Harnessing the Unique Structural Properties of Isolated α-Helices*

The α-helix is a ubiquitous secondary structural element that is almost exclusively observed in proteins when stabilized by tertiary or quaternary interactions. However, beginning with the unexpected observations of α-helix formation in the isolated C-peptide in ribonuclease A, there is growing evidence that a significant percentage (0.2%) of all proteins contain isolated stable single α-helical domains (SAH). These SAH domains provide unique structural features essential for normal protein function. A subset of SAH domains contain a characteristic ER/K motif, composed of a repeating sequence of 4 consecutive basic arginine or lysine (R/K) residues. The ER/K α-helix, also termed the ER/K linker, has been extensively characterized in the context of the myosin family of molecular motors and is emerging as a versatile structural element for protein and cellular engineering applications. Here, we review the structure and function of SAH domains, as well as the tools to identify them in natural proteins. We conclude with a discussion of recent studies that have successfully used the modular ER/K linker for engineering chimeric myosin proteins with altered mechanical properties, as well as synthetic polypeptides that can be used to monitor and systematically modulate protein interactions within cells.

Coiled-coil or Single α-Helical (SAH) Domain?

Until recently, SAH domains in natural proteins were predicted by secondary structure prediction algorithms to form a coiled-coil, in part due to the high concentration of charged and polar residues that are also the hallmark of the coiled-coil motif (1). Among the multiple folds in globular proteins that stabilize α-helices, the coiled-coil motif has been extensively characterized and in general is the most predictable form of tertiary protein structure (2). In the coiled-coil motif, two or more α-helices are individually stabilized by sequence-specific packing at consensus hydrophobic patches. Extensive studies have elicited general sequence and structure rules that govern coiled-coil interactions. Briefly, the amino acid sequence of each α-helix in a coiled-coil is divided into heptads (7 residues) that form nearly two complete α-helical turns and span 1.05 nm along the helical axis. Each amino acid in the heptad is described by its relative position, moving from the N to C terminus, using the nomenclature abcdedef. In canonical dimeric coiled-coils, the a and d positions radiate away from the core of the α-helix, 60° apart and offset by 0.45 nm along the helix length, and are typically occupied by aliphatic hydrophobic residues, whereas polar residues comprise the rest of the positions. As the heptad is repeated, it forms a continuous hydrophobic patch located along a single face of the α-helix compatible with a tight intermolecular interaction between polypeptides with the same or similar heptad pattern (2). However, often polar or charged residues occupy the a and d positions, leading to local destabilization of the coiled-coil motif (2−4). By extension, when most or all of the a and d sites in consecutive heptads are occupied by polar residues, individual α-helices cannot be mutually stabilized through hydrophobic packing. In this event, the polypeptide either will exist as a monomeric random coil or in some cases will form an SAH domain. The SAH domain is a stable, monomeric, extended α-helix that is encoded by its primary amino acid sequence and exists in polar solvent independent of tertiary interactions with other protein motifs.

Stable Synthetic Alanine-based α-Helices

The rules governing the helicity of isolated peptides have been extensively studied. We highlight select studies of particular interest, and refer to Ref. 5 for a more comprehensive review. Prior to work on synthetic peptides from the Baldwin laboratory, including one derived from ribonuclease A (6−8), peptides shorter than 20 amino acids were not expected to show measurable helix formation in aqueous solution based on the statistical Zimm-Bragg model, which focuses on the propagation of spontaneous helicity and does not account for long distance electrostatic interactions between side chains (9). Marqusee et al. (6) found that a synthetic peptide, 16 residues long, containing primarily alanine residues sparsely interspersed with a single Glu or Lys for solubilization, had high helical content (up to 80%) in aqueous solution as measured by CD. This study highlighted the inherent helix-forming potential of alanine in the absence of electrostatic interactions between side chains. In a separate study, Marqusee and Baldwin (7) synthesized peptides containing 16 residues with three pairs of a Glu and a Lys separated by either 3 (i, i + 3) or 4 (i, i + 4) alanines. These peptides were synthesized, in part, to characterize the influence of electrostatic interactions between Glu and Lys on α-helix formation. Both peptides were soluble and monomeric in aqueous solution and had detectable helical content as determined by CD. However, the (i, i + 3) (i.e. (EAAAK)3) spacing yielded significantly higher helicity when compared with (i, i + 3), presumably due to the preferred rotamer configurations of the Glu and Lys side chains. Interestingly,
the measured helicity was preserved at extremes of pH (from 2 to 12) and at high concentrations of NaCl, suggesting that the electrostatic interactions were primarily derived from salt bridges (H-bonded ionic interactions) rather than direct ionic interactions. The helix-stabilizing effects of E-K interactions on an alanine backbone were subsequently extended to D-K, E-R, and D-R pairs (10). A subsequent study with alanine-based peptides also defined the role of capping residues at the N and C termini in stabilizing isolated α-helices. In general, α-helix formation is aided by negatively charged residues at the N terminus and positively charged residues at the C terminus (11). This observation is consistent with an interaction of appropriately charged capping residues with the dipole moment of the protein α-helix, which is directed from its C to N terminus. Further, in the context of peptides, the charged residues at the ends can also undergo stabilizing electrostatic interactions with the free NH₂ and COOH groups (11). These studies of synthetic alanine-based peptides provided precedence and laid the groundwork for identifying and predicting the stability of isolated α-helices.

The (EAAAK)ₙ motif was subsequently incorporated into various synthetically engineered polypeptides. Arai et al. (12) evaluated the utility of (EAAAK)ₙ (n = 2–5) motifs as rigid spacing linkers between a pair of green fluorescent protein variants (EBFP and EGFP). The linkers did not interfere with EBFP and EGFP folding as evaluated from their fluorescence spectra. FRET between EBFP and EGFP decreased, whereas helicity increased with linker length, suggesting that stabilization of the α-helix increased the spacing between the fluorescent proteins fused to the ends of the linker. In a subsequent study, the conformations of the (EAAAK)ₙ linkers were evaluated by small angle x-ray scattering (SAXS). Short linkers (n ≤ 3) showed multimORIZATION, whereas longer linkers (n ≥ 4) remain monomeric even at high concentrations (>25 μM). The radius of gyration increased with linker length and is higher than flexible unstructured linkers (GGGGS)ₙ of the same length (13). Together, these studies support the use of the EAAAK linkers as extended spacers between polypeptides. Utilizing this structural property, the EAAAK linker has been employed to increase expression (14) and bioactivity (15) of fusion proteins. However, of concern for the use of tethering generic peptides with this particular linker, the EAAAK motif has been reported to have autocleavage properties at pH 6–7 (16). Regardless, this SAH domain both demonstrates the feasibility and the highlights potential utility of a modular genetic element to control intramolecular spacing between protein domains.

Stability of ER/K α-Helices

In a parallel vein, helix formation through E-K interactions, independent of alanine, was examined by Lyu et al. (17) with 18-amino acid peptides with either (E₄K₄)ₙ or (E₄K₄)ₙ repeats. Although the composition was exactly the same for both peptides, CD and ¹H NMR data showed that the E₄K₄ peptide has 65% helical content, whereas the E₄K₄ peptide is essentially a random coil. This is consistent with studies in alanine peptides discussed earlier, wherein (i, i + 4) interactions, as in E₄K₄, stabilize the helical conformation, whereas the (i, i + 2) spacing in E₄K₄ cannot facilitate these ionic interactions (Fig. 1, b and c).

These results were independent of peptide concentration in a range of 20–250 μM, indicating that oligomerization was not a factor in augmenting helicity. Additionally, pH and salt titrations showed that both direct ionic (E-K) and salt bridge interactions (H-bonded) likely contribute to the stability of E₄K₄ helices. This is in contrast to (EAAAK)ₙ, which appears to be primarily stabilized by salt bridge interactions (7). Free energy calculations yielded 0.5 kcal/mol stabilization for each (i, i + 4) interaction in E₄K₄, and the contribution from each interaction is proposed to stabilize the isolated α-helical conformation.

The mechanisms that underlie the stability of E₄K₄ helices are also evident in molecular dynamics (MD) simulations. Sivaramakrishnan et al. (18) performed a replica exchange MD simulation on a (E₂K₂)₂ peptide (Fig. 1c). Starting from either a random coil or a fully formed α-helix, both simulations converged on an α-helix. Thermal melting curves derived from the MD simulations matched previous experimentally measured helicities of this peptide (17). The simulations revealed dynamic and continuous interactions between side chains, with preferential i − 4 and i + 3 interactions centered on E(i) residues and i − 3 and i + 4 interactions centered on K(i) residues (Fig. 1c). These computations parallel experimental measurements by Olson et al. (19), who measured the influence of E-R side chain spacing on helicity. Monitoring the distances between the side chains in MD simulations, it was estimated that 45% of the time, direct ionic interactions occur between Glu and Lys, whereas solvent-separated salt bridges occur 37% of the time. This observation is consistent with the presence of both ionic and salt bridge interactions in E₄K₄ inferred from the pH dependence of helicity (17). Additionally, it was observed that the bulky side chains were able to partially shield backbone hydrogen bonds from the polar solvent. Thus, the stability of the α-helical core appears to arise from both shielded backbone hydrogen bonds and ER/K side chain interactions. In this regard, the ER/K motif has been likened to a tensegrity structure that is stabilized by the juxtaposition of “contractile” (backbone hydrogen bonds) and “tensile” (side chain) interactions (20). Overall, the observations in synthetic ER/K peptides have important structural implications in natural proteins as discussed in the next section.

Identification and Characterization of SAH Domains in Natural Proteins

Although the (EAAAK)ₙ motif was the first identified to form SAH domains, SAH domains identified in natural proteins to date more closely resemble the ER/K motif (i.e. (E₄(R/K)₄)ₙ). Smooth muscle caldesmon contains an ~150-residue stretch in its central region that is essentially repeating segments of KAAEEKKAAEEK (21). Wang et al. (22) extensively characterized a 285-residue fragment of caldesmon that encompasses this ER/K stretch. CD revealed ~55% helicity, which is consistent with a near continuous 150-amino acid α-helical region. The sedimentation profile of this polypeptide in ultracentrifugation experiments suggests a monomeric species over a wide range of protein concentrations (0.1–3.5 mg/ml). Rotary shadowed EMs revealed rods with an average length of 35 nm that is near the predicted length of an extended 150-residue α-helix. The rod thickness in EMs was significantly less than coiled-
coils from tropomyosin or the myosin rod segment, again suggesting a single extended α-helix. In addition to stabilizing the α-helix, Wang et al. (22) proposed that the regularly spaced salt bridges may protect this α-helix from proteolytic cleavage. Caldesmon interacts with both actin and myosin through distinct domains located at the ends of its SAH domain. Although muscle caldesmon serves to inhibit the actin-activated ATPase activity of myosins, it does not inhibit the actin-myosin interaction (23). Therefore, the extended single α-helix in caldesmon likely serves as a spacer to allosterically modify this interaction. However, not all SAH domains identified are expected to function solely as spacers, for example the human programmed cell death 5 (PDCD5) protein.

The N-terminal 26-amino acid fragment (GSADDEEARL-RQRLAEALQAKHGDPG) of PDCD5 was demonstrated by Liu et al. (24) to form a single α-helix by both CD and NMR spectroscopic measurements (Fig. 2c). Deletion of the N-terminal α-helix of PDCD5 significantly attenuated the apoptosis-promoting effects triggered by serum withdrawal. Based on the differential nuclear translocation of full-length and truncated PDCD5, Liu et al. (24) propose a role for this SAH domain in the nuclear targeting of PDCD5.

The first high resolution structure of an SAH domain was revealed in a crystal structure of the Bacillus stearothermophilus ribosomal protein L9 (25). This protein contained a rigid and fully extended 34-amino acid linker region between two compact globular domains (Fig. 2d). A subsequent study synthesized the corresponding peptide and found that the peptide was primarily monomeric at concentrations up to 1 mM in analytical ultracentrifugation studies and primarily (70%) α-helical as determined by CD spectroscopy, a much higher degree of helicity than was expected based on its primary sequence (PANLKALEAQKKEQRQAAEELANAKKLKEQLEK) (26). Further, they found that the helicity of the peptide could be disrupted by moderate salt concentrations (~500 mM NaCl), indicative of ionic side chain interactions contributing to the net stability of the α-helix. Although this peptide is far from the ideal E₄K₄ motif, it does have several predicted (i, i + 4) elec-
trostatic interactions. The single α-helix was postulated to act as a rigid spacer between two globular domains of the L9 ribosomal protein such that they are properly positioned to bind RNA. In addition to its structural role in the intact proteins, the authors also suggest that the folding of the α-helix may initiate and stabilize the folding of the two domains at its ends. Specifically, if the α-helix does initiate the folding process, then the folding of the two domains at the ends will be interdependent.

The most extensively characterized SAH domains to date are the ER/K α-helices found in myosin X and VI. A study by Knight et al. (1) investigated a putative coiled-coil motif in murine myosin X that was highly enriched in charged residues in the a and d positions of the heptad repeats. The authors identified this as a peptide that did not conform with typical coiled-coil motifs and coined the term SAH to describe an iso-

FIGURE 2. SAH domains have been observed in natural peptides or full-length proteins with multiple structural techniques. a, from Ref. 1, the putative coiled-coil region of myosin 10 forms an SAH domain. Top, CD of a peptide from murine myosin 10 has a canonical α-helical spectrum at 10 °C with a negative ellipticity at 222 nm (before and after heating to 80 °C) and a random coil spectra (with loss of ellipticity at 222 nm) at 80 °C. Bottom, rotary shadowed transmission electron microscopy of recombinant murine myosin 10 fragments. b, from Ref. 18, the predicted structure of the medial tail (MT) ER/K region (residues 916 –981) of myosin VI docked into its SAXS envelope. c, from Ref. 24, an alignment of 20 solution states, determined by NMR, for the programed cell death 5 protein residues 1–26 following 1H-15N HSQC. PDB, Protein Data Bank. d, from Ref. 25, the x-ray crystal structure of ribosomal protein L9. e, from Ref. 32, the number and percentage of total sequences of putative SAH domains identified from the primary amino acid sequences of the indicated organisms listed in Swiss-Prot and TrEMBL databanks utilizing the CSAH server. b–d, residues in SAH domains are colored in as in Fig. 1.
hydrophobic residues in the a and d sites. After a manual screen of putative coiled-coil domains that were homologous to the bona fide SAH domain in murine myosin X, they found that up to 4% of proteins classified as coiled-coils might indeed be SAH domains instead. This study provides an upper bound of 0.5% of all proteins in the human database that contain an SAH domain (30).

Suveges et al. (31) took a more systematic and analytical approach to identify charged SAH domains. They generated two conceptually different computational models, one a scoring function that identifies characteristic (i, i + 4) or (i, i + 3) salt bridges (SCAN4CSAH), and the second one a fast Fourier transform approach that finds like-charged residues ~1 heptad apart (FT_charge). These methods detected several putative SAH domains by cross-examining the Swiss-Prot database for regions larger than 40 amino acids (31). Both search databases are available at the charged SAH (CSAH) server. Applied to the UniProt database, they provide a conservative estimate of 0.2% of all proteins in an organism as containing CSAH structural motifs (Fig. 2e). Interestingly, Homo sapiens were identified to have the highest number of CSAH-containing sequences in their genome. Of almost 300,000 proteins identified in this search, only one had a published high resolution structure, suggestive of the difficulty of obtaining x-ray crystal structures of CSAH domains. The identified sequences had high overlap with servers, identifying unstructured regions, and coiled-coil motifs. The authors postulate that this motif may be rapidly evolving and that single charge mutations may lead to fine tuning of sequences between SAH, coiled-coil, and disordered segments (32). As an alternative to differentiating between SAH and coiled-coil domains, Sunitha et al. (3) have developed a new computational tool termed COILCHECK+. This web interface informs the user of the strength of a potential coiled-coil interaction at the interface region based on the relative density of both the charged residues and the characteristic repeat pattern of hydrophobic residues. Although these bioinformatics screens require additional experimental verification, it appears that SAH domains are ubiquitous.

Applications for Modular ER/K Motifs in Protein and Cellular Engineering

The ER/K motif has readily found applications in protein engineering. Three separate studies have used chimeric approaches to investigate the interplay of ER/K α-helix mechanical properties on myosin function (Fig. 3). Baboolal et al. (33) demonstrated that an ER/K α-helix can function as a lever arm in myosin V by extending a single myosin stroke nearly as efficiently as its native rigid calmodulin-stabilized lever arm (Fig. 3a). However, in contrast to the native lever, the ER/K α-helix was not able to coordinate the chemomechanical cycles of the two myosin heads within a single dimer, possibly due to its lower bending rigidity. Nagy and Rock (34) demonstrated that the ER/K α-helix from myosin X was necessary and sufficient to engineer preferential processive movement of both myosin X and myosin V on fascin-linked actin bundles. Engineering additional flexibility into the native myosin X abolishes selectivity for actin bundles, suggesting that the ER/K α-helix selectively biases the orientation of the myosin heads within a dimer (Fig. 3b). Hariadi et al. (35) found that ensembles of myosin VI but not myosin V undergo linear directed movement on a dense cellular actin meshwork. Stochastic simulations revealed that myosin lever arm rigidity alone was sufficient to dictate the skewness of movement patterns on cellular actin networks. Swapping a portion of the myosin V lever with the ER/K α-helix from myosin VI was sufficient to linearize myosin V trajectories and vice versa (Fig. 3c). Together, these studies bridge the mechanical properties of ER/K α-helices with specific functions in myosin.

In a radically different approach to protein engineering, the ER/K linker has also been used to tether and dictate the effective concentration of intramolecular protein-protein interactions (Fig. 4a). Sivaramakrishnan and Spudich (36) engineered a single polypeptide sensor containing an ER/K linker with an N-terminal calmodulin (CaM) and CFP variant attached by a flexible (GSG)2 linker and a C-terminal YFP variant attached with a (GSG)2 linker to a peptide known to dimerize with the Ca2+ bound CaM. The calcium-induced intramolecular interaction between CaM and its binding partner was detected by changes in FRET between CFP and YFP variants. In the absence of calcium, no significant FRET was detected with ER/K linkers of 73 amino acids or more (corresponding to > 10 nm in length along the α-helical backbone). A dramatic increase in FRET was observed upon the addition of calcium. Unexpectedly, calcium-stimulated FRET was independent of the concentration of sensor at levels below the bimolecular dissociation constant, indicating that an intramolecular interaction was bringing the FRET pair on either end of the ER/K linker into close proximity. Competitive inhibition of FRET with increasing concentrations of unlabeled CaM was used to quantify the effective concentration of the intramolecular interaction. Regardless of the bimolecular dissociation constant, the effective concentration decreased by about an order of magnitude for each additional 10 nm of ER/K linker. Essentially, changing ER/K linker length from 10 to 30 nm altered the effective concentration of the intramolecular interaction from 10 μM to 100 nM (Fig. 4c) (for reference, the effective concentration of a 60-residue unstructured linker is ~100 μM (37)). Further, similar results are observed when CaM and the CaM binding peptide are replaced by different interacting peptides (Fig. 4b). This trend is in contrast to the expected behavior of an ideal worm-like chain. One possible explanation is that a sensor in the closed state may introduce unfavorable conformations of the ER/K linker, which could increase the off-rate of the CaM-peptide interaction. However, the measured off-rate was found to be independent of ER/K linker length. The authors proposed a structural interpretation of these observations by suggesting that the ER/K linker undergoes rare stochastic breaks in helicity that create pivot points to facilitate interactions between the ends. The frequency of stochastic breaks scales linearly with α-helix length (a L). However, the breaks are unlikely to be spatially coordinated, resulting in far fewer conformations (a 1/L2) that bring the ends in close enough proximity to precipitate CaM-peptide interactions. Although this interpretation needs further testing, it is consistent with a previous SAXS study suggesting that certain α-helical peptides exhibit breaks along their length (38). The use of the ER/K linker to modulate protein-protein interactions was termed Systematic Protein Affinity Strength Mod-
ulation (SPASM). A key feature of the SPASM approach is its modularity, and the individual protein/peptide/fluorophore domains can be easily exchanged with basic molecular biology tools in most research laboratories.

The SPASM approach has since been used to engineer protein-protein interactions in live cells. Single polypeptide FRET sensors based on SPASM have been used to detect G protein-selective conformations of G protein-coupled receptors (39) (Fig. 4d). Modulating effective concentration with varying ER/K linker length has been used to modify the enzymatic activity of focal adhesion kinase (FAK) and dissect the differential effects of kinase activity and domain-domain interactions in controlling cellular migration (40) (Fig. 4e). The ER/K linker provides control over the stoichiometry of expression, with minimal FRET in the absence of an intramolecular interaction. This feature has been used to understand the pH dependence of FAK regulation, while correcting for the effects of pH on the fluorescence levels of GFP variants. Additionally, the ER/K linker has found utility in modulating domain-domain interactions in multidomain proteins, which has yielded a coarse configuration of both intramolecular and intermolecular interactions in protein kinase C (41) (Fig. 4d). Beyond these studies, we propose that the ER/K linker may be readily used in conjunction with other synthetic protein tools, including split proteins and inducible protein interactions, as a means to study individual interactions or to engineer cellular systems (Fig. 4, d–f).
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**Conclusion**

The SAH domain is a structural element found in numerous proteins, where it appears to operate as a semi-rigid structural element that tethers globular domains. Although it has only been extensively characterized in a few natural proteins, its sequence specifications and structural properties allow for its identification distinct from coiled-coils. The ER/K motif is a subset of SAH domains that have been extensively characterized through studies of the myosin family of molecular motors. ER/K α-helices, encoded by this motif, have already found applications for monitoring or systematically modulating protein interactions. The modularity of the ER/K motif makes it a versatile protein structural element in an expanding toolbox of technologies that are being deployed to dissect an integrated cellular signaling network. In addition to informing cellular function, sensors developed on an ER/K platform have direct applications for identifying new small molecule therapeutics.

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REFERENCES

1. Knight, P. J., Thirumurugan, K., Xu, Y., Wang, F., Kalverda, A. P., Stafford, W. F., 3rd, Sellers, J. R., and Peckham, M. (2005) The predicted coiled-coil domain of myosin 10 forms a novel elongated domain that lengthens the head. J. Biol. Chem. 280, 34702–34708

2. Woolfson, D. N. (2005) The design of coiled-coil structures and assemblies. Adv. Protein Chem. 70, 79–112

3. Sunittha, M. S., Nair, A. G., Charya, A., Jadhav, K., Mukhopadhyay, S., and Sowdhamini, R. (2012) Structural attributes for the recognition of weak and anomalous regions in coiled-coils of myosins and other motor proteins. BMC Res. Notes 5, 530

4. Kreuzer, S. M., and Elber, R. (2013) Coiled-coil response to mechanical force: global stability and local cracking. Biophys. J. 105, 951–961

5. Scholtz, J. M., and Baldwin, R. L. (1993) The mechanism of α-helix formation by peptides. Annu. Rev. Biophys. Biomol. Struct. 21, 95–118

6. Marqusee, S., Robbins, V. H., and Baldwin, R. L. (1989) Unusually stable helix formation in short alanine-based peptides. Proc. Natl. Acad. Sci. U.S.A. 86, 5286–5290

7. Marqusee, S., and Baldwin, R. L. (1987) Helix stabilization by Glu−...Lys+ salt bridges in short peptides of de novo design. Proc. Natl. Acad. Sci. U.S.A. 84, 8898–8902

8. Bierzyński, A., Kim, P. S., and Baldwin, R. L. (1982) A salt bridge stabilizes the helix formed by isolated C-peptide of RNase A. Proc. Natl. Acad. Sci. U.S.A. 79, 2470–2474

9. Zimm, B. H., and Bragg, J. K. (1959) Theory of the phase transition between helix and random coil in polypeptide chains. J. Chem. Phys. 31, 526–535

10. Huughyes-Despointes, B. M., Scholtz, J. M., and Baldwin, R. L. (1993) Helical peptides with three pairs of Asp-Arg and Glu-Arg residues in different orientations and spacings. Protein Sci. 2, 80–85

11. Doig, A. J., and Baldwin, R. L. (1995) N- and C-capping preferences for all 20 amino acids in α-helical peptides. Protein Sci. 4, 1325–1336

12. Arai, R., Ueda, H., Kitayama, A., Kamiya, N., and Nagamune, T. (2001) Design of the linkers which effectively separate domains of a bifunctional fusion protein. Protein Eng. 14, 529–532

13. Arai, R., Wriggers, W., Nishikawa, Y., Nagamune, T., and Fujisawa, T. (2004) Conformations of variably linked chimeric proteins evaluated by synchrotron X-ray small-angle scattering. Proteins 57, 829–838

14. Amet, N., Lee, H. F., and Shen, W. C. (2009) Improving the oral efficacy of recombinant fusion protein. Design of the linkers which effectively separate domains of a bifunctional fusion protein. Protein Eng. 14, 529–532

15. Arai, R., Wriggers, W., Nishikawa, Y., Nagamune, T., and Fujisawa, T. (2004) Conformations of variably linked chimeric proteins evaluated by synchrotron X-ray small-angle scattering. Proteins 57, 829–838

16. Amet, N., Lee, H. F., and Shen, W. C. (2009) Insertion of the designed helical linker led to increased expression of TF-based fusion proteins. Pharm. Res. 26, 523–528

17. Bai, Y., and Shen, W. C. (2006) Improving the oral efficacy of recombinant granulocyte colony-stimulating factor and transferring fusion protein by spacer optimization. Pharm. Res. 23, 2116–2121

18. Wu, Y. J., Fan, C. Y., and Li, Y. K. (2009) Protein purification involving a unique auto-clavage feature of a repeated EAAAK peptide. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 877, 4015–4021

19. Lyu, P. C., Gans, P. J., and Kallenbach, N. R. (1992) Energetic contribution of solvent-exposed ion pairs to α-helix structure. J. Mol. Biol. 223, 343–350

20. Sivaramakrishnan, S., Spink, B. J., Sim, A. Y., Doniach, S., and Spudich, J. A. (2005) Dynamic charge interactions create surprising rigidity in the ER/K α-helix: a versatile protein structural motif. Proteins 57, 575–589

21. Olson, C. A., Spek, E. J., Shi, Z., Vologodskii, A., and Kallenbach, N. R. (2001) Cooperative helix stabilization by complex Arg-Glu salt bridges in short peptides of protein activity by fluorescent protein domains. Proc. Natl. Acad. Sci. U.S.A. 100, 22193–22198

22. Nagy, S., and Rock, R. S. (2010) Structured post-IQ domain governs selectivity of myosin X for fascin-actin bundles. J. Biol. Chem. 285, 26608–26617

23. Haridri, R. F., Cale, M., and Sivaramakrishnan, S. (2014) Myosin lever arm directs collective motion on cellular actin network. Proc. Natl. Acad. Sci. U.S.A. 111, 4091–4096

24. Sivaramakrishnan, S., and Spudich, J. A. (2011) Systematic control of protein interaction using a modular ER/K α-helix linker. Proc. Natl. Acad. Sci. U.S.A. 108, 20467–20472

25. Robinson, C. R., and Sauer, R. T. (1998) Optimizing the stability of single-chain proteins by linker length and composition mutagenesis. Proc. Natl. Acad. Sci. U.S.A. 95, 5929–5934

26. Zagrovic, B., Jayachandran, G., Millett, I. S., Doniach, S., and Pande, V. S. (2005) How large is an α-helix? Studies of the radii of gyration of helical peptides by small-angle X-ray scattering and molecular dynamics. J. Mol. Biol. 353, 232–241

27. Malik, R. U., Ritt, M., DeVree, B. T., Neubir, R. G., Sivaramakrishnan, S., and Rock, R. T. (2013) Detection of G protein-selective G protein-coupled receptor (GPCR) conformations in live cells. J. Biol. Chem. 288, 17167–17178

28. Ritt, M., Guan, J. L., and Sivaramakrishnan, S. (2013) Visualizing and manipulating focal adhesion kinase regulation in live cells. J. Biol. Chem. 288, 8875–8886

29. Swanson, C. J., Ritt, M., Wang, W. M., Lang, M. J., Narayan, A., Tesmer, J. J., Westfall, M., and Sivaramakrishnan, S. (2014) Conserved modular domains team up to latch-open active PKCa. J. Biol. Chem. 289, 17812–17829

30. Kerppola, T. K. (2008) Bimolecular fluorescence complementation (BiFC) analysis as a probe of protein interactions in living cells. Annu. Rev. Biophys. 37, 465–487

31. Shekhawat, S. S., and Ghosh, I. (2011) Split-protein systems: beyond binary protein-protein interactions. Curr. Opin. Chem. Biol. 15, 789–797

32. Zhou, X. X., Chang, H. K., Lam, A. J., and Lin, M. Z. (2012) Optical control of protein activity by fluorescent protein domains. Science 338, 810–814

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