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Review

Use of a heterodimeric coiled-coil system for biosensor application and affinity purification

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Abstract

The two-stranded α-helical coiled-coil is now recognized as one of nature's favorite ways of creating a dimerization motif. Based on the knowledge of protein folding studies and de novo design model systems, a novel heterodimeric coiled-coil protein was synthesized. The heterodimeric E/K coiled-coil was constructed with two distinct peptides (E and K) that will spontaneously associate into a full helical coiled-coil structure in solution. Equilibrium CD, NMR and real time biosensor kinetics experiments showed that the E/K coiled-coil is both structurally (ΔG_{unfold}=11.3 kcal/mol) and kinetically ($K_d \approx 1$ n*M*) stable in solution at neutral pH. The engineered coiled-coil had been applied as a dimerization and capture domain for biosensor based applications and used in an expression/detection/affinity chromatography system. Specific test examples demonstrated the usefulness of the E/K heterodimeric system in these applications. The universality of coiled-coil as a dimerization motif in nature and our ability to design and synthesize these proteins suggest a wide variety of applications.

Keywords: Reviews; Coiled-coil system; Biosensors; Affinity purification; Proteins; Peptides

Contents

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1. Introduction

The two-stranded α-helical coiled-coil is a universal dimerization domain used by nature in a diverse group of proteins [1–4]. The existence of such a domain was first predicted by Crick in 1953 based on the X-ray diffraction pattern of α-keratin [5]. He suggested that a coiled-coil is a structural domain where two right-handed amphipathic α-helices wrap around each other to adopt a left-handed super coil. In order for an α-helix to participate in such a motif, several structural and sequence-related parameters are required. Some of these parameters include amphipathicity and placement of appropriate amino acids, mostly non-polar, into a 'knobs-into-holes' type packing at the interfacial area between the amphipathic α-helices [5].

The amino acid sequence of the first coiled-coil was identified initially in 1972 [6,7]. This protein, tropomyosin, was the smallest and simplest protein postulated to contain a coiled-coil structure at that time. Analysis of the primary sequence of tropomyosin revealed a seven-amino acid repeating motif (*a–b–c–d–e–f–g*) where the *a* and *d* positions were occupied by hydrophobic residues. This 3–4 or 4–3 hydrophobic repeat (NXXNXXXNXXN . . .) was shown to be continuous throughout the entire 284-residue polypeptide chain of tropomyosin [8]. A small de novo designed peptide model based on this repeat was first synthesized in 1981 [9] and employed to demonstrate experimentally the requirements of a 3–4 hydrophobic repeat in the formation and stability of a coiled-coil dimer.

Since 1972, coiled-coil sequences have been found in a number of fibrous proteins [10] and intermediate filaments [11], and it is now recognized that the coiled-coil is a common structural domain found in a large range of proteins that play pivotal roles. In 1988, Landscultz et al. [12] compared the primary sequence of a number of DNA binding proteins and recognized a homologous region containing a repeating pattern of leucine residues at every seventh position. They proposed that this domain formed an amphipathic α-helix aligning the leucine residues along one face such that the leucine side chains in adjacent helices interdigitated like teeth in a zipper thus allowing the proteins to dimerize. It was pointed out later that the leucine zipper sequence contained the 3–4 hydrophobic repeat characteristic of coiled-coils [13]. Subsequently, NMR [14] and eventually X-ray crystallographic studies confirmed the molecular interactions initially proposed for coiled-coils [15–18]. These structures confirmed some of the fundamental structural characteristics predicted by Crick some 40 years earlier.

The discovery of coiled-coil motifs in DNA binding proteins tremendously expanded the field of research for this protein structural element. At the same time, the simplicity and elegance of this motif make it an ideal platform for studying protein folding. More recently, it has become a template for rational protein design. We, along with other groups, have also attempted to engineer new coiled-coils for specific biomedical applications [19–31]. In this review, we will describe a de novo designed coiled-coil that is currently being used for biosensor and affinity chromatography purposes. The advantages and limitations of this heterodimeric system are highlighted. This article is divided into several sections. Section 2 details the structural characteristics of a coiled-coil based on the current state of knowledge. Section 3 contains the description of the

designed coiled-coil and its applications to biosensors and affinity chromatographic systems. Section 4 contains a brief description of other common affinity chromatographic systems for comparison. Finally, in section 5, the advantages of the coiled-coil system are described and summarized. The subject of coiled-coils has been reviewed extensively by our laboratory [3,19,32,33] and others [1,4,34,35].

2. The structural characteristics of coiled-coils

2.1. Hydrophobicity and side chain packing in the hydrophobic core

It is obvious from the conserved nature of the 3–4 hydrophobic repeat in native coiled-coils that hydrophobicity is a critical issue in the formation and stability of coiled-coils. The hydrophobic residues at position *a* and *d* of the repeating heptad align their side chains on the same face of the amphipathic helix forming a hydrophobic interface through which two adjacent helices can interact [15,32,33]. The interchain hydrophobic interactions provide the primary driving force for tertiary folding and account for the stability of coiled-coils.

The primary sequence of tropomyosin provided an early indication of the importance of leucine residues in stabilizing the coiled-coil structure [6,7]. In the 284-residue polypeptide chains of tropomyosin homodimers there are 71 residue pairs involved in hydrophobic interactions, 25 of which are L–L interactions, 23 of them are V, M, I, and Y interactions, and the rest are A–A interactions [9]. It was later shown that synthetic coiled-coil model systems consisting of only four or five repeating heptads (28–35 residues) with L at all *a* and *d* positions formed very stable coiled-coils that were considerably more stable than native tropomyosin [36]. More recently, Hodges and co-workers have examined the effect of polypeptide chain length on the stability and formation of coiled-coils when the hydrophobic core was maximally stabilized with I at position *a* and L at position *d* [37,38]. Peptides as short as three heptads (21 residues) employing L at positions *a* and *d* were shown to form stable two-stranded α-helical coiled-coils. It was also suggested that other hydrophobes may be destabilizing relative to the bulky aliphatic side chains of I and L. In addition, the occurrence of charged or polar residues in positions *a* and *d* of the hydrophobic core may be further destabilizing relative to one comprised entirely of leucines [36,39]. Indeed, the consistent presence of leucines in the *d* positions in the short coiled-coil motifs of transcription factors has corroborated its significant role in the hydrophobic core. This requirement for hydrophobicity is demonstrated in the dimerization tendencies of the c-Myc and Max leucine zippers [40]. The *d* positions of the c-Myc leucine zipper are predominantly occupied by leucines, whereas three of the four *a* positions are occupied by charged residues. As a result, one would expect homodimerization of the c-Myc leucine zipper to bring six charged side chains into the coiled-coil interface, and therefore predict the interaction to be unfavorable. Synthetic peptides corresponding to the homodimer of the c-Myc leucine zipper were found to be non-interacting α-helices in benign medium [40,41]. In contrast, synthetic peptides of the Max protein exhibited considerable formation of homodimeric coiled-coils under the same conditions [40]. An explanation for these observations can be drawn from the Max leucine zipper sequence. Apart from two pairs of N residues at two *a* positions and a pair of H residues at a *d* position, the remainder of the *a* and *d* positions are occupied by hydrophobic residues, thereby allowing for more stabilizing tertiary interactions than are possible with the c-Myc homodimer [40]. The presence of polar and charged residues acts to destabilize homodimeric coiled-coil formation and thus promotes the formation of the heterodimeric form. This strategy is particularly effective in short coiled-coils [40,42].

The sequence of tropomyosin demonstrates that both the *a* and *d* positions of the heptad repeat could be occupied by β-branched side chains [7]. On the other hand, coiled-coil (leucine zipper) sequences of DNA binding proteins show a conserved requirement for leucine residues in the *d* position and a distinct tendency for β-branched side chains at position *a* [43]. These observations proved to be puzzling. However, in 1991 the first high-resolution X-ray structure of a coiled-coil protein, the GCN4 leucine zipper, brought much insight into the apparent

preference for a particular type of hydrophobes at position *a* and *d* [15]. The three-dimensional structure clearly indicated that the *a* and *d* positions of the heptad were structurally distinct, with the side chains at these positions having different orientations relative to the dimer axis in parallel and antiparallel coiled-coils [44]. The hydrophobic side chains at position *a* point away from the hydrophobic interface, while those at position *d* point into the dimer interface. The apparent preference of β-branched residues at position *a* may contribute to the stabilization of the hydrophobic core by allowing for closer contacts with adjacent residues than would be possible with a linear aliphatic side chain [15]. Zhu et al. [37] showed that β-branched amino acids, I and V, are preferred at position *a* compared to position *d* in order to maximize coiled-coil stability.

The X-ray three-dimensional structure of GCN4 [15] also confirmed the knobs-into-holes model first proposed by Crick almost 40 years earlier [5]. The hydrophobic interface in parallel two-stranded coiled-coils is formed by two distinct layers which alternate to produce a closely packed hydrophobic core. One layer consists of the side chain of residue *a* which packs between residues *a* and *g* of the corresponding heptad on the opposite helix, and similarly, the alternate layer is composed of the *d* side chain packing between side chains of positions *d* and *e*. In this respect, the leucine side chains at position *d* do not interdigitate, as the leucine zipper proposed [11], but instead pack side to side, as do the side chains at position *a*. Furthermore, the close packing of alternate layers allows the side chain of the *a* residue (knob) to pack into a hole formed by side chains of *a*, *g* and *d* of the preceding and succeeding layer of the opposite helix. Side chains of position *d* pack into the holes formed by consecutive *d* and *e* residues and two *a* residues of the opposing helix. As a result of this close packing, the residues at positions *a* and *d* of the GNC4 leucine zipper sequence were almost totally buried within the dimer interface, with a portion of the buried surface area also coming from the *e* and *g* side chains.

The concept of close packing brings into focus the effects of alanine, relatively large hydrophobes, F, Y, and W, and polar and charged residues in the destabilization of the hydrophobic core [39]. Homodimers containing either small or large hydrophobes (in relation to L, V, and I) could produce packing defects at the interface, potentially destabilizing the coiled-coil structure. Thus, there may be a requirement for heterodimerization with complementary surfaces to achieve close packing and maintain stability. Similarly, polar side chains cannot pack into a solvent inaccessible hydrophobic environment without causing dramatic energetic consequences unless there is a complementary surface/environment to compensate for the disruption.

These results, taken together, establish hydrophobicity and close core packing to be the major driving forces in coiled-coil formation. Local destabilization of the hydrophobic core, created by variations of side chain hydrophobicity and packing effects at the *a* and *d* positions of the heptad, may suggest a mechanism for subtle control of coiled-coil stability that is important in carrying out diverse functions.

2.2. Electrostatic interactions in coiled-coils

Positions *e* and *g* of the heptad repeat flank the hydrophobic interface of the coiled-coil and may contribute to the hydrophobicity of the core by folding over the interface to interact with the hydrophobes through their side chain methylene groups, thereby shielding the core from water [46]. Sequence analysis of coiled-coil domains displayed a preponderance of oppositely charged residues at these positions, which has led to a suggestion that interhelical ionic interactions (*i* to $i'+5$ interhelical interactions) could exist to stabilize the coiled-coil [8,45,46]. Confirmation of the presence of interhelical salt bridges was subsequently found in the three-dimensional structures of the DNA-binding proteins GCN4 [15] and Fos/Jun [18]; and their contributions to stability have been demonstrated [47–52].

It must be noted, however, that the contributions of electrostatic interactions in a protein are variable and dependent on the local micro-environment [53–56]. Electrostatic interactions between largely solvent-exposed areas on surfaces are usually weak and contribute little to stability [57,58]. Conversely, buried ion pairs can be stabilizing [59]. Charged interactions may contribute to coiled-coil stability if the micro-environment surrounding the charged interaction is not entirely aqueous, such as that near the hydrophobic interface [46]. In this light, the

effect of ion pairs on stability is interdependent with the hydrophobicity of the hydrophobic core. The partially non-aqueous micro-environment of the core may allow for the formation of the interchain salt bridge and, in return, formation of the salt bridge may reduce the solvent accessibility of the core. Both effects increase the stability of the coiled-coil structure. Many studies have tried to quantitate the stability contributed by ion pairs to two-stranded α-helical coiled-coils [47–50,54–56,60]. However, a more interesting question may lie in the destabilizing effects that ion pairs can create. This concept is best illustrated by mutagenic studies on the Fos and Jun leucine zippers in which residues of the *e* and *g* positions were grafted onto the leucine zipper of GCN4 [61]. The *e* and *g* positions of the Fos leucine zipper contain mainly negatively charged residues, whereas the Jun leucine zipper harbors positive charges. Mutant GCN4-Fos and GCN4-Jun leucine zippers preferentially formed heterodimers (Fos/Jun) over either homodimer (Fos/Fos or Jun/Jun). The relative instability of the GCN4-Fos homodimer suggested that the repulsion created by four E side chains may have provided the primary driving force for the heterodimerization of the peptides.

In addition to interhelical ionic interactions (*i* to $i'+5$) in coiled-coils, X-ray crystal structures have also shown the presence of intrahelical electrostatic interactions (*i* to $i+3$ and *i* to $i+4$) which may compete for ion pairs between the *e* and *g* positions [18]. Solvent-exposed interactions often do not contribute significantly to overall heterodimer stability [62]. However, it was found that intrastrand ionic interactions which alter the stability of individual helices may also directly influence coiled-coil stability [63].

2.3. Oligomerizaton and chain orientation

Coiled-coil structures in nature can exist as dimeric, trimeric, tetrameric and larger oligomeric assemblies of α-helices with either parallel and/or antiparallel orientations of the peptide chains [44,53,64,65]. Recent evidence for a pentameric coiled-coil has also been obtained [66]. Despite this diversity, all coiled-coil proteins share a common sequence pattern, implying that structural features superimposed on the heptad repeats specify different oligomerization states and alternate topologies [1,4].

Many studies have been carried out in an attempt to characterize the structural requirements that determine the preferred states of oligomerization [35,67]. Harbury et al. [68] investigated the role of residues *a* and *d* of the heptad repeat on oligomerization states. Variations in the hydrophobic side chains at these positions generated two-, three- and four-stranded helices of the GCN4 leucine zipper. They concluded that coiled-coil oligomerization can be based in part on the distribution of β-branched residues at the *a* and *d* positions due to the constraints imposed by distinct local geometries at these positions. In addition, Monera et al. [65] showed by Ala substitution in the hydrophobic core of a model coiled-coil that close packing of hydrophobes to avoid cavity formation also can direct the oligomerization state. The effect of interhelical electrostatic interactions on specifying the heterodimer formation of Fos and Jun has long been recognized [69]. These interhelical electrostatic interactions can have a direct role in the formation of parallel and antiparallel coiled-coils [44,64]. Nautiyal et al. [70] succeeded in designing a heterotrimeric coiled-coil based on the requirements of interchain electrostatic interactions. However, the parameters for oligomeric and orientation topology are not clear cut. A coiled-coil designed to form a dimer was shown to be assembled as a trimer with the helices running up–up–down [71]. Similarly, a 19-residue peptide designed to self associate into a four-helix bundle formed both hexamers and tetramers [72]. Despite the inability to define the structural features specifying oligomerization and chain topology, a recurring conclusion is that hydrophobic side chain packing and electrostatic interactions work in synergy as a finely tuned control for defining the oligomerization states of coiled-coil assemblies.

Coiled-coil (leucine zipper) dimerization of transcription factors brings together two functional domains [73]. Correct alignment (parallel and in register) of their DNA-binding regions is imperative for function [16,74], whereupon another critical parameter in coiled-coil assembly is the determination of chain orientation and alignment. As previously described, positions *a* and *d* are structurally distinct and packing at the hydrophobic core of two parallel

helices is composed of alternating $a–a'$ and $d–d'$ layers [15]. In contrast, for a coiled-coil to pack in the antiparallel orientation [75], hydrophobic side chains of position *a* in one helix must pack against those in the *d* position of the adjacent helix, and vice versa, resulting in mixed pair $a–d'$ and $d–a'$ packing. These packing differences result in altered coiled-coil stability. In fact, synthetic models have demonstrated the increased stability of antiparallel coiled-coils over parallel coiled-coils which have the same hydrophobic interface [44,64]. However, based on the abundance of parallel coiled-coils in natural proteins, other factors such as electrostatic interactions may direct the orientation of helices [44].

The leucine zipper motifs of many b-zip proteins contain a conserved N residue at position *a*16. Based on the X-ray structure of GNC4, O'Shea et al. [15] proposed a role for the conserved residue in positioning the two helices in a parallel and unstaggered orientation. Packing of the polar N residue against nonpolar side chains in the hydrophobic interface may destabilize antiparallel or staggered arrangements and therefore specify for complementary polar groups. A similar strategy appears to be employed for the preferential heterodimerization of the Max and c-Myc leucine zippers [40,42], as will be discussed in the next section.

2.4. Specificity for homo- or heterodimerization

The coiled-coil formation of leucine zipper proteins is critical in mediating interactions of different dimerization partners, thereby not only defining the DNA specificity of the transcription factor but also the combination of regulatory molecules assembled on the gene regulatory element [73]. Having addressed the structural requirements involved in controlling coiled-coil formation and stabilization, we need to examine what governs the selectivity for specific homo or heterodimerization. Whereas hydrophobicity and side chain packing were the major contributors to coiled-coil formation and stability, it has become apparent that electrostatic interactions play the dominant role in determining specificity of helix association. Ionic attractions are not essential for coiled-coil formation but electrostatic interactions can control heterodimerization versus homodimerization and may also control parallel or antiparallel alignment in coiled-coils [40,44,49,70,76–79]. A model example of electrostatically controlled specificity is apparent in the Fos/Jun leucine zippers discussed previously.

Recently, evidence has been obtained to support the role of charged residues within the actual core of the hydrophobic interface in specifying for heterodimerization [40]. The c-Myc family of the b-HLH-zip transcription factors represent a group of regulatory proteins implicated in controlling genes whose functions are important in the machinery of cell growth and proliferation [80]. The primary sequences of the coiled-coil (leucine zipper) region in these proteins display a number of charged residues in the *a* position of the heptad repeat. Of particular interest is a conserved acidic side chain at an equivalent a position in one heptad of c-Myc, N-Myc, L-Myc, Mxi1 and Mad, which appears to align with a conserved H residue in the corresponding heptad of the Max leucine zipper [40]. Lavigne et al. have investigated the role of these ionizable residues in the specific recognition of heterodimerization in this sub-family of b-HLH-zip DNA-binding proteins [40,42]. Their results support the presence of specific electrostatic interactions in the hydrophobic core of the heterodimeric c-Myc and Max coiled-coil. In the folded heterodimeric protein, the ionizable groups are fully engaged in hydrogen bonding and salt bridges. This arrangement negated the destabilizing effect of bringing a charged group from a polar solvent environment into a non-polar protein core. It also has a positive energetic contribution since the salt bridges are shielded from the solvent. In addition, these ionic interactions within the protein core serve to specify correct dimerization partners. Selective heterodimerization, mediated by a specified coiled-coil macromolecular interaction, can define the ultimate function for a given protein completely, and thus play a significant role in cellular regulation.

3. A de novo designed heterodimeric coiled-coil

3.1. Design features

A helical wheel representation of the designed heterodimeric coiled-coil is presented in Fig. 1. This protein was designed for biosensor-based and affinity

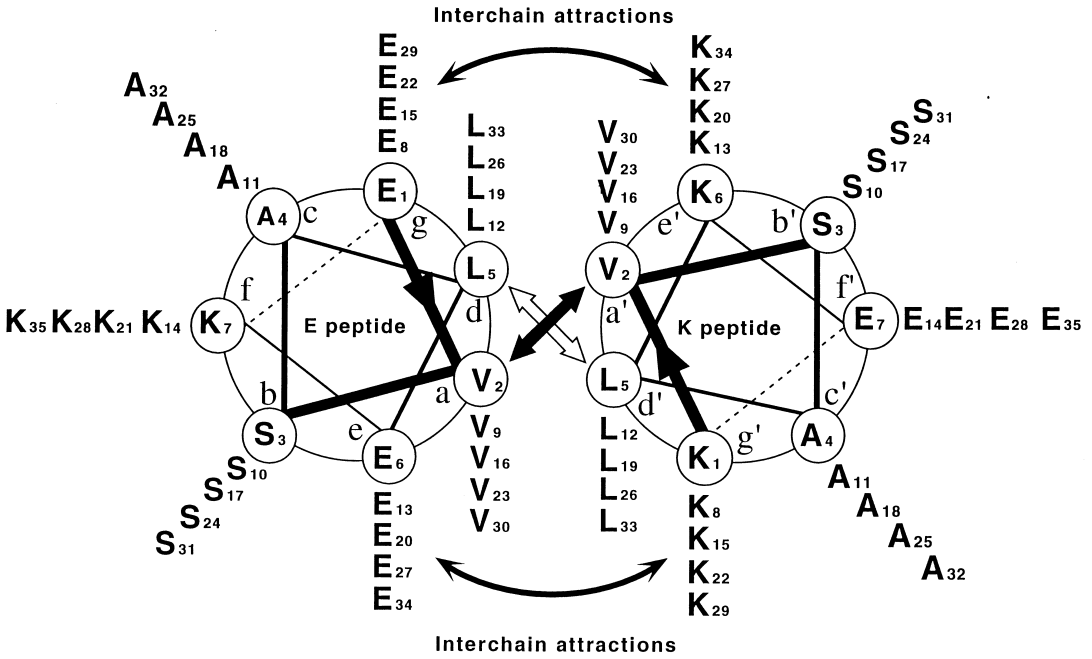

Fig. 1. Helical wheel representation of the heterodimeric parallel E/K coiled-coil. The polypeptide chain propagates into the page from the N- to the C-terminus. The K peptide is made up of five heptad (g'–a'-b'–c'–d'–e'–f') repeats of the sequence K–V–S–A–L–K–E and the E peptide consists of the same number of heptad repeats with the sequence E–V–S–A–L–E–K (g–a–b–c–d–e–f). The hydrophobic residues at positions a, a', d and d' interact and are mainly responsible for the formation and stability of the coiled-coil. Potential electrostatic attractions at positions g–e' or g'–e provide additional stability to the system by encouraging E and K salt bridge formation. Formation of these interchain salt bridges can also shield the non-polar core from solvent, furthering stabilizing the E/K coiled-coil. In addition to a role in protein stability, the E/K interaction also specifies heterodimerization rather than homodimerization in pH neutral buffer.

chromatographic applications after considering parameters that are important for coiled-coil formation, protein stability and chemical peptide synthesis. Some of these design features include: (1) selecting amino acids that are non-disruptive to helical structure; (2) placement of complementary non-polar residues that can form a stable hydrophobic core (positions a and d); (3) choosing appropriately charged amino acids for the e and g positions that will promote heterodimeric rather than homodimeric coiled-coil formation; (4) inclusion of charged amino acids at the f positions for solubility; (5) balancing the net charge on the individual α-helices by having opposite charged residues at positions f compared to positions e and g; (6) selecting an optimal protein sequence length for the designed applications which can still be made efficiently by synthetic means; (7) maintaining a balance of the stability of the hydrophobic core which drives homodimer formation with electrostatic repulsions which prevent homodimer formation in order to favor the heterodimeric form. Several designs were considered before selecting the E/K coiled-coil as our prototype.

The E/K coiled-coil is made of two distinct peptides, each with a unique repeating heptad sequence. The heptad sequence for the E peptide is E–V–S–A–L–E–K (position g–a–b–c–d–e–f) and the sequence for the K peptide is K–V–S–A–L–E–K. Each heptad is repeated five times in the respective peptide. When the two peptides associate, a dimeric protein of 70 amino acids in size with a molecular mass of 7682 is formed. The E and K peptides are amidated and acetylated at the C and N termini, respectively, to increase helix-forming propensity and to reduce the likelihood of proteolysis. Furthermore, this limits the available charged groups to those of the amino acid side chains only.

The hydrophobic core of the E/K coiled-coil is

made of V and L residues at the respective *a* and *d* positions. These amino acids are commonly found in natural and synthetic coiled-coil sequences, and coiled-coils with this hydrophobic core tend to favor a dimeric conformation [37,48,67,79]. Although a V and L hydrophobic core would be less stable than an all-leucine one or an I and L core, it is important to note that a weaker core will reduce the likelihood of forming unwanted higher order aggregates in solution [37,68,71] and prevent homodimerization in the presence of electrostatic repulsions. In line with choosing commonly observed non-polar residues for the hydrophobic interface, the same strategy was used in selecting residues for the *e* and *g* inter-helical salt bridge positions. In this case, glutamate and lysine are logical candidates since they are frequently observed to occupy these positions in natural coiled-coils [47,67]. Studies also showed that they can form energetically favorable $e-g'$ interhelical salt bridges in coiled-coils [48,81]. Furthermore, the methylene group of the K side chain can contribute to the overall stability of the protein by interacting with the partially exposed hydrophobic interface during salt bridge formation [15]. In addition, at neutral pH, the ionized side chains of E and K will discourage formation of homodimeric E/E or K/K coiled-coils as a result of like-charge repulsion, thus promoting the formation of heterodimeric E/K coiled-coil [48–52].

3.2. Physical characterization of the E/K heterodimeric coiled-coil

The E and K peptides preferred to form a heterodimeric coiled-coil under benign conditions and at moderate protein concentrations, as designed [82]. Neither peptide adopted significant structure on its own, suggesting that the designed E/K salt bridges are important for protein folding. As shown in Fig. 2, the CD spectra of a solution of E or K peptide is largely random in character. In contrast, a solution containing both peptides displays a CD profile that is characteristic of a helical structure, with spectral minima near 208 and 222 nm. The ratio of $[\Theta]_{222}$ vs. $[\Theta]_{208}$ of the heterodimer is 1.07, which is consistent with previous empirical observations that a coiled-coil structure will have a ratio greater than unity [36,79].

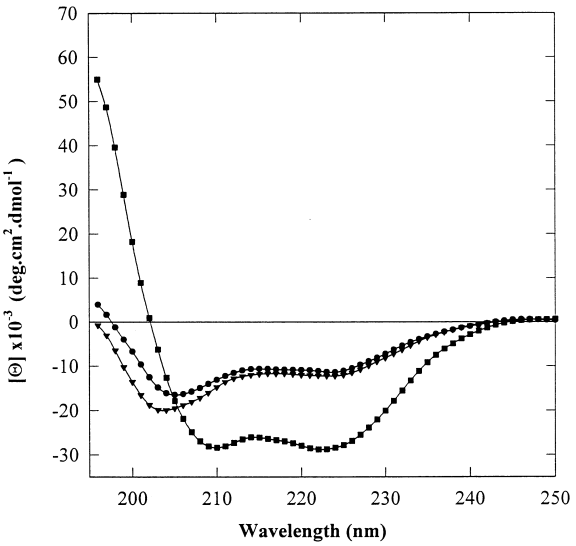

Fig. 2. CD spectra of the E peptide (▼), K peptide (●) and the E/K coiled-coil (■) recorded at 20°C. Protein concentrations were 20 μ*M* for each sample. Sample buffer was 50 m*M* phosphate, 100 m*M* KCl, pH 7.0. Each sample was scanned four times from 250 to 195 nm at a rate of 5 nm/min. Data were sampled at 0.1 nm intervals and averaged (figure adapted from Chao et al. [82] with permission).

In order to corroborate the data from CD experiments, the K peptide was also selectively labeled with [^{13}C]α-L at position 19 (central heptad of the hydrophobic core) and analyzed by NMR experiments. In the absence of the E peptide, the chemical shift value of the leucine α-proton was 4.31 ppm and the α-carbon was 52.81 ppm from a 2D ^{1}H–^{13}C heteronuclear multiple quantum filtered correlation (HMQC) experiment [83]. Both of these values are consistent with the leucine residue being in a random or rapidly interconverting conformation [84]. Titration of the E peptide into this solution changes the respective shift values to 4.17 ppm (^{1}H) and 53.41 ppm (^{13}C). When compared with the idealized averaged values of 3.98 and 55.6 ppm for the ^{1}H and ^{13}C atoms for a leucine residue in a fully helical environment [84], the NMR data are consistent with the CD observations that the K peptide will adopt a helical conformation when the E peptide is present.

The stability of the E/K coiled-coil was also studied by both chemical and temperature denaturation experiments as monitored by CD spectroscopy [82]. The E/K coiled-coil was resistant to heat

denaturation up to 85°C or urea denaturation. More than 50% of the observed helicity was retained at 8 *M* urea (Fig. 3). A combination of heat (85°C) and 5 *M* urea also was unable to denature the E/K coiled-coil (Fig. 3). Guanidine HCl (GdnHCl) was the only agent tested which could denature the E/K coiled-coil completely (Fig. 3). A $GdnHCl_{1/2}$ of 3.9 *M* was observed and a ΔG_{GdnHCl} of unfolding of 11.3 kcal/mol was estimated based on data fitting to a dimer ⇄ monomer model (E/K=E+K). The differential ability of urea and GdnHCl to denature the E/K coiled-coil is consistent with the energetic importance of glutamate and lysine salt bridges and that GdnHCl is more efficient in denaturing highly charged proteins [85].

The ability of the E/K coiled-coil to remain stable in a biological medium was also tested. E and K peptides and E/K coiled-coil were incubated in human serum over an extended period of time at 37°C. The integrity of the individual peptides and the coiled-coil was assessed by reversed-phase HPLC and mass spectrometry, and compared to controls incubated in a phosphate buffer. As shown in Fig. 4, the coiled-coil and the individual peptides are stable in this medium for 50 h with no significant degradation when compared to controls. Thus the highly charged random coil peptides or the folded coiled-coil are very resistant to proteolysis.

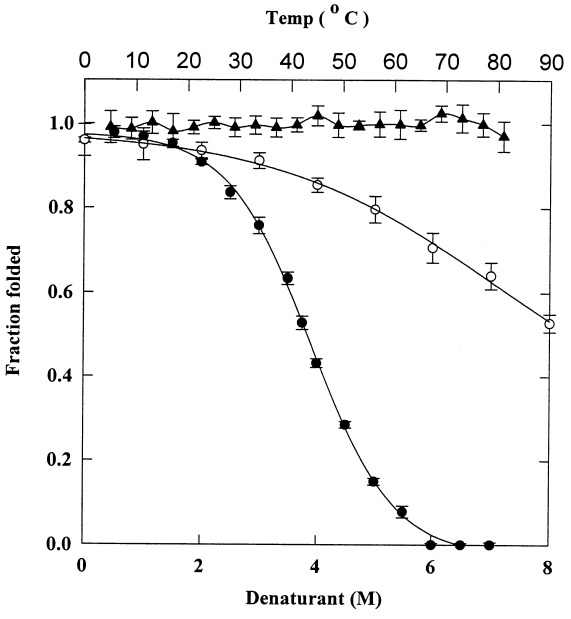

Fig. 3. Urea (○), GdnHCl (●) and heat denaturation (▲) profiles of the E/K coiled-coil [82]. The protein concentration was 20 μ*M* for each experiment. The buffer used for the chemical denaturation experiments was 50 m*M* phosphate, 100 m*M* KCl, pH 7.0. In the case of heat denaturation, the buffer also contained 5 *M* urea. The progress of the experiment was monitored at 222 nm by CD spectroscopy. Each data point is the average of eight samplings.

3.3. E/K coiled-coil formation and dissociation kinetics

The good stability of the E/K coiled-coil points to a small equilibrium dissociation constant in solution. Sedimentation equilibrium data suggested that the E/K coiled-coil remained predominantly a dimer at concentrations as low as 0.4 mg/ml [82]. Based on the well-known relationship of free energy of unfolding and equilibrium dissociation constant ($\Delta G = -RT \ln K$), the K_d was estimated to be 3.53×10^{-9} *M* (Table 1) [82]. This value is consistent with a value of 2.45×10^{-9} *M* obtained from a protein dilution experiments [82].

The association and dissociation behavior of the E/K coiled-coil was further studied on a Pharmacia BIAcore™ biosensor in order to obtain the individual association and dissociation rate constants [82]. The BIAcore™ biosensor monitors the changes in the solution refractive index near a gold metal surface as a result of molecular interaction near that surface in real time. Therefore, it allows the experimenter to quantitate the binding event over a finite period. In the E/K coiled-coil experiments, the K peptide was modified with an N-terminal 'C-G-G-G' linker. The terminal thiol group provides an anchoring site for the K peptide to a derivatized biosensor surface in order to capture the E peptide during a binding experiment. In a typical kinetic experiment, a solution of the E peptide was injected over a K peptide-coated surface at a fixed flow-rate over a defined period of time. This measures the binding of the E peptide to the immobilized K peptide. The slow release of the K peptide from the sensor surface is observed by injecting buffer over a defined period. These types of experiments were repeated with several different concentrations of the E peptide and with sensor surfaces that had been functionalized with different amounts of the K

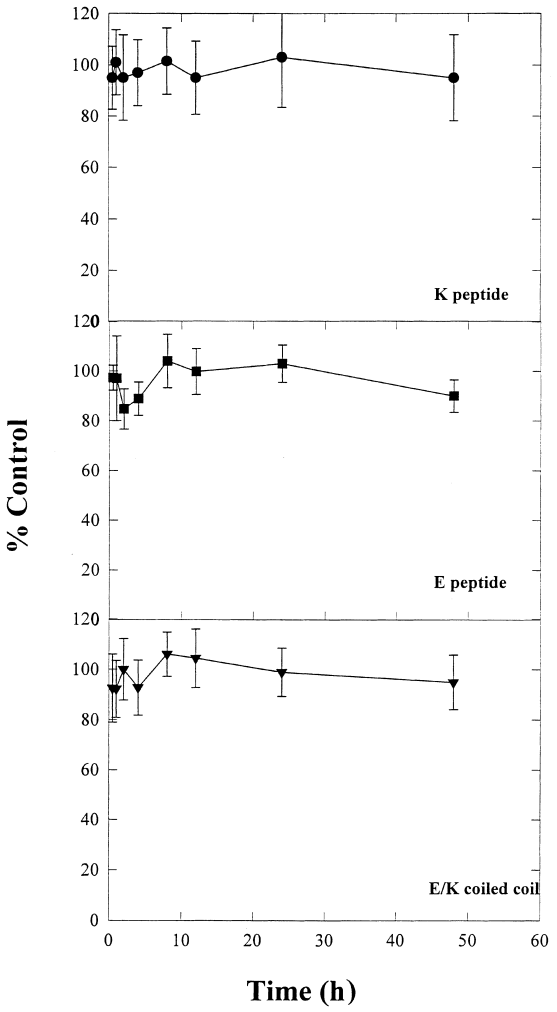

Fig. 4. Stability of the E/K coiled-coil (▼), E peptide (■) and K peptide (●) in human serum. Protein was dissolved in human serum to a concentration of 1 mg/ml or 0.26 m*M* and the samples were incubated for a total of 50 h at 37°C. Aliquots were removed at various time intervals and extracted with dichloromethane. The sample was further clarified by the addition of acetonitrile to a concentration of 50% (v/v) and centrifuged (13 000 *g*, 10 min) to remove precipitates. The peptides E, K and E/K coiled-coil, which remained in the supernatant, were stored at −20°C. The integrity of the E/K coiled-coil and peptides were analyzed by reserved phase HPLC and later by mass spectrometry. Data were compared to controls that were run in parallel. For the controls, proteins were incubated in 50 m*M* phosphate, 100 m*M* KCl at pH 7.0. Each data point is the average of three samplings. The standard deviation is also displayed.

Table 1
Summary of equilibrium dissociation and rate constants by CD-based and biosensor-based methods (adapted from Chao et al. [82] with permission)

K_d ($\times 10^{-9}$ *M*)		
GdnHCl denaturation[a]	Dilution study[b]	Biosensor[c]
3.53±0.48	2.45±0.71	0.50±0.13

[a]Dissociation constant estimated from the GdnHCl denaturation data. The K_d was calculated by the relationship $\Delta G = -RT \ln K$, where ΔG is the free energy of unfolding, R is the gas constant, T is the temperature (K) and K is the equilibrium dissociation constant.
[b]Estimated equilibrium dissociation constant from protein dilution experiments as monitored by CD spectroscopy.
[c]Calculated dissociation constant from the BIAcore™ biosensor study. The individual on and off rate constants were derived by fitting a monomer to dimer model to the sensor data. The presented K_d is the ratio of the k_{off} vs. k_{on} observed.

peptide in order to get a proper sampling of the data. The reversed experiment, that is an E peptide-modified sensor surface and a K peptide-containing mobile phase, was also done to rule out artifacts caused by immobilization. In some experiments, the mobile phase peptide, E or K, was conjugated with a biotin/avidin complex in order to enhance the response signal.

Representative plots of the biosensor experiment are shown in Fig. 5. Both the injection or association phase (0–300 s) and the buffer or dissociation phase (300–600 s) are presented. During the injection phase, the signal (response unit) rises quickly which is indicative of a fast interaction. A slow signal decay over the buffer phase suggests a slowly dissociating system. Analysis of the individual runs provided a set of k_{on} and k_{off} rate constants that were in good agreement with each other. The averaged k_{on} was $4.3\pm0.8\times10^{5}$ *M* and the averaged k_{off} was $2.21\pm0.2\times10^{-4}$ s^{-1}. This corresponded to a calculated equilibrium dissociation constant ($K_d = k_{off}/k_{on}$) of 0.5×10^{-9} *M*. Current analyses of these data by global analyses (simultaneous fitting of all data) and with more complex kinetic models suggest that the K_d value is probably slightly larger and therefore closer to those estimated by CD-based equilibrium methods (M. O'Connor-McCourt, personal communication).

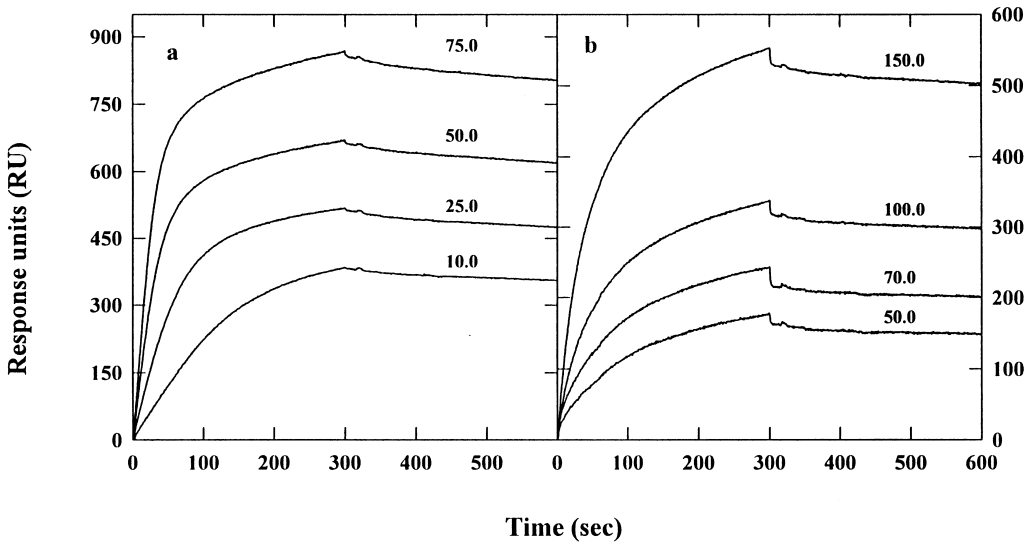

Fig. 5. Overlaid sensorgrams showing the binding of the streptavidin/biotinylated K peptide to an E peptide-coated sensor surface (panel a) and the binding of streptavidin/biotinylated E peptide to a K peptide-coated sensor surface (panel b) [82]. Each kinetic cycle involved a 300-s peptide injection phase (0–300 s), a 300-s dissociation phase (300–600 s) and a 60-s regeneration phase (not shown). An injection of 5 μl of 10 m*M* NaOH followed by buffer was used to dissociate the E/K coiled-coil and to regenerate the sensor surface. The concentrations of the biotinylated K peptide used on the E-coated sensor surface were 10, 25, 50, and 75 n*M*. For the K surface, the concentrations of the biotinylated E peptide used were 50, 70, 100, and 150 n*M*. Experiments were performed at 20°C, and sensor signals were recorded in real time with a sampling interval of 0.5 s. The data are presented after subtracting the baseline and repositioning the time of injection to the origin. Each panel represents the result from one set of experiments performed on the same sensor surface. All experiments were preformed on a Pharmacia BIAcore™ instrument. The CM5 sensor chip was used and peptide was immobilized on the surface via a terminal cysteine according to the manufacturer's thiol immobilization procedure (diagram modified from Chao et al. [82] with permission).

3.4. The use of E/K coiled-coil for biosensor applications.

The inherent stability of the E/K coiled-coil, the fast association kinetics and the relatively slow dissociation kinetics of the polypeptide chains make this protein ideal for biosensor applications that require a stable dimerization and capture domain. In this scenario (Fig. 6), one strand of the heterodimeric coiled-coil (e.g., K peptide) is first covalently attached to the biosensor surface. This surface is then charged with the E peptide that has been modified with a ligand for which its interaction with its receptor is to be studied. A significant advantage of the use of coiled-coils is that a single chemistry is used for attachment of one of the coiled-coil peptides to the surface of the biosensor. The particular chemistry for attachment of the other coiled-coil peptide to a variety of different molecules can be developed in solution rather than with the biosensor surface, which lacks the chemical flexibility.

In order for this application to be successful, the conjugated coiled-coil peptide in the mobile phase must be accessible to the anchored peptide. A test was performed on the Pharmacia BIAcore™ biosensor to address this issue. In this experiment, the E peptide was first immobilized on the sensor surface via a C-terminal C residue. The surface was then challenged with a solution containing a monoclonal antibody that was covalently linked to K peptides (K-mAb). The peptide to antibody conjugation ratio was estimated to be 3:1. Fig. 7 displays a representative diagram of one such experiment. The results of five K-mAb injections with different protein concentrations are shown. Qualitatively, the association phase (0–300 s) and the dissociation phase (300–600 s) kinetics are very similar to those observed previously for the E and K peptides alone. A fast on

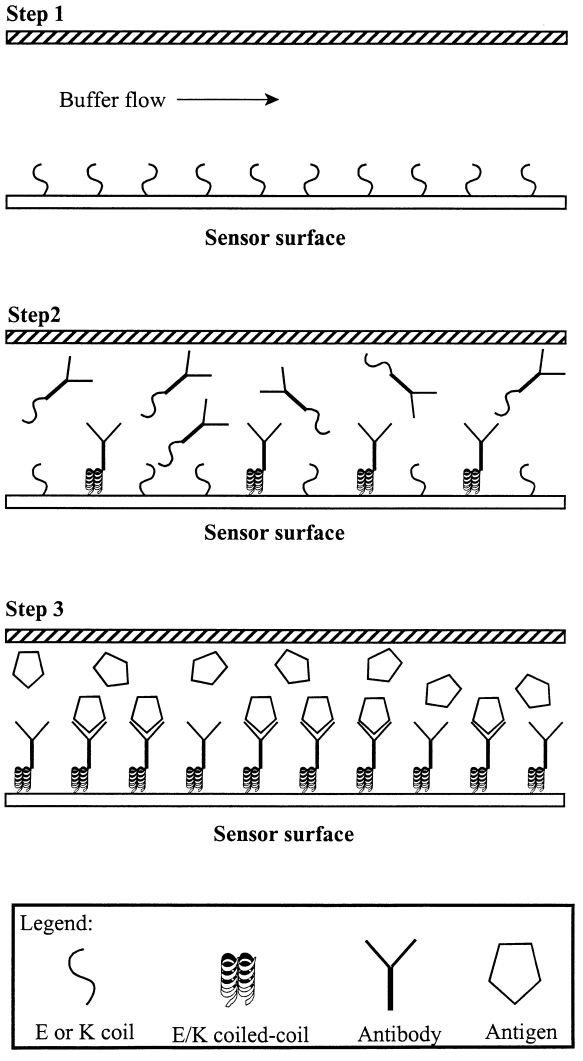

Fig. 6. Utility of the E/K heterodimerization domain for biosensor experiments. The E/K coiled-coil is used as a capture and dimerization domain. In this application, one of the two peptides (step 1) is first immobilized on the sensor surface. The second peptide is conjugated to an antibody, for instance, and injected over the sensor surface (step 2). Formation of the heterodimeric coiled-coil generates an antibody surface for biosensing (step 3). In theory, the sensor surface can be used repeatedly as long as it is charged with a sufficient amount of the coiled-coil peptide conjugate. A test of this application is currently being done on the Pharmacia BIAcore™ Biosensor.

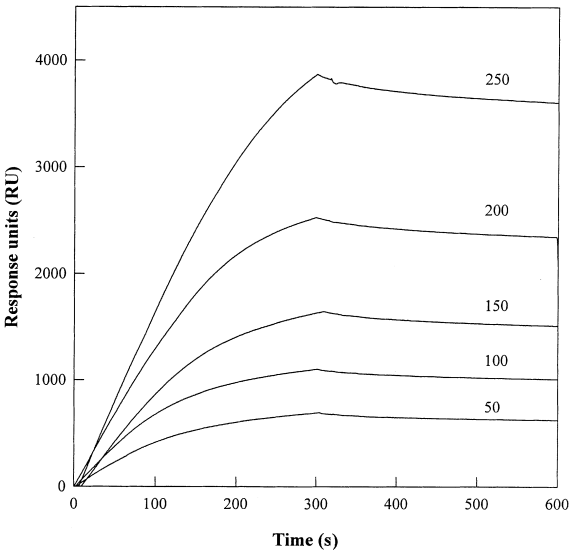

Fig. 7. BIAcore™ biosensor experiments demonstrating the utility of the E/K coiled-coil as a capture and dimerization domain on a biosensor surface. A CM5 chip was coated with E peptide via a terminal cysteine according to the manufacturer's instructions. The coated chip is then charged with a K peptide monoclonal antibody conjugate (K-mAb). Representative sensorgrams showing the charging of a E-coated surface with five different concentrations (50, 100, 150, 200 and 250 n*M*) of K-mAb are shown in overlay format. Each run consisted of an injection or charging phase (0–300 s) and a buffer phase (300–600 s). It is during the buffer phase where the desired interaction (e.g., antibody and antigen) would be run. The buffer phase can be extended significantly to accommodate most kinetic experiments. For this set of experiments, the surface was regenerated with a pulse (5 μl) of 10 m*M* NaOH to remove the K-mAb. Surface regeneration can be performed eight to 10 times without significantly affecting the ability of the E-coated sensor surface to be recharged.

(association) and a slow off (dissociation) kinetics regime consistent with the behavior of the E/K coiled-coil was observed.

The interaction of the anchored E peptide and the K-mAb was specific. Injecting a free K peptide solution prior to the delivery of an K-mAb solution over the E-coated sensor surface prevented the binding of K-mAb conjugate. A similar result was observed when free E peptide (equimolar concentration) was added to the K-mAb solution prior to injection. Antibody that had not been conjugated with the K peptide did not interact with the E peptide sensor surface. Addition of up to 10 mg/ml bovine

serum albumin (BSA) in the K-mAb solution had no effect on the anchored E peptide and K-mAb interaction. These data suggest that despite the presence of a conjugated molecule (i.e., mAb) that is over 35 times larger in its mass, or placement in a complex medium (i.e. BSA), the ability of the E and K peptides to associate is not significantly impaired.

It should be noted that current analysis of the aforementioned data does reveal a more complex E/K kinetic picture. A simple one-on-one model can no longer satisfy the observed data. In fact, the result is more consistent with models that describe multiple-site interactions. This perhaps should not be surprising since the injected mAb is conjugated with more than one copy of the K peptide. It should be mentioned that even though the underlying E/K formation kinetics has become more complex, it does not significantly affect one's ability to use the E/K coiled-coil for biosensor-based studies since the coiled-coil dissociation phase is sufficiently slow and occurs at a constant rate. Therefore, it is possible to correct for the background signal of the E/K coiled-coil mathematically.

3.5. A heterodimeric coiled-coil affinity matrix protein tag system

The versatility of the E/K heterodimeric coiled-coil as a dimerization and capture domain led us to explore the possibility of using this protein as an affinity purification tag (Fig. 8) [86]. In this application, a recombinant bacterial expression vector pRLDE was engineered to contain a copy of the E peptide at the C-terminus of the newly expressed peptides or proteins [86]. The plasmid contained an IPTG inducible promoter for selective recombinant protein expression, a multiple cloning site for gene insertion, an ampicillin resistance gene for clone selection and a signal sequence to direct the newly made protein to the periplasmic space to simplify purification. The complementary HPLC affinity matrix was prepared by the conjugation of the K peptide to an insoluble silica (Fig. 9). The entire system would allow the researcher to express an E peptide fusion protein and to purify the protein from crude cell lysate in one step by an on-line HPLC K peptide affinity column. Detection of the fusion products can be carried out at any stage in the process by detecting the fusion tag using a free labeled coiled-coil strand in an immunoblot-type or even biosensor-based experiment. The E peptide can be removed easily by a conventional cyanogen bromide cleavage if necessary.

A test of this concept has been demonstrated and reported [86]. In this specific example, a gene encoding the 17-amino acid pilus epithelial cell-binding domain of the *Pseudomonas aeruginosa* strain K pilin (PAK-pilin) with a methionine flanking the C- and N-termini was inserted upstream of the E peptide-coding region of pRLDE. Positive clones were selected and cultured as single colony isolates. Recombinant protein synthesis was induced by the addition of IPTG to cells grown to an A_{550} of 0.5–0.6 in LB medium with carbenicillin as the selecting antibiotic for 3 h at 25°C. Cells were then harvested by centrifugation and the fusion protein was released from the periplasmic space by osmotic shocks. The supernatant which contained the E fusion protein was clarified by passage through a 0.22-μm filter and stored in the cold until ready for the K affinity column [86].

Several affinity matrix supports were considered initially for the K affinity column. The selection was narrowed down to an aminopropyl control pore glass resin (CPG, 125 μm particle size, 500 Å pore size; Sigma Chemical Company, St. Louis, MO, USA). This glass bead was chosen because of the desire to generate a matrix that is compatible with an HPLC system. For the preparation of the K affinity matrix, CPG was washed in dimethylformide and dichloromethane. The glass beads were then neutralized in a solution of 5% diisopropylethylamine–dichloromethane (Fig. 9). The aminopropyl functional group on the glass bead was bromoacetylated by adding to the glass beads a solution of bromoacetic acid dissolved in dicyclohexylcarbodimide/dichloromethane and dimethylformamide. The functionalized glass beads were washed with organic solvents and coupling was verified by a qualitative ninhydrin test. Free silanol groups on the resin beads were capped by chlorotrimethylsilane in pyridine and dichloromethane. Capped resin was rinsed thoroughly to conjugate the K peptide.

The conjugation of the K peptide to the functionalized glass beads was done at pH 8.0 and at room

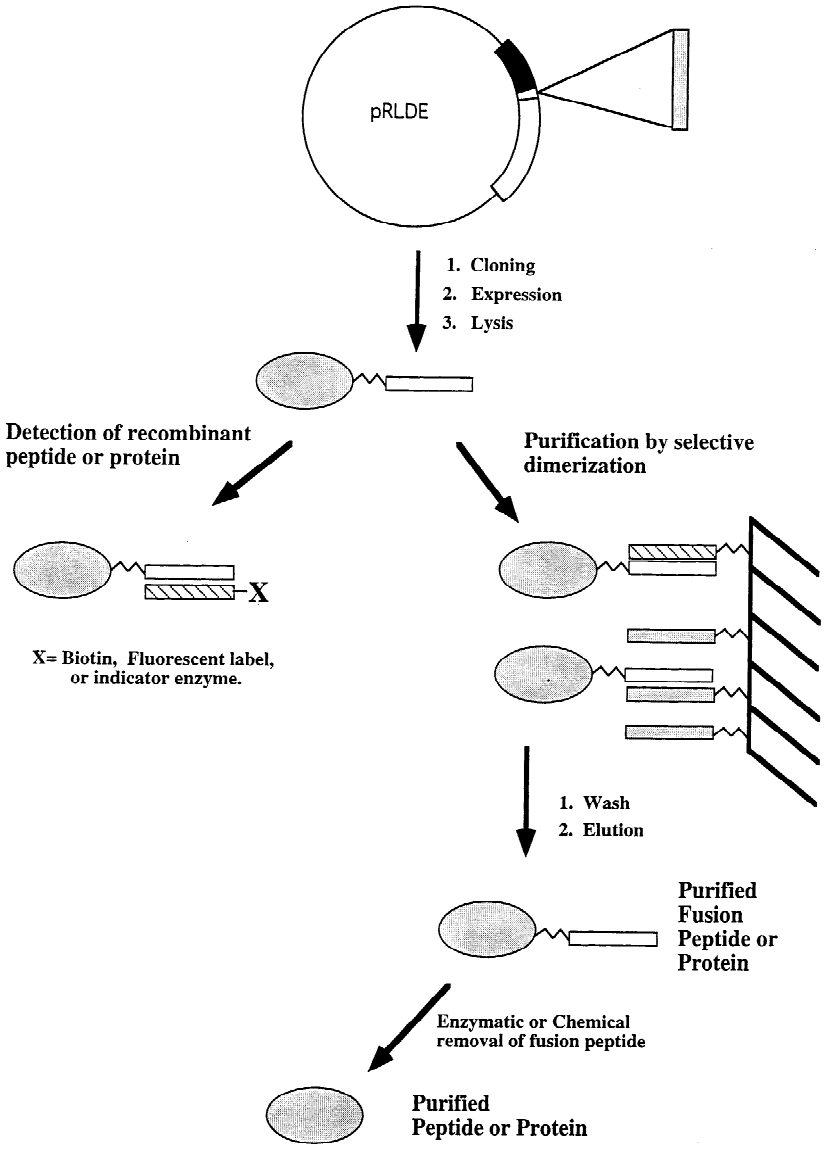

Fig. 8. The use of the E/K coiled-coil in an expression and purification system for recombinant proteins [86]. The system consists of a expression vector pRLDE and a K-peptide affinity column. The expression vector pRLDE codes for one copy of the E peptide with a signal peptide that directs the fusion product to the periplasmic space of the bacteria. The fusion protein can be released from the bacteria by osmotic shock and the lysate can be loaded onto the K-affinity column directly after clarification by centrifugation or filtration. The loaded column is then washed with appropriate buffer and the desired product can be eluted. If necessary, chemical or enzymatic cleavage can be carried out to removed the tag (diagram modified from Tripet et al. [86] with permission).

temperature. A solution containing 100 mg (24 μmol) of the K peptide was added to 1 g of the activated glass and stirred for 30 min. The K peptide was synthetically made and was modified with a C-terminal G–G–G–norleucine–cysteine linker. After the reaction was completed, β-mercaptoethanol

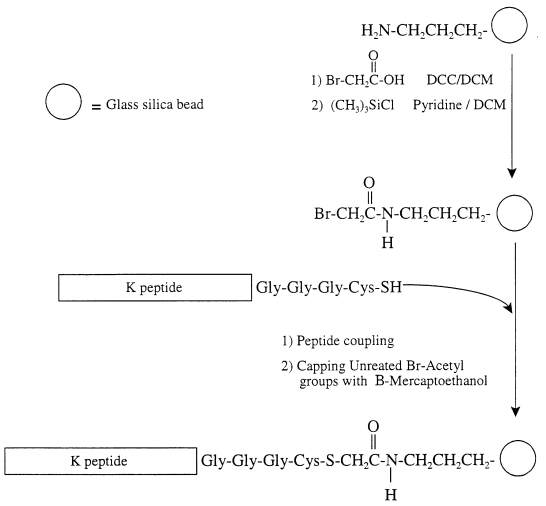

Fig. 9. Schematic representation of the steps involved in the formation of the K peptide affinity matrix. The glass beads are acetylated with bromoacetic acid and the free silanol groups are capped with trimethylchlorosilane. E peptide modified with a C-terminal cysteine is then added to the matrix slurry to be coupled to the resin. Residual bromoacetyl groups are quenched with the addition of β-mercaptoethanol. The affinity matrix is washed and equilibrated in standard phosphate buffer. DCC and DCM denote dicyclohexylcarbodiimide and dichloromethane, respectively (diagram adapted from Tripet et al. [86] with permission).

was added to quench the remaining bromoacetyl groups. The K affinity resin was washed in water, ethanol, and acetone, and then air dried. A substitution ratio of 12.5 μmol of peptide to 1 g of resin was obtained.

The dried resin (1 g) was packed in a stainless steel HPLC column (200×4.6 mm I.D.) and washed at a flow-rate of 0.5 ml/min with water, 5.0 *M* GdnHCl in 50 m*M* KH_2PO_4, pH 7.0, 50% aqueous acetonitrile containing 0.1% TFA and then with water again. The column was stored in 50% aqueous acetonitrile until used.

During the purification run, the filtered bacterial lysate containing the PAK-pilin E fusion protein was applied to the K column at a flow-rate of 0.2–0.5 ml/min with 10 m*M* KH_2PO_4, pH 6.0, as the running buffer. The loaded column was first washed with 0.5 *M* KCl in the running buffer and the fusion protein was eluted with 50% aqueous acetonitrile and 0.1% TFA. Representative chromatograms from the reversed-phase HPLC analysis of the crude bacterial lysate, the 0.5 *M* KCl wash, and the eluted product are shown in Fig. 10a–c, respectively. Mass spectrometry analysis of the final eluate revealed a homogeneous product that is indistinguishable from the expected PAK-pilin E fusion protein (data not shown) [86].

The unique design of the E/K coiled-coil permitted the use of high-ionic strength wash buffer during the purification process. In the presence of 0.5 *M* KCl, which would remove most of contaminants from the affinity matrix, the E fusion protein remained bound on the column. This is because the presence of salt strengthened the hydrophobic interaction of the E and K peptides despite the competition with interchain *e* and *g* salt bridges. Similarly, moderate amounts of organic solvent can also be used to wash the bound column with little effect on the E/K dimerization state. In this case, the presence of the *e* and *g* interchain salt bridges compensated for the weakened hydrophobic core in the presence of organic solvent. For this reason the elution procedure required both a lowering of the pH and the use of an organic buffer. The former condition is needed to remove the stabilizing effect of the ionic interactions while the latter condition disrupts the hydrophobic core and the bound material is eluted.

The recombinant expression and on-line affinity chromatography system has now been expanded to provide more flexibility. Recently, we have successfully generated a K fusion expression vector and a complementary E affinity column. Experiments are in progress to generate expression vectors where the K or E coding region is placed upstream of the multiple cloning site. Enzymatic cleavage site(s) other than the existing methionine chemical cleavage are being tested in the linker region to provide more efficient fusion tag removal. We are also experimenting with new affinity resins to order to increase the versatility of the purification system. Some of these modifications include the use of uniformly sized non-porous glass beads which might be suitable for batch-type purification. Liquid chromatography-compatible resins that are suitable for in situ chemical peptide synthesis are also being tested in an attempt to streamline the production of the affinity chromatography system.

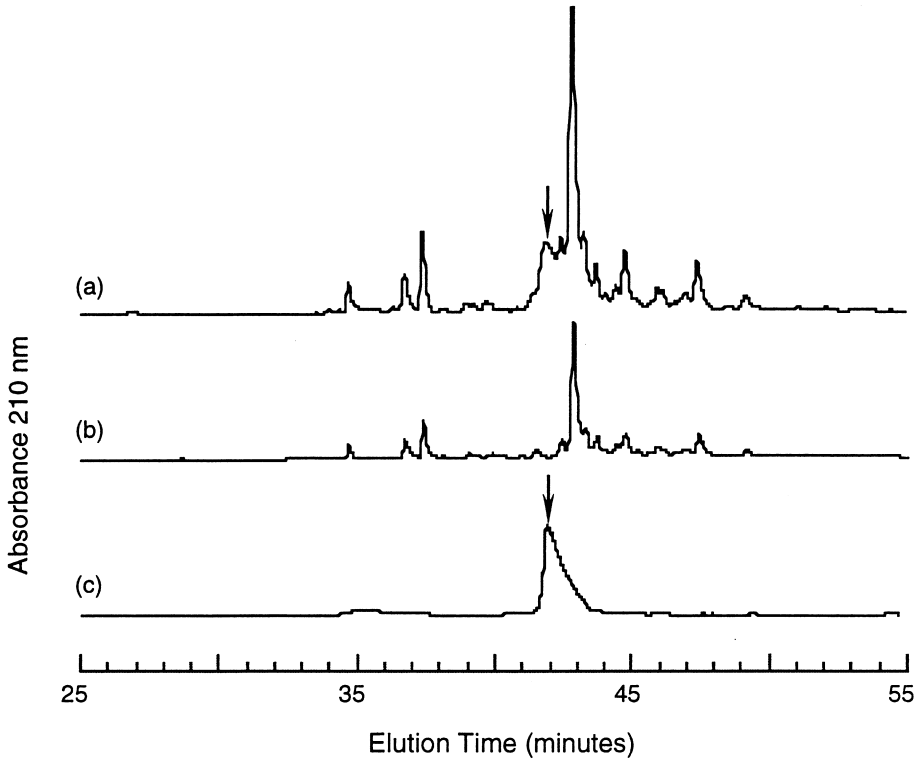

Fig. 10. Reversed-phase HPLC analysis of the eluates from each step of the affinity purification of the recombinantly expressed PAK-pilin-E coil using the K coil affinity column: (a) crude periplasmic extract; (b) 0.5 *M* KCl wash; (c) product eluted with 50% acetonitrile and 0.1% TFA. All runs were performed on an HP 1090 HPLC with a Zorbax RP-C8 column (250×4.6 mm I.D., 6.5 μm particle size, 300 Å pore size). A linear AB gradient (1% B/min), where solvent A is 0.05% TFA in water and solvent B is 0.05% TFA in acetonitrile with a flow-rate of 1 ml/min was applied. The runs were monitored at 210 nm.

4. Other affinity chromatographic systems

The E/K coiled-coil affinity chromatography module is a flexible and versatile purification unit. When coupled with the recombinant expression module, the entire system significantly economizes and streamlines an often labor-intensive multi-step process. The coiled-coil system, in our opinion, offers a good alternative to existing affinity purification systems. The following section describes briefly several affinity systems that are used today. Their advantages and limits are also highlighted.

4.1. Immunoaffinity chromatography

The recognition of antibodies by their antigens is highly specific and usually of high affinity. This has been the basis of many biodetection techniques, including ELISAs and immunoaffinity chromatography. The antibody can be immobilized on the matrix to purify the antigen or vice versa. In 1951, Campbell et al. [87] were the first to do this when they immobilized bovine serum albumin on aminobenzylcellulose in order to purify antibodies that were raised against the protein. Polyclonal antibodies can be used as immunoaffinity ligands, but the development of monoclonal antibodies has made this technique much more popular. Monoclonal antibodies are generally preferred because they can be obtained reproducibly and a particular strain can be selected for the appropriate affinity.

The kinetics of the antibody–antigen interaction determine how well affinity chromatography will work. The antibody must have high affinity for its ligand, indicated by a small equilibrium dissociation constant (K_d). The dissociation rate constant (k_{off})

must be finite, or the Ab–Ag complex will be too difficult to dissociate [88]. Ideally, the K_d should be between 10^{-4} and 10^{-8} *M* for affinity chromatography. If the K_d is greater than 10^{-4} *M*, the affinity is too weak, leading to loss of product. If it is less than 10^{-8} *M*, the complex will be very difficult to dissociate, making elution difficult [89].

The antibody–antigen complex is stabilized by the same forces which stabilize protein structure: electrostatic interactions, hydrogen bonding and hydrophobic interactions. This makes dissociation very difficult, and the elution conditions will frequently denature the target protein. The two most common elution methods are a change in pH and the introduction of chaotropic salts. Many stabilizing forces, such as electrostatic interactions, are disrupted at low pH by the protonation of acidic groups and at high pH by the deprotonation of basic groups. Typical elution buffers include 0.1 *M* glycine (pH 3) [90], 0.1 *M* acetic acid (pH 3.0) [88], and 0.1 *M* triethylamine HCl (pH 11) [91]. In contrast, chaotropic salts disrupt the structure of water which decreases hydrophobic interactions and so weakens the antibody–antigen complex. Examples include CCl_3COO^-, CF_3COO^-, ClO_4^-, I^-, and Cl^- at concentrations between 1.5 and 8 *M*. The most common elution buffer is 3 *M* NaSCN [89]. Chaotropic salt elution tends to be less denaturing than pH elution.

Despite the popularity of immunoaffinity chromatography, it has significant disadvantages. Ironically, the greatest strength of immunological techniques, the high specificity of antibody/antigen recognition, is also its greatest disadvantage. No general purification strategy exists because a new antibody must be raised against each protein, which can be time consuming and expensive, and the elution conditions must be optimized. This process is particularly difficult and time consuming if the protein of interest has never been purified before. The denaturing elution conditions are also a problem, because it is usually desirable to have a protein in its native conformation, especially if its activity is to be studied and in some cases where in vitro refolding is not possible. Antibodies may be structurally altered or functionally inactivated while being coupled to the matrix. There are problems with antibodies leaching from the matrix. Additionally, immobilization of either the antibody or the antigen can interfere with their interaction due to steric hindrance [92]. Many variations of immunoaffinity chromatography have been developed in an effort to overcome these problems.

A more general technique takes advantage of the ability of Protein A and Protein G to bind to the constant heavy chain of the Fc region of immunoglobins. Protein A, from *Staphylococcus aureus*, and Protein G, from Group G streptococci, are specific for different types of Ig molecules. Both proteins are stable, specific and can be obtained at relatively low cost. Either Protein A or G can be coupled to the chromatography matrix for monoclonal antibody purification. Alternately, Protein A can be used to anchor the antibody to the resin in the correct orientation for the subsequent purification of the antigen [88]. Recently, Yang et al. [90] have used the Fc domain of human immunoglobin as a C-terminal affinity tag in the purification of recombinant acetylcholine receptor-inducing activity protein. The fusion protein was immobilized on a Protein G–agarose column, the impurities were washed away, and the protein was eluted with 0.1 *M* glycine.

Berry and Davies [93] developed a single-step purification of turkey lysozyme using the Fv fragment of a monoclonal antibody raised against the enzyme. The resulting lysozyme was highly pure and recovered in high yields. There are several advantages to the use of the Fv fragments. Unlike complete antibodies, Fv fragments can be recombinantly expressed in bacteria, which is cheaper and more dependable than producing monoclonal antibodies. They are small enough to be immobilized within the pores of the silica matrix, and have more binding sites per mg of protein – thereby increasing the efficiency of the purification. This technique could be useful if a monoclonal antibody with a known Fv sequence were available for the protein to be purified. The interaction between the protein and the Fv fragment is still very strong, and hence elution may still be a problem.

Immunoaffinity tags have been developed as a generalized approach to the purification of recombinant proteins. The target protein to be purified is expressed as a fusion protein with a short antibody epitope at either the N- or C-terminus. The epitope must be a linear peptide, not a conformational

epitope [88]. This technique was used by Templeton [94] in the purification of retinoblastoma susceptibility gene product. They used the hemagglutinin antigen epitope of the influenza virus as a C-terminal affinity tag. The recombinant protein was purified with an anti-hemagglutinin antibody coupled to Protein A Sepharose. After washing, the protein was eluted by a competing peptide ligand (YPYDVPDYA). A protease cleavage site can be engineered between the protein and the peptide tag to remove the peptide.

4.2. Affinity tags

Affinity tags were designed to make affinity chromatography simpler and standardized. A peptide with a known affinity for a particular ligand is genetically engineered onto the N- or C-terminus of the target protein. A standardized purification scheme can then be used, with minimal adaptation, for any recombinant protein. This greatly increases the speed with which an expression system can be evaluated and optimized. The time required for purification is also reduced, especially since it can often be accomplished in a single step. Many affinity-tags are commercially available, along with the necessary reagents. This means that the same affinity resin and column can be used in the purification of many proteins, a significant advantage over individually tailored monoclonal antibodies. The same tag can then be used to anchor the protein directly to a microtiter well for assays. Finally, proteins which are truncated due to an error in synthesis can be extremely difficult to remove. When affinity tags are used, the truncated protein will not carry the tag and will not bind to the affinity matrix [88].

There are limitations to the use of affinity tags that one must be aware of. Occasionally, the unnatural amino acids at one terminus of the protein may interfere with the expression or folding of the protein. It is also possible for the activity of the protein to be affected by the presence of a tag. These problems can frequently be alleviated by switching the tag from one terminus to the other. If physical characterization or structural determination is to be done with the recombinant protein, the native amino acid sequence is preferred. In this case it would be best to include a protease cleavage site between the tag and the protein sequence [88]. Despite these potential problems, affinity tags have become extremely popular in the last several years.

4.3. Biotin/streptavidin affinity system

One of the best known examples of molecular recognition is that of streptavidin for biotin. Biotin, or vitamin H, is a small cofactor required by enzymes involved in CO_2 transfer. Streptavidin, a protein isolated from *Streptomyces avidinii*, binds biotin with a high affinity. Each protein molecule has four binding sites for biotin and is highly specific for it. Because many biodetection assays make use of the interaction between biotin and streptavidin, various types of biotinylation reagents and derivatized streptavidin are commercially available [95].

The affinity of streptavidin for biotin is one of the highest known, with a K_d equal to 10^{-15} *M*. This is 10^3–10^6 times greater than the affinity of antibodies for their antigens. The resulting complex is very stable and is unaffected by pH, chaotropic salts, and washing steps. Dissociation requires harsh, denaturing conditions, such as heating in 2% SDS or treatment with 70% formic acid [96]. This has seriously limited the usefulness of this system in affinity chromatography.

Cleavable biotin analogs have been introduced to allow the elution of proteins with their native structure. Thiele and Fahrenholz [96] attached a photocleavable biotin analog to the cholecystokinin (CCK) peptide. This peptide was incubated with an anti-CCK antibody and then with streptavidin agarose. After a wash step, the mixture was irradiated (λ>320 nm) to cleave the antibody–CCK complex from the resin. They succeeded in purifying the antibody 360-fold, but the peptide was still bound to the resin.

The Strep-tag was developed by Schmidt and Skerra [97] as an alternate streptavidin ligand. Through the use of a peptide library, they isolated a nine residue peptide (AWRHPQFGG) that bound to streptavidin. It was used as a C-terminal affinity tag for the recombinant expression of an antibody Fv fragment. In a single-step purification process, the protein was immobilized on streptavidin agarose and eluted with 1 m*M* iminobiotin, a competing ligand. The mild elution conditions allowed the Fv fragment

to be purified as a heterodimer. The equilibrium dissociation constant between streptavidin and the Strep-tag was found to be 3.7×10^{-5} *M* by isothermal titration calorimetry [98]. The Strep-tag is a small hydrophillic peptide which is resistant to proteolysis in vivo. It did not interfere with the expression and secretion of the Fv fragment. It also binds competitively with biotin.

4.4. Histidine-Tag purification system

The Histidine-Tag is one of the most common affinity-tags in use. This is a form of immobilized-metal affinity chromatography (IMAC). An oligohistidine peptide is engineered into a recombinant protein as an affinity tag. The histidine residues bind to Ni^{2+}, which has been immobilized onto a column by covalently bound chelating agents. This system has become extremely popular due to its flexible conditions and mild elution. The His-Tag and Ni^{2+} columns are commercially available.

This technique is based on the principle that transition metal ions will coordinate to electron donating groups on the protein surface [99,100]. This binding is dominated by the imidazole group of histidine residues, but cysteine and tryptophan can also play a role under some conditions. Different metal ions have been used, and they are listed in the order of their affinity for imidazole: $Cu^{2+}>Ni^{2+}>Zn^{2+}\cong Co^{2+}$. An average single histidine residue has a $K_d=2.2\times10^{-4}$ *M* for Cu^{2+}, while that for Ni^{2+} is 15 times weaker [101].

The chelating agents are small molecules covalently linked to the matrix. The two most common ones are iminodiacetic acid (IDA) and nitrilotriacetic acid (NTA). Since IDA is a tridentate chelator, three of the six Ni^{2+} coordination sites bind to IDA and three are available to bind to the protein. This means that the protein is bound relatively strongly, and 250 m*M*–1 *M* imidazole, a competing ligand, is required for elution. Ni^{2+} is bound more strongly to NTA, a tetradentate ligand, and hence there is less problem with the metal 'bleeding' from an NTA column. As only two metal coordination sites remain, proteins are bound less strongly and can be eluted with 100–200 m*M* imidazole [100].

Proteins are coordinated through the ϵ- or δ-nitrogen of surface-accessible histidine residues. Few proteins have more than a couple of surface histidines, as the residue is only found at about 2% of the positions in proteins, only half of which will be surface accessible [101]. This makes the engineered His-Tag, which is usually six to eight histidine residues in length, a very specific binding domain. Ni^{2+}–NTA has only two free coordination sites and so can only bind to two histidine residues. Optimal binding occurs when the two histidines are found on subsequent turns of an α-helix. This can be achieved by engineering a –His–X_3–His tag, but this will only bind to Ni^{2+} when correctly folded and presented. Better results can often be obtained by a tag of five or six histidine residues in a row. Only two will bind, but there is a better chance of there being two in the correct orientation. The pK_a of surface histidines is from 6 to 7. As it is the unprotonated form which binds to Ni^{2+}, the best metal binding occurs at pH 6–8.

There are three common ways to elute His-tagged proteins from a Ni^{2+} column. The first is by a pH gradient. A drop to pH 4–5 is generally sufficient to protonate the histidine residues and elute the protein. This is dependent on the number of surface-accessible histidines, and hence proteins with higher number of histidines require a lower pH for elution. The second method is to introduce a competing ligand, usually imidazole. Imidazole elution is generally milder, especially for proteins which are not stable at low pH. The third technique is to flush the column with a chelating agent such as EDTA. Unlike with the first two methods, EDTA elution will provide no resolution between species with different affinities to the resin – everything will elute at once. This would be a disadvantage if there were a Ni^{2+} binding impurity that might have been separated by pH or imidazole elution. In addition, the chelating agent will strip the column of Ni^{2+} ions [99–101].

There are only a few constraints on the conditions for His–Ni^{2+} purification. First, the pH should be in the range of 6–8. Since this pH range encompasses the physiological pH, this is rarely a problem. Second, a high salt buffer, typically 0.5–1.0 *M* NaCl, is required to reduce nonspecific binding through an ion exchange effect. Finally, chelating agents must be avoided for they will strip the Ni^{2+} from the column. The most common include EDTA, EGTA, β-mercaptoethanol, dithiothreitol, and citrate. Other

than this, Ni^{2+} chromatography is compatible with a wide range of conditions, including many salts, detergents, and denaturants [100].

There are some advantages to carrying out His–Ni^{2+} purification under denaturing conditions. If the target protein has a low solubility or tends to associate with other proteins, extraction and purification in the presence of 6 *M* guanidine HCl will disrupt those interaction without interfering with the Ni^{2+} chromatography. However, there may be more impurities due to the unfolding of proteins with internal histidines which can then bind to Ni^{2+}.

The His–Ni^{2+} system can also be used to purify noncovalently bound protein complexes. If a single member of the complex has a His-Tag, the entire complex can be immobilized on the matrix. This can be done with protein complexes formed either in vivo or in vitro. Obviously denaturing conditions should be avoided and imidazole elution would be preferred to a pH gradient [100].

There are several advantages to using the His-Tag for the purification of recombinant proteins. It is a small tag which rarely alters the properties and functions of the target protein. Therefore, proteolytic cleavage to remove the tag is seldom necessary. The small chelating agents are stable over a wide range of temperature and solvent conditions. The resin is capable of high metal loading, allowing high protein capacities (2–10 mg protein per ml resin) [102]. The elution process is simple and allows the native structure to be maintained. Should the resin be stripped of Ni^{2+}, it can be regenerated by simply pumping a $NiSO_4$ solution through the column. Finally, the entire system is quite inexpensive [100–102]. Despite the general usefulness of the His-Tag, there are some instances where the histidine residues interfere with the expression or folding of the protein. In addition, some proteins, such as Fv fragments, tend to dissociate in the high salt conditions which are necessary [97].

5. The advantages of the E/K coiled-coil system

The heterodimeric E/K coiled-coil system is a comprehensive and versatile research tool. It integrates the laborious processes of recombinant protein expression, product detection, biosensor functional assays and protein purification into a common platform. The E/K coiled-coil serves as a capture and dimerization domain for the biosensor; a detection and an affinity tag for protein expression and purification. The E/K system allows the researcher to produce, detect, purify and analyze the protein product in an integrative manner. The efficiency and convenience of this system are the main reasons for its usefulness as a research tool.

The versatility of the E/K coiled-coil system stems from the ability of a coiled-coil to be used as a dimerization domain and our ability to design these proteins with specific dimerization characteristics. In theory, any native heterodimeric coiled-coil such as Fos/Jun or Myc/Max can be used in this system. However, the use of these native proteins has several disadvantages. Firstly, natural coiled-coils such as those found in transcription factors usually have a modest dimerization affinity (10^5–10^8 *M*). Although this may be an important characteristic of the transcription factor from a regulatory perspective, the weak affinity reduces the usefulness of these coiled-coils as capture domains. The weak affinity can place a limit on the sensitivity of the biosensor-based assays as it reduces the number of captured acceptors presented on the surface to be studied. This may also mean a lower overall yield from the purification process because it probably constrains the type and number of washes one can do during the affinity purification process. Secondly, many of the native proteins that form coiled-coils can dimerize with multiple partners. A good example is the c-Myc/Max family as described previously. From an application perspective, the apparent lack of specificity will be a disadvantage since the presence of complementary protein dimer-forming partners in the biological medium other than the anticipated one can compromise the expression system, the biosensor-based assay and even the affinity purification process. Thirdly, naturally occurring proteins are usually degraded rapidly in serum or cell extracts. This will no doubt reduce the overall efficiency of the system and increase the cost associated per analysis.

A good way to overcome these disadvantages is the use of de novo designed coiled-coils. This is a practical alternative because of the large pool of knowledge that is currently available in the field to design a coiled-coil. Theoretically, the current stage

of knowledge will permit the design of a coiled-coil with specific dimerization affinity, unique specificity, good stability and appropriate size. We along with other groups have demonstrated that such efforts are feasible. The E/K coiled-coil is a specific example of such an effort.

The E/K coiled-coil is designed to be very stable in solution. Its resistance to urea and heat denaturation demonstrated this characteristic. Yet the E/K coiled-coil can be dissociated under specific conditions which ensure its usefulness in affinity chromatography. The ability of the E and K peptides to dimerize, even when one is fused with a large protein (e.g. antibody), showed that it is a useful purification tag. The unique amino acid sequence of the E and K peptides enables them to dimerize specifically and minimizes the probability of interference from naturally occurring coiled-coils. The fast formation and slow dissociation kinetics of the dimeric coiled-coil also permit the construction of a stable capture domain to be used in the biosensor-based applications. Lastly, its apparent stability in serum suggests that the system should be useful in a variety of applications.

The E/K coiled-coil is only one of the many possible designs that can be used with the system described. Given the inherent flexibility of chemical peptide syntheses, and the number of structural variations that are possible as learned from nature and ongoing research, the heterodimeric coiled-coil system can truly be tailor-made to the exact needs of the researcher.

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