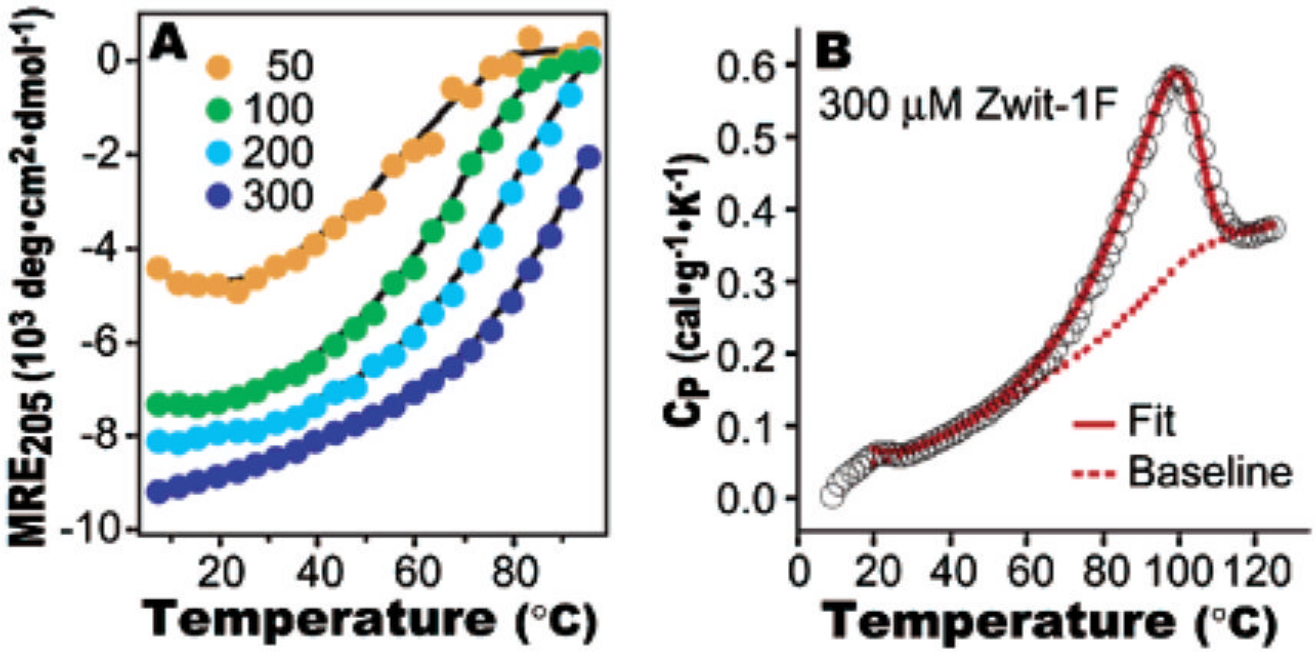

Figure 2.

(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]

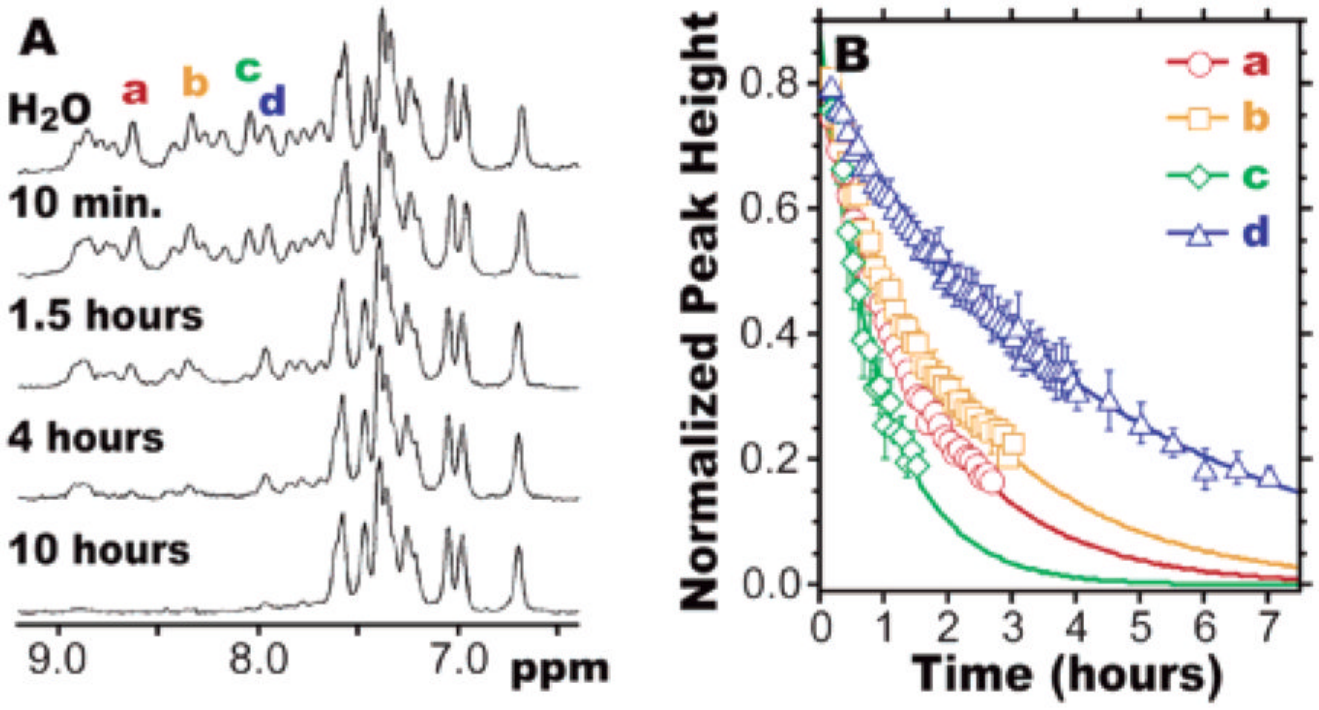

Figure 3.

(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.

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	$_{\geq 3.0}13$

[a] ΔG_{area} values in units of cal· mol^{-1}·$Å^{-2}$. Interaction surface areas and ΔG_{area} calculated as described in Supporting Information.