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Enhancing β^3-Peptide Bundle Stability by Design

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We reported recently that certain β^3-peptides self-assemble in aqueous solution into discrete bundles of unique structure and defined stoichiometry. The first β-peptide bundle reported was the octameric Zwit-1F, whose fold is characterized by a well-packed, leucine-rich core and a salt-bridge-rich surface. Close inspection of the Zwit-1F structure revealed four nonideal interhelical salt-bridge interactions whose heavy atom–heavy atom distances were longer than found in natural proteins of known structure. Here we demonstrate that the thermodynamic stability of a β-peptide bundle can be enhanced by optimizing the length of these four interhelical salt bridges. Combined with previous work on the role of internal packing residues, these results provide another critical step in the "bottom-up" formation of β-peptide assemblies with defined sizes, reproducible structures, and sophisticated function.

We reported that certain β^3-peptides self-assemble spontaneously in aqueous solution into discrete bundles of defined stoichiometry (β-peptide bundles) whose kinetic and thermodynamic metrics are virtually indistinguishable from those of natural proteins.[1] The β-peptide bundles we reported undergo cooperative, all-or-none folding transitions and exclude hydrophobic dyes; the structures of three such bundles have been defined at high resolution.[1a,c,2] These initially reported quaternary structures were designed, without the benefit of high-resolution structural data, to contain both a leucine-rich hydrophobic core and a salt-bridge-rich exterior. The structures now in hand provide the opportunity to evaluate and optimize these interactions to better understand and control β-peptide-bundle structure, and the relationship of this structure to kinetic stability, thermodynamic stability, and the cooperativity of unfolding. In previous reports, we focused on the role of aliphatic side-chain identity and volume on β-peptide bundle stoichiometry[1a,b,e,g] and the contribution of aromatic side chains, included initially to aid concentration determination, to bundle stability.[1c] Here we focus on the salt-bridge-rich exterior, and evaluate the extent to which β-peptide stability depends on and can be enhanced by improved salt bridge interactions on the bundle surface.

The octameric β-peptide bundle fold, exemplified by Zwit-1F,[1a] is characterized by parallel and antiparallel helix interfaces, a well-packed leucine-rich core, and a bundle surface replete with 16 paired salt bridge interactions (Figure 1 A).[1a,c]

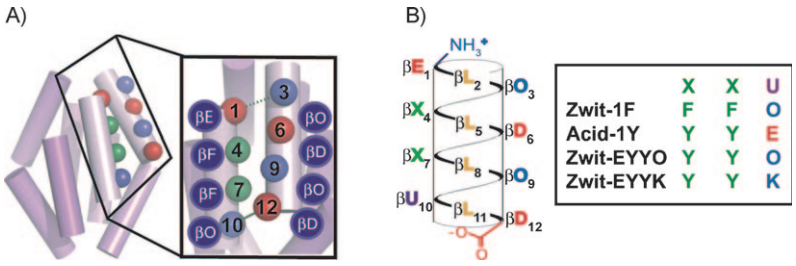

Figure 1. A) Structure of the Zwit-1F octamer[1a] highlighting one of four symmetry-related, parallel-helix interfaces and identifying the imperfect interhelical salt bridge between $\beta hOrn_{10}$ and $\beta hAsp_{12}$. B) Helical net diagram of β-peptides studied and discussed herein.

Eight of these paired salt bridges occur between side chains on parallel helix interfaces, of which there are four, and involve residues on both the aromatic and the salt-bridge faces of the Zwit-1F monomer (Figure 1 A). Close examination of the Zwit-1F bundle structure revealed that, with four exceptions, these salt bridges possess heavy atom–heavy atom distances that coincide with those in natural proteins—2.6 to 4.6 Å.[3] The exception—repeated four times per octamer—is the interhelix salt bridge between $\beta hOrn_{10}$ on one β-peptide helix and $\beta hAsp_{12}$ on its parallel partner (Figure 1 A). Here, the distance varies between 5.6 and 5.8 Å, well outside the 5 Å length observed for salt bridges in proteins of known structure.[3] Previous work has shown that side-chain lengths contribute to *intra*molecular salt-bridge strength in both short α-helical peptides[4] and β^3-peptides,[5] and to *inter*molecular salt-bridge strength in coiled coils.[6] Herein we demonstrate that, as in coiled coils, the thermodynamic stability of a β-peptide bundle can be enhanced significantly by optimizing the lengths of interhelical salt bridges.

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We prepared two analogues of Zwit-1F to evaluate the extent to which β-peptide bundle stability could be improved by optimizing the length of the $\beta hOrn_{10}$–$\beta hAsp_{12}$ salt bridge: Zwit-EYYO and Zwit-EYYK (Figure 1B). Both contained βhTyr in place of βhPhe at positions 4 and 7, as previous work suggested this change would both increase stability and improve water solubility;[1c] one analogue contained βhOrn at position 10, while the other contained βhLys. We hypothesized that the additional methylene group in the βhLys side chain relative to βhOrn would facilitate formation of a shorter salt bridge with $\beta hAsp_{12}$ and increase bundle stability. Circular dichroism (CD) spectroscopy revealed that both Zwit-EYYO and Zwit-EYYK exhibited the concentration dependent increase in mean residue ellipticity (MRE) between 208 and 215 nm that characterizes β-peptide bundles, and which is characteristic of Zwit-1F assembly. In both cases the CD data could be fit to an ideal monomer–octamer equilibrium characterized by $\ln K_a$ values of 87.1 ± 0.5 (Zwit-EYYO) and 94.2 ± 0.3 (Zwit-EYYK); the corresponding value for Zwit-1F is 70.5 ± 1.9 (Figure 2A).[1e,7] The differences between the $\ln K_a$ values of the Zwit-EYYO and Zwit-EYYK bundles and that of the Zwit-1F bundle correspond to free energy changes ($\Delta\Delta G$) of −9.83 and −14.03 kcal mol^{-1}, respectively; this emphasizes the previously reported impact of the βhTyr substitution.[1c] The corresponding $\Delta\Delta G$ between the stabilities of the Zwit-EYYK and Zwit-EYYO bundles, −4.20 kcal mol^{-1}, is smaller but still significant. These differences in stability translate into real changes in the concentration dependence of bundle assembly: a 15 μM solution of Zwit-EYYK is 90% assembled into an octameric bundle, whereas the corresponding values for Zwit-EYYO and Zwit-1F are 77 and 0.5%, respectively.

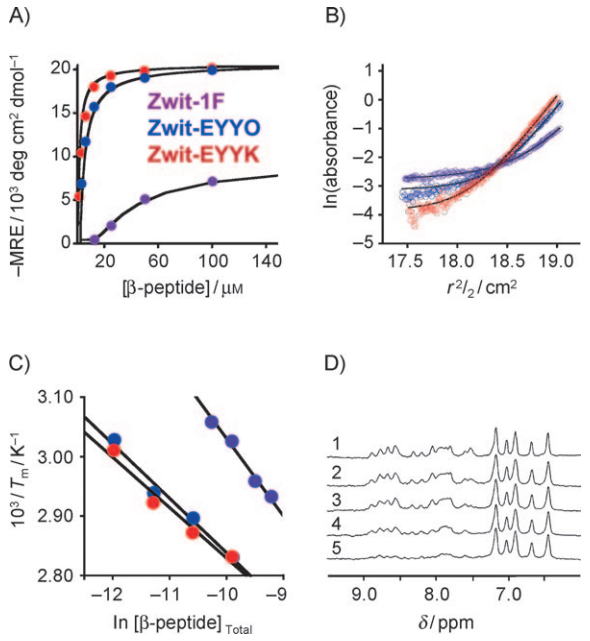

Figure 2. Biophysical characterization of β-peptide bundles formed from Zwit-EYYO and Zwit-EYYK. A) The value of the negative MRE at 205 nm for Zwit-1F (purple) and 208 nm of Zwit-EYYO (blue) and Zwit-EYYK (red) increases with respect to concentration; lines indicate fits to monomer–octamer equilibria. B) SE-AU data for solutions of Zwit-1F (purple), Zwit-EYYO (blue), and Zwit-EYYK (red) acquired at 60000 rpm, plotted as ln(absorbance) vs $r^2/2$ in order to highlight the differences in sedimentation. Circles represent data, and lines represent fits to monomer–octamer equilibria. C) van't Hoff plots showing the relationship between T_m and concentration for Zwit-1F (purple), Zwit-EYYO (blue), and Zwit-EYYK (red). D) NMR spectra of the amide N–H region of Zwit-EYYK at $t = 0$ (1), 15 min (2), 30 min (3), 2 h (4), and 6 h (5) after addition of D_2O to lyophilized β-peptide. The amide N–H resonances appear between 7.5 and 9.0 ppm, while the reference aromatic peaks appear between 5.9 and 7.25 ppm.

Additional evidence for the octameric stoichiometry and relative stability of the Zwit-EYYO and Zwit-EYYK bundles was sought from sedimentation equilibrium analytical ultracentrifugation (SE-AU). Zwit-EYYO and Zwit-EYYK sedimented as ideal species, with molecular weights of 12845 and 13389, respectively. These values correspond to oligomeric states of $n = 7.70$, and 7.96, thus confirming the octameric assembly suggested by the CD data. The equilibrium association constants of the octamers derived from the SE-AU data correspond to $\ln K_a$ values of 71.0 ± 0.9, 89.9 ± 0.7, and 94.5 ± 1.4 for Zwit-1F, Zwit-EYYO, and Zwit-EYYK (Figure 2B, and Figure S3 in the Supporting Information), respectively, which are in excellent agreement with values derived from CD spectra (70.5 ± 1.9, 87.1 ± 0.5, and 94.2 ± 0.3).[8] Taken together, these SE-AU and CD studies indicate that Zwit-EYYO and Zwit-EYYK β-peptide bundles are both more stable than Zwit-1F, and that Zwit-EYYK is more stable than Zwit-EYYO.[1e] The thermodynamic stabilities of Zwit-EYYO and Zwit-EYYK surpass that of the naturally occurring homooctamer hemerythrin ($\ln K_a = 84.0$).[9]

Two different metrics were applied to assess whether the relative thermodynamic stabilities of Zwit-EYYK, Zwit-EYYO, and Zwit-1F, as determined by CD and SE-AU, would be reflected in the relative cooperativity of unfolding. First, we examined the temperature-independent van't Hoff enthalpies (ΔH_{vH}) of the three bundles, determined from the concentration dependence of their T_m values. These values correspond to 147, 152, and 164 kcal mol^{-1} octamer for Zwit-1F, Zwit-EYYO, and Zwit-EYYK, respectively (Figure 2C). Thus, the Zwit-EYYK bundle unfolds with the greatest cooperativity, and the improvement relative to Zwit-1F is contributed largely by the introduction of βhLys in place of βhOrn, with a smaller contribution from the introduction of βhTyr in place of βhPhe. Next, we examined the width at half-maximum of the derivative of the temperature-dependent CD signal ($\delta MRE_{Minimum}/\delta T$); this value corresponds to 40, 24, and 22 °C for Zwit-1F, Zwit-EYYO, and Zwit-EYYK, respectively, at 50 μM. According to this metric, introduction of βhTyr has a greater effect on the cooperativity of unfolding than the introduction of βhLys.[10] Combining these two effects leads to values that are analogous to those of α-helix coiled-coil proteins such as GCN4 (20 °C)[11] and ROP (15 °C).[12] Thus, according to commonly applied metrics, even conservative substitutions at the bundle surface can significantly impact bundle stability and the cooperativity of unfolding.

To establish whether the increased thermodynamic stability of Zwit-EYYK was accompanied by an increase in kinetic stability, we analyzed the rate of amide H/D exchange by NMR spectroscopy (Figure 2D). The amide N–H resonances in the ^{1}H spectrum of Zwit-EYYK, under conditions in which the sample

is $\geq$95% octameric, span 1.5 ppm, a value comparable to that for Zwit-1F (1.4 ppm).[1e] Surprisingly, Zwit-EYYO was insoluble under these conditions and could not be analyzed. When a lyophilized sample of Zwit-EYYK was redissolved at 500 μM in D_2O, eight of eleven resolvable peaks required more than six and one-half hours to become indistinguishable from the baseline (Figure 2D). This corresponds to exchange rate constants of between 1.05×10^{-4} and 9.95×10^{-5} s^{-1}. The protection factors (P) for these amide protons, calculated as k_{rc}/k_{ex} (where k_{rc} is the exchange rate constant for a random-coil peptide and k_{ex} is the measured exchange rate constant), range from 2.3×10^4 to 6.4×10^5; the corresponding values for Zwit-1F are 6.0×10^3 to 2.9×10^4. Thus, the protection factors for the amide N–H resonances of the Zwit-EYYK bundle are, on average, four times greater than those for Zwit-1F; this is consistent with the increased thermodynamic stability of the former.

Finally, in order to evaluate whether we could attribute the increase in thermodynamic and kinetic stability of the Zwit-EYYK bundle to the presence of a shorter interhelical salt bridge, we solved the structure of Zwit-EYYK to 1.9 Å resolution using X-ray crystallography. As expected, the overall Zwit-EYYK fold is similar to those of other octameric β-peptide bundles (Figure 3A); the backbone carbon atoms of Zwit-EYYK

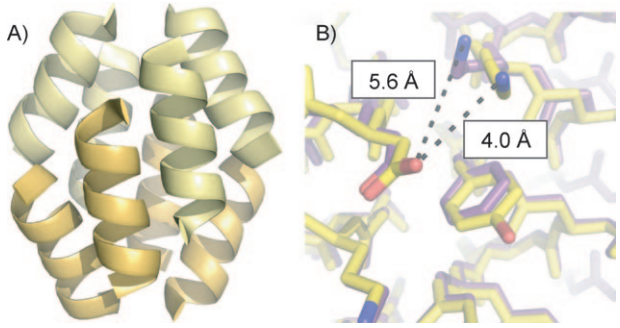

Figure 3. Structure of the Zwit-EYYK β-peptide bundle at 1.65 Å resolution. A) Ribbon diagram of the Zwit-EYYK octamer as determined by X-ray crystallography. B) Close-up of the parallel interhelical interface highlighting the salt bridge between $\beta hAsp_{12}$ on one helix and $\beta hLys_{10}$ on its parallel partner for Zwit-EYYK (yellow), and the equivalent $\beta hAsp_{12}$–$\beta hOrn_{10}$ salt bridge in Zwit-1F (purple).This salt bridge spans 5.6 Å in the Zwit-1F bundle; in the Zwit-EYYK bundle the length is 4.0 Å.

align with those of Zwit-1F with a root mean square deviation of 0.11 Å.[1a] More importantly, the Zwit-EYYK structure shows clear evidence of a salt bridge between βhLys at position 10 and βhAsp at position 12 of its parallel partner (Figure 3B). The heavy atom–heavy atom distance between $\beta hAsp_{12}$ and $\beta hLys_{10}$ spans 4.0 Å, well within the 5.0 Å limit for analogous interactions in proteins containing α-amino acids.[3]

There is considerable interest currently in the design of higher-order, nonproteinaceous assemblies possessing defined oligomeric states,[13] as materials with these properties have potential as nanomaterials[14] and catalysts.[15] Here we show that the stability of a β-peptide bundle can be tuned by controlling the length of a solvent-exposed surface salt-bridge interaction. Combined with previous work on the roles of internal packing residues,[1a,b,d,f] these results provide another critical step in the "bottom-up" assembly of β-peptide assemblies with defined sizes, reproducible structures, and sophisticated function.

Experimental Section

Details on esperiments, CD spectroscopy, analytical ultracentrifugation, and H/D exchange NMR as well as tables containing data collection and refinement statistics for crystallography may be found in the Supporting Information. CCDC 804687 (Zwit-EYYK bundle) contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

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