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Reversed-phase liquid chromatography as a useful probe of hydrophobic interactions involved in protein folding and protein stability

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Abstract

We have evaluated the potential of reversed-phase liquid chromatography (RPLC) as a probe of hydrophobic interactions involved in protein folding and stability. Our approach was to apply RPLC to a *de novo* designed model protein system, namely a two-stranded α-helical coiled coil. It was shown that the reversed-phase retention behaviour of various synthetic analogues of monomeric α-helices and dimeric coiled-coil structures correlated well with their stability in solution, as monitored by circular dichroism during guanidine hydrochloride and temperature denaturation studies. In addition, an explanation is offered as to why amphipathic coiled coils, an important structural motif in many biological systems, are more stable at low pH compared to physiological pH values. The results of this study suggest that not only may RPLC prove to be a useful and rapid complementary technique for understanding protein interactions, but also the *de novo* designed coiled-coil model described here is an excellent model system for such studies.

1. Introduction

One of the most difficult and important challenges currently facing biochemists is understanding protein folding and stability; specifically, how does the amino acid sequence of a protein determine its three-dimensional structure and the pathway of folding, as well as its resultant stability? The goal of predicting polypeptide and protein conformation from primary structure information, including the interactions responsible for stabilizing this conformation, is being pursued by many researchers, using a variety of methodologies.

A very promising approach to such studies is the utilization of reversed-phase liquid chromatography (RPLC) as a physicochemical model of biological systems. Hydrophobic interactions are the major driving force for protein folding and stability; the hydrophobic interactions between peptides or proteins and the non-polar stationary phase upon which this chromatographic mode depends [1] may well reflect similar interactions between the non-polar residues that stabilize the folded or three-dimensional structure of the native protein molecule.

Several previous studies have attempted to equate conformational stability of proteins with

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their reversed-phase retention behaviour. However, these studies have tended to focus on such concerns as protein conformational changes due to denaturation during RPLC [2–8], thermodynamics of protein unfolding during RPLC [3,6,9,10] or simple observations of protein conformational changes under RPLC conditions [11], *i.e.*, generally little attempt is made to correlate protein primary structure with protein folding or stability of the folded protein molecule. Even studies where such correlation between peptide [10] or protein [7] retention behaviour with protein folding is attempted, tend to offer limited insight into the contribution of individual interior amino acid side-chains to protein stability.

In the authors' view, the best approach to gauging the effectiveness of RPLC as a probe to further our understanding of the hydrophobic forces responsible for protein folding and stability is to focus on work conducted with defined model peptide and protein systems, the results of which can then be extrapolated to peptides and proteins as a whole. A promising candidate for such a model system is represented by *de novo* designed model two-stranded α-helical coiled-coils. Hodges and co-workers [12–26] have carried out extensive studies on the stability and properties of such synthetic coiled coils, and the credentials of this biologically important motif for the purposes of the present study appeared excellent. For example, such a model system fits well with the proposed mechanism of protein folding which involves the collision of secondary structural elements, resulting in the progressive association of these elements which leads to the native conformation [27]. In addition, about 50% of all α-helices in soluble globular proteins are amphipathic [28,29], *i.e.*, with opposing polar and non-polar faces; such structures are also found in many fibrous proteins, *e.g.*, myosin [30,31] and tropomyosin [32] as well as smaller molecules such as polypeptide hormones [33,34], polypeptide venoms [35,36] and polypeptide antibiotics [37,38], reflecting their wide distribution in secondary structural motifs. Indeed, by their very nature, amphipathic α-helices play an important role in the hydrophobic interactions involved in protein folding and stability. The coiled-coil structure itself is also a widespread protein quaternary structure, with more than 200 proteins thought, at present, to contain the coiled-coil motif [39] and many more are expected to be discovered.

From a practical point of view, the model amphipathic α-helical peptides described by Hodges and co-workers [12–26] are large enough to form a stable three-dimensional structure capable of tolerating sequence changes, yet small enough for easy chemical synthesis of analogues. Further, the hydrophobic domain of these model amphipathic peptides will bind preferentially to a reversed-phase packing, *i.e.*, only this part of the peptide sequence would be expected to have a major effect on peptide retention behaviour during RPLC. Thus, it is reasonable to expect that even subtle variations in hydrophobicity of the preferred binding domain will be of a magnitude able to be expressed as a variation in RPLC retention time. Relative retention behaviour of various model peptide analogues could then be extrapolated to the way in which such amphipathic structures interact with each other within native protein molecules. Although other researchers have utilized synthetic amphipathic peptides in conjunction with RPLC for protein stability studies [40,41], the model peptides employed (consisting of only lysine and leucine residues) lacked the biological relevance of those reported in the present study.

It is interesting to note that, as long as 18 years ago, Horváth *et al.* [42] postulated that the hydrophobic stationary phase of reversed-phase packings may be a useful probe for investigation of amphipathic helices induced or stabilized in hydrophobic environments. We believe we are now in a position to test more fully and further develop this hypothesis with our peptide/protein model. Thus, the present study describes our initial evaluation of RPLC as a probe of biological systems by attempting to correlate the stability of model single and double-stranded amphipathic α-helical peptides with their chromatographic retention patterns.

2. Experimental

2.1. Materials

HPLC-grade water and acetonitrile were obtained from J.T. Baker (Phillipsburg, NJ, USA). ACS-grade orthophosphoric acid and triethylamine (TEA, redistilled before use) was obtained from Anachemia (Toronto, Canada). Trifluoroacetic acid (TFA) was obtained from Aldrich (Milwaukee, WI, USA). Trifluoroethanol (TFE) was obtained from Sigma (St. Louis, MO, USA). Guanidine hydrochloride (Gdn · HCl) was obtained from Schwarz/Mann Biotech, Cleveland, OH, USA.

2.2. Instrumentation

Peptide synthesis was carried out on an Applied Biosystems peptide synthesizer Model 430A (Foster City, CA, USA). Crude peptides were purified by an Applied Biosystems 400 solvent-delivery system connected to a 783A programmable absorbance detector.

The analytical HPLC system consisted of an HP1090 liquid chromatograph (Hewlett-Packard, Avondale, PA, USA), coupled to an HP1040A detection system, HP9000 series 300 computer, HP9133 disc drive, HP2225A Thinkjet printer and HP7460A plotter.

Amino acid analyses of purified peptides were carried out on a Beckman Model 6300 amino acid analyzer (Beckman Instruments, Fullerton, CA, USA).

The correct primary ion molecular masses of peptides were confirmed by time-of-flight mass spectroscopy on a BIOION-20 Nordic (Uppsala, Sweden).

Circular dichroism spectra were recorded on a JASCO J-500C Spectropolarimeter (Easton, MD, USA) attached to a JASCO DP-500N data processor and a Lauda (Model RMS) water bath (Brinkman Instruments, Rexdale, Canada) used to control the temperature of the cell. The instrument was routinely calibrated with an aqueous solution of recrystallized *d*-camphorsulphonic acid at 290 nm. Constant nitrogen flushing was employed.

2.3. Columns

Analytical HPLC runs were performed on a Zorbax 300-SB C_8 reversed-phase column (250 × 4.6 mm I.D., 5-μm particle size, 300-Å pore size) from Rockland Technologies, Wilmington, DE, USA.

Crude peptides were purified on a semi-preparative SynChropak RP-P C_{18} reversed-phase column (250 × 10 mm I.D., 6.5 μm, 300 Å) from SynChrom, Lafayette, IN, USA.

2.4. Peptide synthesis

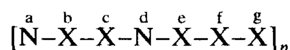
Peptides were synthesized on co-poly(styrene–1% divinylbenzene) benzhydrylamine hydrochloride resin (0.92 mmol/g resin). All amino acids were protected at the α-amino position with the *tert.*-butyloxycarbonyl (Boc) group and the following side-chain protecting groups were used: 4-methylbenzyl (Cys), benzyl (Glu) and 2-chlorobenzyloxycarbonyl (Lys). All amino acids were single coupled as preformed symmetrical anhydrides (with the exception of Gln, which was coupled as the hydroxybenzotriazole active ester) in dichloromethane. Boc groups were removed at each cycle with an 80-s reaction with TFA–dichloromethane (33:67, v/v), followed by a second reaction with TFA–dichloromethane (50:50, v/v) for 18 min. Neutralizations were carried out using 10% (v/v) diisopropylethylamine in dimethylformamide. N-Terminal residues were acetylated using acetic anhydride–dichloromethane (25:75, v/v) for 10 min. The peptides were cleaved from the resin by treatment with anhydrous hydrogen fluoride (20 ml/g resin) containing 10% (v/v) anisole and 2% (v/v) 1,2-ethanedithiol for 1 h at −4°C. The resins were then washed with diethyl ether (3 × 25 ml) and the peptides extracted with neat acetic acid (3 × 25 ml). The resulting peptide solutions were then lyophilized prior to purification.

3. Results and discussion

3.1. Synthetic model protein

The model amphipathic peptide analogues described in this study were based on a repeating pattern of hydrophobic residues, first identified by Hodges *et al.* [43], throughout the entire length of the 284-residue polypeptide chain of the two-stranded α-helical coiled-coil protein, rabbit skeletal α-tropomyosin. These workers proposed that tropomyosin and other two-stranded α-helical coiled-coil proteins were stabilized by hydrophobic residues at positions 2 and 5 of a repeating heptad sequence, X–N–X–X–N–X–X–X–N–X–X–N–X–X–X–N . . . . , where N is a non-polar residue. This pattern of hydrophobes is often referred to as a 3–4 or 4–3 repeat.

The synthetic peptide analogues employed for the present study (Fig. 1, top) are, thus, polyheptapeptides of 35 residues based on the repeating sequence:



where the hydrophobic residues are denoted N. The high α-helix-forming potential of this sequence has been well documented [12–26,43]. Fig. 1 (bottom) illustrates a cross-section of two molecules of synthetic analogue "L", represented as helical wheels. The amphipathicity of this sequence is very apparent, with the leucine residues at positions a and d forming the hydro-

1 2 6 8 12 13 15 16 19 20 22 26 27 29 g a b c d e f g a d e g a d e g a d e g a d	Denotion	Figure(s)
Ac-K-C-E-A-L-E-G-K-L-E-A-L-E-G-K-L-E-A-L-E-G-K-L-E-A-L-E-G-K-L-E-A-L-E-G-NH2	L	6,7
Ac-----------------------V-------------V-------------V-------------------NH2	LVd	2-5
Ac-----------------------I-------------I-------------I-------------------NH2	LId	2-5
Ac-------------------------------I-----I---------------------------------NH2	I	7
Ac-------------------------------V-----V---------------------------------NH2	V	7
Ac-------------------------------F-----F---------------------------------NH2	F	6,7
Ac-------------------------------Y-----Y---------------------------------NH2	Y	7
Ac-Q---------K-Q-----------K---Q-A-------K---Q---------K---Q-------------NH2	EKQ	8-10
Ac-E---------K-E-----------K---E-A-------K---E---------K---E-------------NH2	EKE	8-10

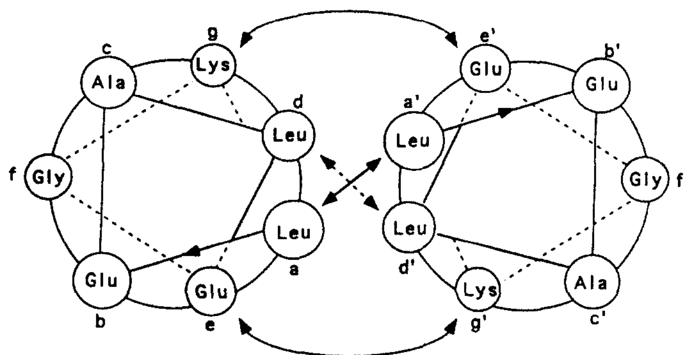


Fig. 1. Amino acid sequences of synthetic model peptides used in this study. Top: primary sequences of peptide analogues, where the N-terminal amino acid of each chain is acetylated (Ac–) and the C-terminal carboxyl group is amidated (–NH_2). Peptide L may be considered the "native" peptide structure, where all of the a and d positions (save residue 2) responsible for stabilizing the coiled coil, are occupied by leucine. The only residues shown for the other analogues are those where a substitution has been made in the "native" sequence. The residue number and heptad position of substituted residues are also shown. Bottom: helical wheel representation of a cross-section, looking from the N-terminal end, of two helices in a coiled coil containing the "native" heptapeptide sequence Leu–Glu–Ala–Leu–Glu–Gly–Lys. The α-helices descend into the page on proceeding from residues a to g. The chains are in-register and parallel. The non-polar residues in positions a and a′ and d and d′ interact and are responsible for the formation and stabilization of the coiled coil. Possible interchain ionic interactions could occur between g and e′ and between g′ and e.

phobic face. A coiled coil is formed by the two molecules wrapping around each other at their hydrophobic faces. Based upon the high resolution X-ray structure by O'Shea *et al.* [44] in 1991, these hydrophobes are 83% buried in the coiled coil.

From Fig. 1 (bottom), the coiled coil is stabilized by hydrophobic interactions between the individual α-helices with residue a interacting with a′ and residue d with d′. This coiled coil also exhibits interchain ionic interactions between lysine and glutamic acid residues at positions g and e′ (i to i′ + 5 or, for example, K1 in chain 1 with E6 in chain 2). There are also intrachain repulsive interactions along the α-helix, *e.g.*, glutamic acid at e with glutamic acid at b; these are $i \rightarrow i+4$ interactions or glutamic acid at b with glutamic acid at e; these are $i \rightarrow i+3$ interactions.

The 35-residue lengths of the analogues was based on the work of Lau *et al.* [14], who demonstrated that, with leucine occupying all the hydrophobic a and d positions in the repeating heptad, two-stranded α-helical coiled coils were formed in benign medium at pH 7.0. The presence of a disulphide bridge at position 2–2′ (hydrophobic position a in the first heptad) in the peptide sequence confers further stability to the two-stranded coiled coil [18].

3.2. Retention behaviour of amphipathic α-helices in RPLC

On binding to a reversed-phase column, the high hydrophobicity of the stationary phase disrupts the interchain hydrophobic interactions of a coiled coil such as that shown in Fig. 1 (bottom), whilst stabilizing the secondary (α-helical) structure. In fact, Zhou *et al.* [17] showed that such amphipathic peptides remain α-helical when bound to the reversed-phase column and, due to the preferred binding domain created by the non-polar face of the α-helix, are much more retentive than peptides of the same composition but lacking the preferred binding domain. Similar results were reported by Steiner *et al.* [45], who examined the reversed-phase retention behaviour of a six-helix bundle template-assembled synthetic protein (TASP) molecule and its amphipathic building blocks.

3.3. Correlation of single-stranded amphipathic α-helical peptides with their stability in solution

Fig. 2 shows the sequences of peptide analogues LVd and LId (Fig. 1, top) represented as helical nets, with the hydrophobic faces of the peptides (made up of hydrophobic residues in positions a and d of the heptad repeat) lying between the dotted lines. The only difference between the two analogues lies in the substitution of valine at residue numbers 12, 19 and 26 (all at position d of the heptad sequence) in

Fig. 2. Sequences of amphipathic α-helical peptides LVd and LId represented as α-helical nets. The radius of the α-helix is taken as 2.5 Å with 3.6 residues per turn, a residue translation of 1.5 Å, and thus a pitch of 5.4 Å. The area between the dotted lines represents the hydrophobic domain of the amphipathic α-helix containing hydrophobic (circled or boxed) residues only. These two peptides differ only in the boxed residues within this domain, containing either valine (LVd) or isoleucine (LId) at the positions shown.

peptide LVd (Fig. 2, left) by isoleucine in peptide LId (Fig. 2, right). The hydrophobicity of isoleucine, as expressed by hydrophobicity coefficients obtained from RPLC peptide retention data [46], is greater than that of valine at pH 2 and pH 7. Thus, it would be expected that peptide LId would have a more hydrophobic face along the helix than peptide LVd, and be more retentive on binding to a reversed-phase column.

Fig. 3 shows the reversed-phase elution profiles of the two peptides, under reducing conditions (to ensure no disulphide bridge formation), at pH 2 and pH 7. As expected, in both cases the isoleucine-substituted analogue, LId, was retained longer on the column than the valine-substituted analogue, LVd. The peptides are eluted earlier at pH 7 compared to pH 2 due to the increased overall hydrophilicity of the peptides as the glutamic acid residues are deprotonated (become negatively charged) at the higher pH value. Temperature denaturation of the individual α-helical analogues in 50% TFE (a

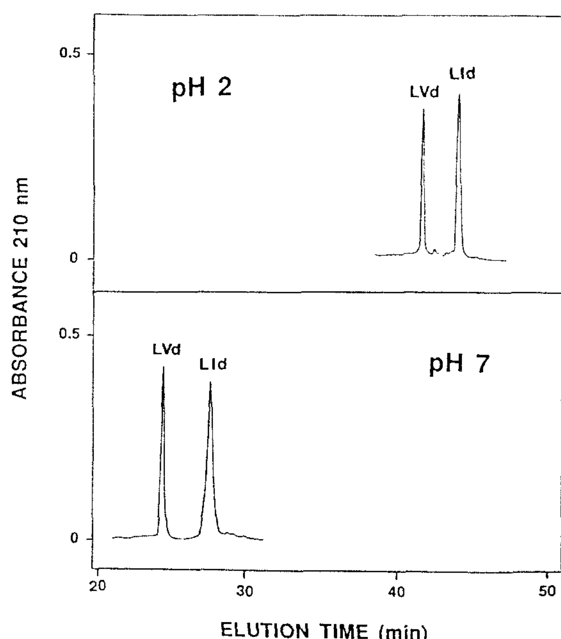


Fig. 3. Reversed-phase chromatography of single-stranded amphipathic α-helical peptides at pH 2 (top panel) and pH 7 (lower panel). The sequences of peptides LVd and LId are shown in Figs. 1 and 2.

helix-inducing solvent) was now carried out (this denaturation monitored by circular dichroism) and the results compared to their reversed-phase retention times. Apart from its helix-inducing properties, the presence of 50% TFE also serves to disrupt coiled-coil structure and maintain the peptides as monomeric α-helical strands. From the thermal denaturation profiles shown in Fig. 4, peptide LId is clearly more stable in solution than peptide LVd, with $t_{1/2}$ values (the temperature at which the α-helicity of the peptides is reduced by 50%) of 42.2°C (LId) and 36.7°C (LVd). Hence, the temperature denaturation behaviour of the two amphipathic α-helical analogues (Fig. 4) correlates very well with their comparative retention behaviour during RPLC (Fig. 3); or, to put it another way, the hydrophobicity of the non-polar faces of the peptides, as expressed by their retentiveness during RPLC, correlates well with their helical stability.

3.4. *Correlation of stability of two-stranded amphipathic α-helical coiled coils with retention behaviour during RPLC*

Since hydrophobicity is a general parameter of protein folding and stability, it might be supposed that the relative hydrophobicity of an amphipathic helix as expressed by its strength of adsorption to a hydrophobic stationary phase may reflect the strength of interchain interactions in the native protein, *i.e.*, the more hydrophobic or amphipathic the helix, the greater the stability of interchain hydrophobic interactions. Since, from Fig. 3, the hydrophobicity of the non-polar face of LId was shown to be of a greater magnitude than that of LVd, the stability of the LId–LId coiled coil would be expected to be more stable than the LVd–LVd coiled-coil structure. Fig. 5 shows the Gdn·HCl denaturation profiles of the two oxidized coiled coils at pH 7. Clearly, as expected, the stability of the isoleucine-substituted coiled coil is more stable than the valine-substituted analogue, with $[\text{Gdn}\cdot\text{HCl}]_{1/2}$ (Gdn–HCl concentration required for 50% loss of helicity) values of 4.8 and 1.5 *M*, respectively.

The concept of probing the stability of such

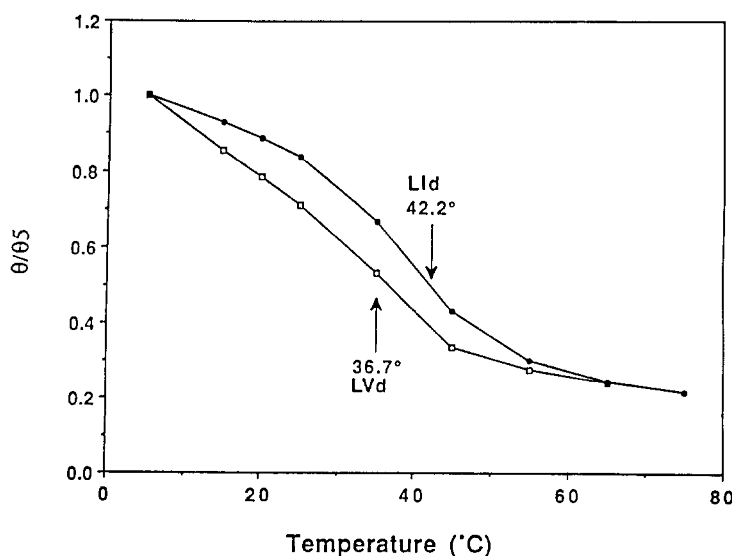


Fig. 4. Thermal melting profiles of single-stranded amphipathic α-helical peptides in 0.1 *M* KCl + 50 m*M* potassium phosphate buffer–trifluoroethanol (1:1, v/v) at pH 7. The sequences of peptides LVd (□) and LId (●) are shown in Figs. 1 and 2. θ/θ_5 represents the ratio of the ellipticity at 220 nm at the indicated temperature to the ellipticity at 5°C.

model coiled coils by RPLC was now taken a step further by examining the retention behaviour of a series of synthetic peptide analogues, where two residues at a time were substituted in the hydrophobic face of the amphipathic helix. Thus, two leucines at residue numbers 16 and 19 (positions a and d, respectively, of the central heptad) were replaced by hydrophobic amino acids isoleucine (peptide I), valine (V), phenylalanine (F) or tyrosine (Y). The leucine-

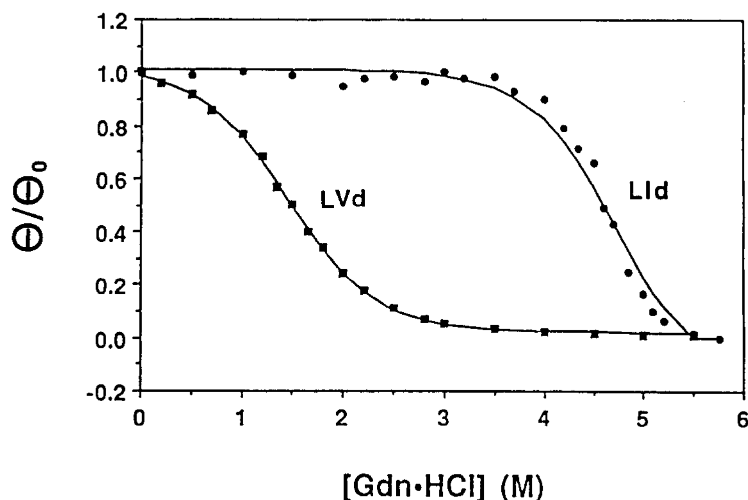


Fig. 5. Guanidine hydrochloride (Gdn · HCl) denaturation profiles of two-stranded α-helical coiled coils of amphipathic peptides LVd (■) and LId (●) in 0.1 *M* KCl, 50 m*M* potassium phosphate buffer at pH 7 and 20°C. θ/θ_0 represents the ratio of molar ellipticity at 220 nm at the indicated molarity of Gdn · HCl to the ellipticity in the absence of Gdn · HCl. The oxidized peptide pairs of LVd and LId (sequences shown in Figs. 1 and 2) are linked by a disulphide bridge at position 2 and 2′ of the sequence.

substituted ("native") analogue was denoted L (Fig. 1).

Fig. 6 compares the reversed-phase elution profiles of the leucine (top) and phenylalanine (bottom) analogues under reducing conditions (L_r, F_r) or under oxidized conditions (L_o, F_o). Under reducing conditions, the two analogues L_r and F_r bind as monomeric α-helices. Under oxidized conditions, it may be expected that the disulphide-bridged dimers (L_o, F_o) would be eluted later than their respective single-stranded α-helices (assuming denaturation of the coiled-coil structure by the hydrophobic stationary phase), since the overall hydrophobicity has been doubled. However, from Fig. 6, it can be seen that the disulphide-bridged dimers are, in fact, being eluted prior to their monomeric forms. This observation can be accounted for if there is little denaturation of the coiled-coil structure, the stability of which is considerably enhanced by the presence of the disulphide bridge at

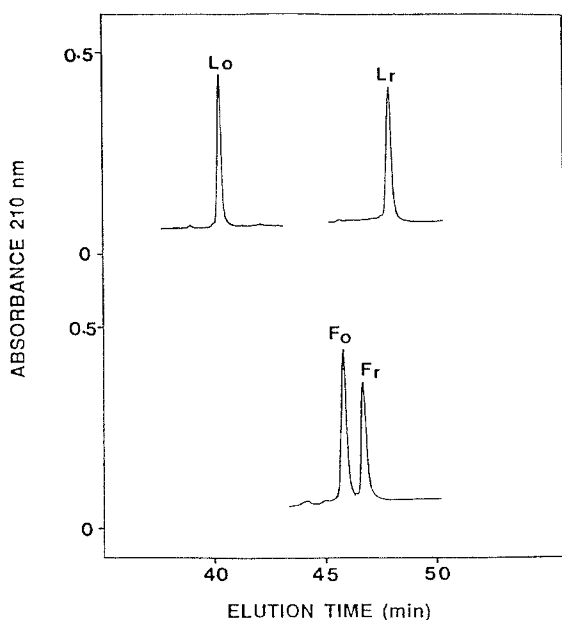


Fig. 6. Comparison of reversed-phase chromatography of single-stranded amphipathic α-helical peptides and double-stranded α-helical coiled-coil peptides at pH 7. The sequences of peptides L and F are shown in Fig. 1. The subscripts "o" and "r" denote oxidized (double-stranded, *i.e.*, linked by a disulphide bridge) and reduced (*i.e.*, single-stranded) forms of the peptides.

position 2 of the peptide sequence. Thus, there is incomplete exposure of the hydrophobic residues on the interacting hydrophobic faces of the amphipathic helices to the reversed-phase matrix, *i.e.*, the hydrophobes are buried in the coiled coil making the dimers less hydrophobic than the respective reduced monomers. The more stable the coiled coil, the more hydrophobic surface area of each α-helix is buried and, hence, the greater the difference in retention time between the single-stranded α-helix and the disulphide-bridged coiled coil. Thus, in the case of the Phe analogue coiled coil (F_o), it is less stable than the Leu analogue coiled coil (L_o), reflecting the preferred hydrophobic interactions of leucine compared to phenylalanine; thus, the *difference* in retention time between the single-stranded amphipathic α-helix and its respective two-stranded oxidized coiled coil is less for F compared to L, since the coiled-coil structure of the former (F_o) is unfolded to a greater extent than the latter (L_o) by the hydrophobic stationary phase and, hence, exposes more of its hydrophobic surface area to the reversed-phase matrix.

Similar observations of the relative RPLC retention times of the oxidized two-stranded dimers *versus* those of the respective monomeric α-helices were now made for the I, V and Y analogues (Fig. 1). Fig. 7 shows a plot of the free energy of unfolding (ΔG) for each coiled-coil protein (derived from Gdn · HCl denaturation data) *versus* the difference in reversed-phase retention time between reduced monomeric α-helix and the oxidized coiled coil for peptides L, F, I, V, Y. The ΔG term represents the stability of the coiled-coil protein and the excellent correlation of the ΔG values for each coiled-coil analogue with the difference in retention times of respective dimeric and monomeric analogues is quite clear. It should be noted that the unfolding of these model coiled coils is a reversible process [26]; furthermore, through thermodynamic characterization of the structural stability of the coiled-coil region of the basic region leucine zipper (bZIP) transcription factor GCN4, it has been indicated that the unfolding of this structural motif is a two-state process [47].

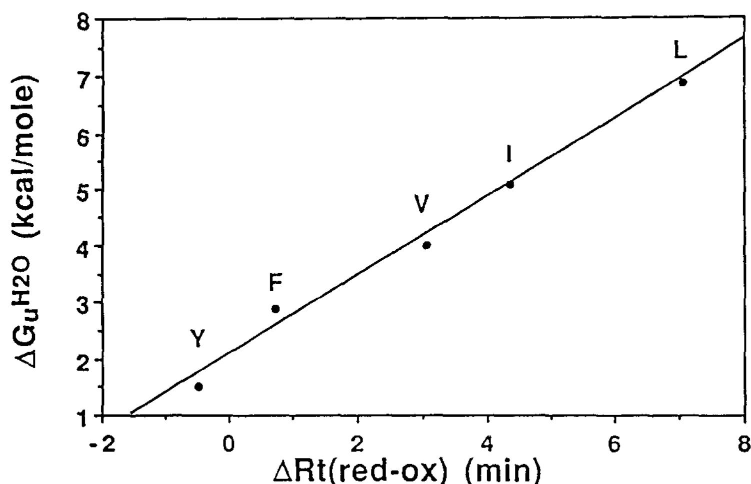


Fig. 7. Free energy of unfolding of model proteins *versus* the difference in reversed-phase retention time between the reduced monomeric amphipathic α-helical peptides and the oxidized dimeric α-helical coiled coils [ΔRt (redox)]. $\Delta G_u^{H_2O}$ is the free energy of unfolding in the absence of guanidine hydrochloride and is estimated by extrapolating the free energy of unfolding at each individual concentration of guanidine hydrochloride (ΔGu) to zero concentration assuming that they are linearly related [51]. The sequences of peptide analogues denoted Y, F, V, I and L are shown in Fig. 1.

3.5. Role of RPLC in explaining the effect of pH on protein stability

For more than 25 years, there has been no explanation as to why two-stranded α-helical coiled-coils are more stable at low pH (*e.g.*, pH 2) compared to pH 7 [14,16,19,48,49]. To investigate this problem with our model system, two peptide analogues, denoted EKQ and EKE (Figs. 1 and 8), were synthesized and their reversed-phase retention behaviour compared with their stability in solution at low and neutral pH.

Fig. 8 (left) shows a cross-section of the peptide analogues presented as helical wheels. The hydrophobic (leucine) residues on the non-polar face of each amphipathic α-helix in positions a and d are identical, *i.e.*, the hydrophobic surfaces of each α-helix are identical. The only difference between the two helices is the glutamic acid (peptide EKE) or glutamine (EKQ) residue at position g on the helix (denoted by arrow). The lysine and glutamic acid residues at positions e and b, respectively, serve to stabilize the individual α-helices by intra-chain attractions. It has been demonstrated previously [50] that ion pairs may form between glutamic acid and lysine residues in i, i + 3 or i + 4 positions along the α-helix, with the i + 4 interactions being dominant. From Fig. 8 (right) which presents the analogues as helical nets, it can be seen that the glutamic acid (EKE) and glutamine (EKQ) residues at position g on the helix (Fig, 8, left) are closely adjacent to the hydrophobic face of the helix (between the dotted lines). The arrows denote the intrachain attractions along the hydrophilic face of each helix.

The retention behaviour of the two peptide analogues during RPLC was now investigated at pH 2 and pH 7, the results of which are shown in Fig. 9. At pH 2, peptide EKE is eluted later than peptide EKQ; in contrast, at pH 7, the elution order is reversed with peptide EKE now being eluted considerably earlier than peptide EKQ. In addition, change in pH had little effect on the retention time of peptide EKQ. These results can be summarized as follows: in order of decreasing retention time, *i.e.*, decreasing hydrophobicity of the peptide hydrophobic face interacting with the reversed-phase matrix,

$$\text{EKE,pH2} > \text{EKQ,pH2} = \text{EKQ,pH7} > \text{EKE,pH7}$$

It is interesting to note that, if only the hydro-

Fig. 8. Left: cross-sections of amphipathic α-helical peptides EKQ and EKE represented as helical wheels, looking from the N-terminal end. The dotted lines represent potential intrachain electrostatic interactions between positively charged lysine at position e and a negatively charged glutamic acid at position b. Right: sequences of amphipathic α-helical peptides EKQ and EKE represented as α-helical nets (see Fig. 2 for details). The arrows represented potential intrachain electrostatic interactions between lysine and glutamic acid as noted above. The substituted (glutamine and glutamic acid) residues at position g (see helical wheel presentation) are circled. The linear sequences of peptides EKQ and EKE are shown in Fig. 1.

phobic areas between the dotted lines on the helical nets (Fig. 8, right) were interacting with the hydrophobic stationary phase, then a substitution of glutamine for glutamic acid at position g on the helix (Fig. 8, left) should have no effect on retention behaviour of the peptide analogue at low pH, *i.e.*, the retention times of EKE and EKQ should be the same at pH 2, where glutamic acid, as well as glutamine, is electroneutral. Clearly, since the two peptides are well separated at pH 2 (Fig. 9), the residue at position g on the helix is contributing to the retention process, indicating that the surface area of the helices interacting with the hydrophobic stationary phase is larger than at first surmised. In fact, O'Shea *et al.* [44], during studies on the X-ray structure of the GCN4 leucine zipper coiled coil, noted that the methylene groups of the residue side-chains at positions e and g also contact the hydrophobic side-chains at positions a and d, protecting the hydrophobic interface from solvent. Thus, the hydrophobic interface is actually formed by side-chains of four residues at positions a, d, e and g of the heptad repeat. The retention behaviour during RPLC of the two peptides can thus be rationalized by considering the effect of pH on glutamic acid. At low pH, a protonated glutamic acid residue is more hydrophobic than glutamine [46], which agrees with the longer retention time of peptide EKE compared to peptide EKQ at pH 2 (Fig. 9). However, at pH 7, an ionized glutamic acid residue is considerably more hydrophilic than a glutamine residue [46], which now agrees with peptide EKE being eluted prior to peptide EKQ at pH 7 (Fig. 9). It should be noted that protona-

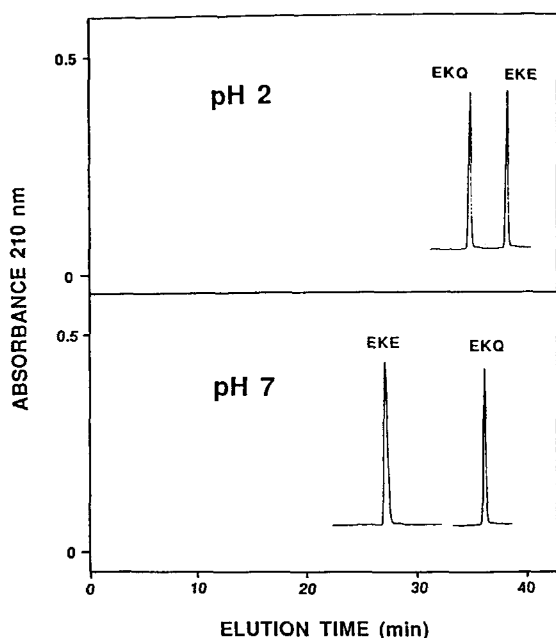


Fig. 9. Effect of pH on reversed-phase chromatographic profiles of single-stranded amphipathic α-helical peptides. The sequences of peptides EKQ and EKE are shown in Figs. 1 and 8.

tion or deprotonation of the glutamic acid residue at position b on the helix (Fig. 8, left), and which is considerably distant from the preferred binding domain, has little or no effect on the retention behaviour of the peptide analogues, as evidenced by the almost identical retention behaviour of peptide EKQ at both pH 2 (neutral, protonated Glu residues) and pH 7 (ionized Glu residues).

The stability of the single-stranded α-helices was now investigated at low and high pH, by temperature denaturation studies of the α-helices in 50% TFE. Loss of helical content with increasing temperature was again monitored by circular dichroism. The results, shown in Fig. 10, can be summarized as follows: in order of decreasing stability,

$$\text{EKE,pH3} > \text{EKQ,pH3} = \text{EKQ,pH7} > \text{EKE,pH7}$$

Clearly, these results correlate very well with the retention behaviour of the peptides illustrated in Fig. 9, *i.e.*, increasing hydrophobicity of the peptides, as expressed by increasing retention time, corresponds with increasing peptide stabili-

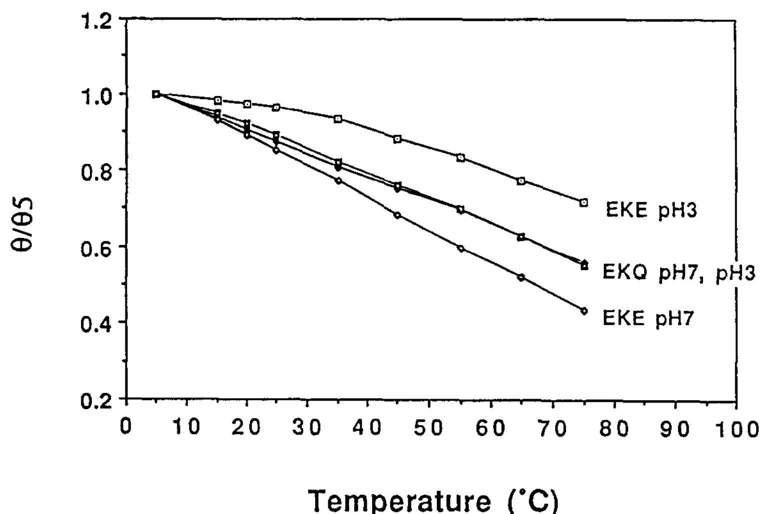


Fig. 10. Thermal melting profiles of single-stranded amphipathic α-helical peptides in 0.1 *M* KCl + 50 m*M* potassium phosphate buffer–trifluoroethanol (1:1, v/v) at pH 3 and pH 7. The sequences of peptides EKQ and EKE are shown in Figs. 1 and 8. θ/θ_5 represents the ratio of the ellipticity at 220 nm at the indicated temperature to the ellipticity at 5°C.

ty in solution. Thus, the protonation of the glutamic acid residue adjacent to the hydrophobic face of peptide EKE (Fig. 8, right) at pH 2 is stabilizing the helix, *i.e.*, the amphipathicity of the helix is being increased. Again, it should be noted that the glutamic acid residue in position b on the helix (Fig. 8, left) which is involved in an ion pair (at pH 7) with lysine at position e, and which is not adjacent to the hydrophobic face of the helix (Fig. 8, right), has no effect on peptide stability with a change in pH, as shown by the essentially identical temperature stability profiles of peptide EKQ at both pH 3 and pH 7 (Fig. 10).

Considering the importance of interchain ionic interactions (e–g′ and e′–g) to stabilizing a parallel and in-register structure of a two-stranded α-helical coiled coil [44], it may be expected that a reduction in pH to pH 2, thereby protonating glutamic acid residues taking part in interchain ion pairs, would have a destabilizing effect on coiled-coil stability. However, from the present study, it is clear that the loss of these ionic interactions between the two helices is more than compensated for by the increase in hydrophobicity of the hydrophobic interface between the chains as glutamic acid in this interface is protonated.

4. Conclusions

In summary, this study represents our initial survey on the potential of RPLC for use as a probe of hydrophobic interactions involved in protein folding and protein stability. By applying RPLC to a *de novo* designed model protein system, namely a two-stranded amphipathic α-helical coiled coil, it was shown that the reversed-phase retention behaviour of various synthetic analogues of monomeric α-helices and dimeric coiled-coil structures correlated well with their stability in solution. In addition, light has now been shed on the long-standing question of why coiled coils, an important structural motif in many biological systems, are more stable at low pH compared to physiological pH values. While not suggesting that RPLC of amphipathic α-helices and the corresponding dimeric coiled-coil structures is an infallible reflection of all hydrophobic interactions stabilizing native proteins, this technique nonetheless shows great promise as a useful (and extremely rapid) complementary technique for understanding peptide/protein interactions.

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