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Assessing the Structural Stability of Designed β-Hairpin Peptides in the Cytoplasm of Live Cells

Zihao Cheng and Robert E. Campbell*[a]

Ribosomally synthesized peptides and proteins capable of specific molecular recognition in the cytoplasm or nucleus of a living cell are valuable biological research tools that enable the modulation of biochemical pathways[1] or localization of cellular components.[2] The majority of research on such reagents has focused on recombinant antibody fragments (intrabodies) that retain the binding specificity of the intact antibody.[1] Two major limitations of intrabodies are their large size and their notoriously poor folding efficiency in the reducing environment of the cytoplasm.[3] One approach to circumventing these problems has been to look to alternative protein structures with more favorable properties and engineer them to have "unnatural" molecular-recognition functions.[4] The proteins best suited for conversion into molecular-recognition domains have been designated generic protein scaffolds: proteins in which the residues that confer structural stability are distinct from the residues appropriated as the "unnatural" binding site.[5] In an effort to add a new minimal motif to the growing repertoire of validated non-immunoglobulin binding proteins, we have undertaken the development and validation of a generic protein scaffold based on a single β-hairpin that can fold efficiently in the cytoplasm.

β-Hairpin peptides of the "tryptophan zipper" (trpzip) type[6] are promising candidates for a minimal generic protein scaffold. Notably, trpzip peptides are only 16 to 20 amino acids in length, monomeric, and highly soluble. The remarkable stability of these peptides arises from two interdigitating cross-strand Trp–Trp pairs on one face (the Trp face) of the hairpin (Figure 1).[6] We have hypothesized that the identity of residues with side chains directed towards the non-Trp face is much less important to the structural stability of trpzip β-hairpins. If so, we expect that it should be possible to select structured β-hairpins that are preorganized for target binding from phage-displayed[7] libraries in which multiple non-Trp face residues have been randomized. Related β-hairpin sequences that contain Trp–Lys cross-strand pairs on the Trp face have recently been shown to selectively bind ATP,[8] flavin,[9] and ssDNA.[10] Polypeptides composed of multiple linked β-hairpins could

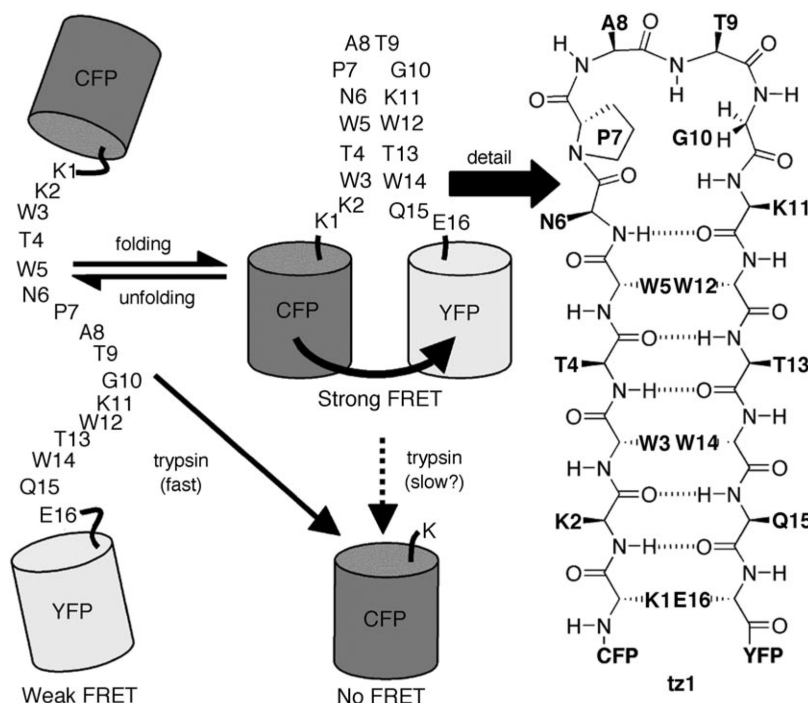

Figure 1. Schematic illustration of how the FRET efficiency and rate of trypsin digestion depend on the amount of β-hairpin structure in the peptide portion of the N_{term}-CFP-peptide-YFP-C_{term} proteins.

serve as the basis for an unnatural class of multivalent binding proteins that would be suitable for use in the reducing environment of the cytoplasm. As such, these proteins could serve as an intracellular complement to "avimers", the recently reported class of high-affinity binding modules based on multiple linked domains of ~35 amino acids each.[11] These multivalent proteins are capable of subnanomolar binding affinities against a variety of protein targets but are stabilized by disulfide bonds and are therefore unsuitable for use in the cytoplasm.

A generic protein scaffold must tolerate multiple amino acid substitutions without significant structural destabilization. To address the tolerance of trpzip β-hairpins for destabilizing mutations on the non-Trp face, we have developed a versatile method for rapidly evaluating the β-hairpin structure of recombinant peptides both in vitro and in vivo. Our strategy is based on the presumption that attaching a cyan fluorescent

[a] Z. Cheng, Prof. R. E. Campbell
Department of Chemistry, University of Alberta
Edmonton, Alberta T6G 2G2 (Canada)
Fax: (+1) 780-492-8231
E-mail: robert.e.campbell@ualberta.ca

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protein (CFP) and a yellow fluorescent protein (YFP) to the ends of an intervening peptide sequence would provide a convenient fluorescence resonance energy transfer (FRET)-based probe of the end-to-end distance (Figure 1). This approach is conceptually analogous to the “molecular beacon” approach for in vitro detection of specific nucleic acids.[12] We anticipated that the FRET efficiency would be higher for peptides that exist in a predominantly β-hairpin conformation than for unstructured peptides of the same length. A major advantage of using a genetically encoded FRET pair is that new peptide sequences could be rapidly created and screened through the combination of molecular biology and fluorescence imaging of bacterial colonies. A logical extension of such a method would be the high-throughput screening of large libraries of peptide sequences for β-hairpins that are highly structured in live cells.

To validate our approach, we have designed, expressed, and purified six proteins each consisting of an N-terminal CFP and a C-terminal YFP flanking a peptide sequence provided in Table 1. The parent peptide in protein **tz1** is identical in sequence to the previously reported trpzip peptide **HP5W4**, which has been optimized for fold stability and reported to be >96% folded at 298 K.[13] The peptide portions of **tz2** through **tz5** were designed to have varying degrees of destabilization due to the introduction of amino acids with low β-sheet propensity[14] or low occurrence at particular hairpin positions.[13] The peptide portion of **tz6** has all four Trp replaced with Gly and was expected to be unstructured. The intrinsically ratiometeric design of the **tz** series CFP/YFP fusion proteins allowed us to obtain accurate and reproducible measurements of the FRET efficiencies by measuring the intensity of the donor CFP fluorescence before and after treatment with trypsin. Under the conditions of the proteolysis reaction, the linker region is cleaved and FRET is abolished, while the CFP remains intact (see Figure S1 in the Supporting Information). As shown in Table 2, the experimental FRET efficiencies determined for the **tz** series of proteins range from 47% for **tz6** to 59% for **tz1**. With the exception of **tz3** (discussed below), all proteins gave a distinct and characteristic FRET efficiency that was correlated with the β-hairpin stability, as subsequently determined by orthogonal methods.

Table 1. Sequences of peptides flanked by CFP and YFP.

Protein	Peptide sequence[a]
tz1	KKWTWNPATGKWTWQE
tz2	KKWAWNPATGKWAWQE
tz3	KAWAWNPATGKWAWAE
tz4	KKWAWAPAAGAWAWQE
tz5	KAWAWAPATGAWAWAE
tz6	KKGTGNPATGKGTGQE

[a] Differences relative to **tz1** are underlined.

The FRET efficiency of 59% determined for **tz1** corresponds to a calculated interchromophore distance of 4.7 nm.[15,16] This distance is consistent with molecular models of the full-length protein with a folded β-hairpin and the assumption of random chromophore orientations. In support of this assumption, the **tz** series of proteins was designed with hydrophilic linkers of five residues in length connecting the inserted peptide to each of the flanking fluorescent proteins and with a CFP and YFP pair that does not dimerize.[17] The FRET efficiency of 47% determined for **tz6** corresponds to an interchromophore distance of 5.1 nm. It is important to emphasize that the experimental FRET efficiencies are ensemble averages for populations of linked CFP and YFP pairs with a distribution of interchromophore distances.[18] CFP and YFP pairs with increased flexibility of their linkers probably have much broader distributions of interchromophore distances, yet the increase in the mean distance is apparently slight. Fortunately, in the **tz** series proteins, these small changes in the mean distance are at approximately the Förster distance ($R_O = 5$ nm),[15] at which the corresponding change in FRET efficiency is maximal.[16]

Table 2. FRET efficiencies, rates of proteolysis, and exciton strengths for proteins **tz1** through **tz6**.

	FRET efficiency [%][a]	Relative rate of trypsinolysis per lysine[b]	Relative exciton strength from CD[c]
tz1	59.0±0.7%[d]	1.00±0.08[d]	1.0
tz2	55.5±1.0%	2.39±0.22	1.0
tz3	63.1±1.6%	2.48±0.15	0.80
tz4	51.4±1.7%	18.5±1.7	0.37
tz5	49.4±1.0%	31.8±2.4	0.04
tz6	47.2±1.4%	14.6±0.4	<0.01

[a] FRET efficiency (E) was determined from $E = 1 - F_{DA}/F_D$, where F_{DA} and F_D are the intensities of CFP before and after trypsinolysis, respectively. [b] Initial velocity of proteolysis divided by the number of lysine residues and normalized to **tz1**. [c] Baseline-corrected exciton strength in difference CD spectra relative to **tz1**−trypsinized **tz6**. [d] Errors for ≥3 averaged measurements are reported as ±standard deviation.

During the course of the FRET measurements, we noted dramatic differences in the rates of trypsin digestion. A detailed investigation of the kinetics of proteolysis revealed that the rate of digestion is inversely correlated with the FRET efficiency (Figure S1). The rates of proteolysis divided by the number of lysines in each peptide (1, 2, or 3) and normalized to **tz1** are shown in Table 2. Our interpretation of the observed trend is that highly folded peptides are poorer substrates for trypsin. This led us to conclude that both the FRET efficiency and rate of proteolysis are probing the structural stability of the β-hairpin peptide.

To further characterize the β-hairpin structure in the context of the **tz** proteins, we employed circular dichroism (CD) spectroscopy. The far-UV CD spectrum of **tz1** is dominated by the primarily β-sheet structures of CFP and YFP. However, trpzip peptides have a unique CD signature with strong bands at 215 and 229 nm due to exciton coupling of the indole side chains of Trp.[6] We have found that the exciton component is visible as a positive contribution at ~227 nm in the CD spectrum of **tz1** when compared to **tz6** (Figure S2). Subtraction of the CD spectrum of trypsinized **tz6** from the **tz1** spectrum clearly reveals the positive component of the Trp exciton (Figure 2). The negative component at 215 nm is poorly defined due to the

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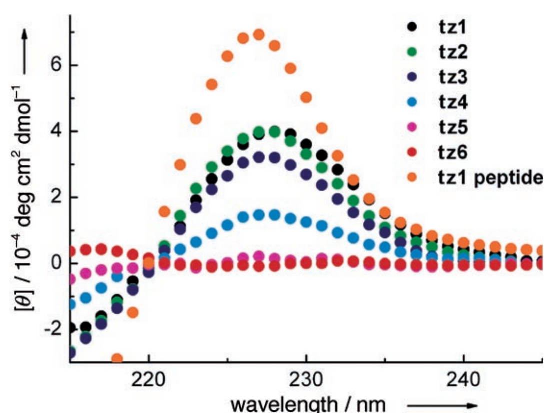

Figure 2. Difference CD spectra obtained by subtracting the CD spectrum of trypsinized **tz6** from each of **tz1** through **tz6**.

strong contribution of the β-sheet structure at this wavelength. The exciton strengths (Table 2) provide a reliable measure of the relative amount of β-hairpin structure in the context of the full-length proteins. To assess the effect of the linked CFP and YFP proteins on the stability of the intervening β-hairpin structure, we measured the CD spectra for **tz1**-peptide, the peptide portion of **tz1** without linked CFP and YFP. As shown in Figure 2, the magnitude of the **tz1** exciton is 61% of that measured for **tz1**-peptide; this demonstrates that the linked CFP and YFP cause only moderate destabilization of the intervening hairpin structure. This destabilizing effect should be constant across the **tz** series of proteins and irrelevant to measurements of relative stability.

To assess whether FRET between CFP and YFP can reliably report on the structural stability of an intervening β-hairpin peptide in vivo, each of the **tz** proteins was expressed in *E. coli* and, by using a custom-built digital imaging system, the YFP/CFP ratio in individual colonies was determined. Figure 3 shows a plot of the YFP/CFP emission ratio (excitation at 436 nm) versus YFP intensity (excitation at 500 nm) for six different populations of bacterial colonies, each of which is expressing a different member of the **tz** series of proteins. Each population exhibits a distinct and characteristic average ratio that is in qualitative agreement with the ranking of the in vitro FRET efficiencies provided in Table 2. It is interesting to note that the proteins with less structured peptides tended to accumulate at higher levels and that, within a given population, colonies with higher levels of protein tended to have higher ratios. To test whether protein aggregation was contributing to the observed differences in "in colony" ratio, we measured the ratio for proteins **tz1** and **tz6** in individual colonies that had accumulated intracellular protein concentrations of between 3 and 18 μM (Figure S3). Over this range of concentrations, colonies expressing either **tz1** or **tz6** exhibited a slight dependence of ratio on concentration; this suggested that intermolecular energy transfer was taking place. To investigate this possibility, we constructed and purified versions of the **tz1** protein in which either the YFP was replaced with CFP (CFP-**tz1**-CFP) or the CFP was replaced with YFP (YFP-**tz1**-YFP). We reasoned that if intermolecular associations were contributing to the observed YFP/CFP ratios, we should be able to observe FRET when CFP-**tz1**-CFP and YFP-**tz1**-YFP were mixed. Indeed, when we measured the emission spectra (excitation at 436 nm) for mixtures containing up to 15 μM of each protein, we did observe a concentration-dependent increase in the YFP emission peak (Figure S4). However, this apparent energy transfer could not be abolished with trypsin and so could not be attributed to interactions between the peptide sequences. Rather, the concentration-dependent changes in YFP/CFP ratio observed both in vitro and in vivo are consistent with the inner filtering of the CFP emission by the YFP chromophore. Inner filtering was not observed at the concentrations (less than 100 nM) used for our FRET and trypsinolysis measurements. Our data strongly support the conclusion that the "in colony" ratios are determined by the amount of β-hairpin structure rather than potentially confounding effects such as proteolysis or aggregation. A previous FRET-based approach for determining the stability of antibody VL domains found that the dramatic differences between the range of FRET intensities observed in vitro (~10%) and in vivo (~300%) could only be explained by differences in proteolytic susceptibility.[19]

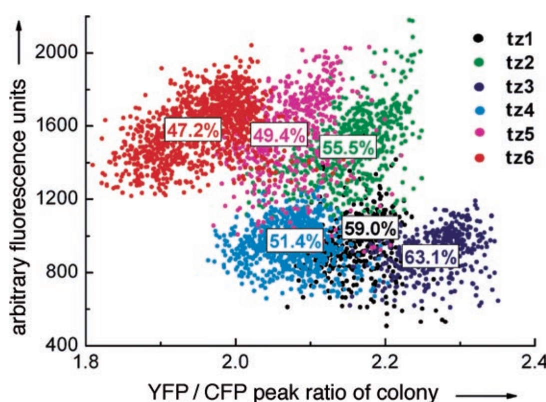

Figure 3. Scatter plot of "in colony" YFP/CFP fluorescence ratio (excitation at 436 nm) against "in colony" YFP fluorescence intensity (excitation at 500 nm) extracted from digital images of hundreds of bacterial colonies (1 data point per colony) grown on LB/agar (1 Petri dish per **tz** protein). All plates were prepared and imaged under identical conditions. Superimposed on each set of data points is the in vitro FRET efficiency from Table 2. The fluorescence intensity of YFP (directly excited at 500 nm) is proportional to the amount of protein present in the colony and depends on colony age and camera settings.

The results described above have led us to conclude that the relative β-hairpin structural stabilities of the peptide portions of **tz1** through **tz6** are the same both in vitro and in vivo and decrease in the order **tz1** > **tz2** > **tz3** > **tz4** > **tz5** > **tz6**. This conclusion is strongly supported by the CD data, is reinforced by the proteolysis data, and is consistent with the fluorescence data. The FRET and proteolysis data sets are almost certainly biased by sequence-dependent factors, such as fluorescent-protein orientation and trypsin substrate preference, which are irrelevant to fold stability yet difficult to account for. These factors could explain the anomalous ranking of **tz3** in the FRET data set and **tz6** in the proteolysis data set. While we must remain cognizant of these caveats, they do not undermine the fact that this approach is perhaps the most effective and versa-

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tile of the few reported methods for assessing the relative structural stability of a systematically modified series of peptides in live cells.[20] A corollary of these results is that genetic fusion of two relatively large proteins to the termini of a small peptide does not necessarily preclude proper folding of the peptide sequence.

Does the peptide portion of **tz1** qualify as a generic protein scaffold? Our CD results show that the presence of five destabilizing mutations, as in **tz4**, decreases the amount of folded structure by about half (Table 2). Since Ala has a less than average β-sheet propensity,[14] we are probably overestimating the destabilizing effects of random substitutions. With this provision in mind, we propose that the **tz1** peptide is an imperfect generic hairpin scaffold and that an average member of a library with random substitutions (except Pro) at positions 4, 6, 9, 11, and 13 (as in **tz4**) would be approximately 50% folded. These results are extremely encouraging and have inspired us to attempt development of a new β-hairpin variant with an improved tolerance for destabilizing mutations. To reach this goal, we are currently employing a combination of the in vivo FRET-based screening and the in vitro proteolysis assay to select highly stable β-hairpins from large libraries expressed in live cells.

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