Advanced approaches for the characterization of a de novo designed antiparallel coiled coil peptide

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We report here an advanced approach for the characterization of the folding pattern of a de novo designed antiparallel coiled coil peptide by high-resolution methods. Incorporation of two fluorescence labels at the C- and N-terminus of the peptide chain as well as modification of two hydrophobic core positions by Phe/[15N,13C]Leu enable the study of the folding characteristics and of distinct amino acid side chain interactions by fluorescence resonance energy transfer (FRET) and NMR spectroscopy. Results of both experiments reveal the antiparallel alignment of the helices and thus prove the design concept. This finding is also supported by molecular dynamics simulations. Electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry (ESI-FTICR-MS) in combination with NMR experiments was used for verification of the oligomerization equilibria of the coiled coil peptide.

Introduction

The coiled coil folding motif is one of the most widespread structural motifs in nature.1 Approximately 3% of amino acids in naturally occurring peptides and proteins are involved in the formation of coiled coil structures.2 Usually it consists of two to five amphipathic helices which are wrapped around each other in a parallel or antiparallel manner with a slight superhelical twist.3 A schematic model of an antiparallel coiled coil dimer is shown in Fig. 1. The sequence is characterized by a seven amino acid heptad repeat pattern which is denoted a–g. Positions a and d are usually occupied by apolar amino acids, forming a 3–4 hydrophobic repeat aligned with a “knobs-into-holes” packing in the coiled coil.4 Positions e and g are frequently occupied by charged amino acids which direct the parallel or antiparallel helix orientation forming interhelical electrostatic interactions.5 7 Furthermore, introducing specific buried polar interactions within the hydrophobic core provides an additional possibility for directing the helical alignment more efficiently.4 Although the design principles described above are very well accepted, many de novo designed coiled coil peptides used as model systems have never been investigated concerning their folding specificity using high resolution structure determination methods.

Various attempts have been made to characterize and investigate the relative helix alignment of various de novo designed antiparallel coiled coil peptides in detail. Circular dichroism (CD) spectroscopy and thermal denaturation experiments are the most common methods used for characterization of coiled coil folding motifs. Unfortunately, these approaches do not provide any information about the relative helix alignment of coiled coil peptides.

Studying coiled coil peptides by fluorescence resonance energy transfer allows investigation not only of the process of coiled coil formation itself but also the determination of the parallel or antiparallel orientation of the helices. However, many of the established FRET labels are very large and finding labels that are easy to introduce and small enough for application in short peptides up to 50 amino acids remains a challenge. Using voluminous labels may significantly affect the stability of the specific coiled coil. Another approach to investigate the relative helix alignment is cysteine labelling at the C and/or N termini of the peptide. The orientation can than be determined by disulfide exchange experiments and HPLC.8,9,10 The advantage of this approach is that the range of parameters such as concentration, ionic strength etc. that can be used are solely limited by the detection limit of the HPLC system.

Beyond these relatively simple methods determination of the relative helix orientation would be possible applying high resolution methods such as nuclear magnetic resonance spectroscopy (NMR) and X-ray diffraction. Unfortunately, obtaining detailed information by high resolution methods is time consuming and involves the challenging interpretation of the spectra, expensive instrumentation and the need for relatively large amounts of peptide material in the case of NMR. Nevertheless, different labelling approaches enable the simplification of complex peptide NMR spectra.11

Fig. 1 Schematic model of an antiparallel coiled coil dimer. Yellow circles represent hydrophobic, red and blue circles electrostatic interactions.

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NMR in particular provides a wide range of strategies for the study of coiled coil peptides. Beside the determination of complete high resolution structures of dimeric\(^2\) and trimeric\(^3\) coiled coils as well as the verification of a trimeric coiled coil’s α-helical structure composition,\(^4\) different labelling approaches have been used to provide a variety of additional information. Holtzer and co-workers showed that selective \(^13\)C\(_{\text{a}}\)-labelling of certain amino acids in the 190–254 region of α-tropomyosin and the GCN4 leucine zipper represents an effective strategy to study the thermal unfolding of coiled coil peptides by NMR spectroscopy.\(^{5,6}\) Furthermore, spin inversion labels have been used to investigate thermodynamics and kinetics of the folded and unfolded coiled coil state.\(^7\) Differences in H–D exchange rates of amide protons within the hydrophobic core have been studied as well to characterize the equilibrium between an unstructured monomer and a folded dimeric coiled coil.\(^8\)

The oligomerization state is another important parameter for the characterization of coiled coil peptides.\(^9\) Common methods to address this issue are analytical ultracentrifugation\(^{10,11}\) and size exclusion chromatography.\(^{12}\) Beside these well established methods it is possible to analyze the noncovalent coiled coil peptide complex transferring it as an intact aggregate into a mass spectrometer. In order to maintain the integrity of the complex during the complete ionization and transfer process it is necessary to apply the soft electrospray ionization method, which additionally provides the opportunity to work with solutions under nearly physiological conditions.\(^{13,14}\) Przybylski and co-workers have succeeded transferring the leucine zipper-like homodimer complex of autoantigen L7 and varying leucine zipper model peptides as intact units into the gas phase of different mass spectrometers.\(^{15,16}\) Even though the direct comparison of gas phase behaviour with properties of peptides in solution is controversial, it is nonetheless possible to determine the oligomerization state of coiled coil peptides by ESI-MS.\(^{17}\)

In addition to the experimental approaches described above, theoretical modeling methods have also been used to shed light on the folding and association of coiled coil forming peptides. In particular a number of molecular dynamics simulations of coiled coils have been reported,\(^{18-23}\) which examine the stability or attempt to model the formation of different complexes although none have focussed directly on the question of peptide association or attempt to model the formation of different complexes.\(^{19}\) Differences in H–D exchange rates of amide protons within the hydrophobic core have been studied as well to characterize the equilibrium between an unstructured monomer and a folded dimeric coiled coil.\(^{20}\)

The basis for our investigations is a 41 amino acid coiled coil peptide which has been designed to fold exclusively in an antiparallel manner. The antiparallel folding is dictated by the interhelical electrostatic interactions between position e and g. Repulsive electrostatic interactions between these residues should completely disfavour parallel alignment. In case of antiparallel folding solely attractive electrostatic interactions would stabilize the arrangement.

To prove the antiparallel relative helix orientation of the peptide different labels for the investigation with fluorescence resonance energy transfer (FRET) and nuclear magnetic resonance spectroscopy (NMR) were introduced. The synthesized sequences and the helical wheel illustration for the basis antiparallel coiled coil (AP), the antiparallel coiled coil labelled for FRET (APFRET) and the antiparallel coiled coil modified for NMR (APNMR) are shown in Fig. 2. The coiled coil structure for all peptides has been proven by CD spectroscopy (data not shown).

Furthermore, the enormous potential of ESI-FTICR-MS based on a combination of soft electrospray ionization combined with a high resolution mass analyzer which enables the transfer of intact noncovalent complexes into the gas phase was used to investigate the oligomerization state of peptide APFRET. The de novo designed system is further characterized by molecular dynamics simulations.

**FRET measurements**

Fluorescence quenching by resonance energy transfer\(^{21}\) has been used previously to investigate the formation of a leucine zipper polypeptide. Two different fluorescence labels were introduced at the N-terminal end of two peptide chains which form a heteromeric coiled coil.\(^{22}\) In contrast to this strategy we introduced both, the donor as well as the quencher within one peptide chain to avoid the formation of heteromers and to keep the FRET system as close as possible to the basic antiparallel coiled coil structure of AP. The fluorescent donor 4-amino-3-methylcarboxyanine (\(\lambda_{\text{em}} = 530 \text{ nm} ; \lambda_{\text{abs}} = 420 \text{ nm} \)) was introduced at a lysine sidechain amino function at the C-terminal end of the peptide chain while the fluorescence quencher 3-nitrotroisine (\(\lambda_{\text{em}} = 420 \text{ nm} \)) was introduced as an additional amino acid at the N-terminal end of the basic sequence of AP.\(^{23}\) As the...
helical wheel in Fig. 2 shows, these labels are introduced in $f$ position of the coiled coil heptad repeat and are, thus, expected not to affect the folding and dimerization. Furthermore, the big advantage of these labels is their amino acid like structure which prevents problems like disturbing side reactions of the labels, solubility of the modified peptide and stability of the resulting coiled coil dimer. The characteristic Förster radius $R_0$ of this donor–acceptor pair is approximately 29–31 Å.\(^{25,26}\) To exclude quenching effects within one peptide strand a model of the structure of peptide AP was used to determine the average distances for the folded state. Within a single coiled coil helix the distance between donor and acceptor is approximately 60 Å which is double the Förster radius and is, therefore, expected to cause no internal quenching effects.\(^{21}\) In case of formation of an antiparallel coiled coil dimer the distance between the donor and quencher is circa 20 Å which is 2/3 of the Förster radius. Therefore, an intense quenching is expected for the antiparallel orientation of the coiled coil helices. The obtained spectra of labelled peptide APFRET at different concentrations of denaturating GdnHCl is shown in Fig. 3A. The denaturation process was followed independently by CD spectroscopy at the same GdnHCl concentrations and is shown in Fig. 3B. The resulting fluorescence spectra clearly show a considerable increase in fluorescence intensity at rising concentrations of denaturating agent. During denaturation the helices separate from each other resulting in a decrease in quenching and an increase of the overall fluorescence intensity. Furthermore, the melting curves at Fig. 3B show that the denaturation process is almost complete at 7 M GdnHCl and room temperature. This finding matches the results of the fluorescence spectra which also show the strongest intensity increase between 6 M and 7 M GdnHCl. These data clearly prove the antiparallel orientation of the coiled coil helices in peptide APFRET.

In addition, the enormous stability of the coiled coil secondary structure of APFRET, which is intact up to a concentration of 5 M GdnHCl, should be noted. Folding stability is certainly a function of peptide lengths and number of heptad repeats in particular, but also results from the perfect match of charged amino acids in positions $e$ and $g$ of the heptad repeats.

**ESI-FTICR-MS measurements**

To determine the oligomerization state of the noncovalent complexes, a 50 μM solution of APFRET in 10 mM ammonium acetate buffer (pH 5) was subjected to ESI-FTICR-MS. In order to maintain the complex intact it is necessary to use nearly physiological and soft conditions. Thus, a buffered solution was used and the declustering potential as well as the desolvation temperature were carefully adjusted. Analogous investigations were described recently by Przybylski et al.\(^{25,26}\) According to them, unequivocal identification of an oligomer composed of identical polypeptide chains is obtained by a molecular ion with a non-integer charge number, when divided by the number of complex components. Hence, homodimers of peptides are characterized by all odd-charged ions. A spectrum of APFRET at low declustering potential (DCS = 60 V) is shown in Fig. 4.

**NMR-measurements**

Two different labels were incorporated in the basis sequence of peptide AP to study the conformation of the coiled coil system and to prove the antiparallel orientation by NMR spectroscopy (Fig. 2). The leucine residue at position 9 was replaced by phenylalanine which can easily be assigned by its chemical shifts because there are no other aromatic amino acids in the basis sequence of AP. Furthermore, substituting

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**Fig. 3** (A) Fluorescence spectra of 10 μM APFRET and (B) CD denaturation curves of 50 μM APFRET at different GdnHCl concentrations. (●) 0, (○) 1 M, (■) 2 M, (□) 3 M, (▲) 4 M, (△) 5 M, (●) 6 M, (●) 6.5 M, (○) 7 M GdnHCl.

**Fig. 4** ESI-FTICR-MS spectra of peptide APFRET. (M) Monomer, (D) Dimer.
leucine by phenylalanine within the hydrophobic core does not substantially affect the coiled coil stability, however, might affect the oligomerization equilibria. The second label introduced at position 33 was a uniformly $^{13}$C and $^{15}$N isotope labelled leucine which can be detected independently by $^{13}$C- and $^{15}$N-NMR, respectively. Antiparallel alignment results in a close packing of these labels within the hydrophobic core and, thus, enables the investigation by nuclear Overhauser enhancement spectroscopy (NOESY).

In $^{1}$H, $^{15}$N HSQC spectra two peaks were detected. Therefore we performed temperature and concentration experiments with peptide APNMR and monitored the methyl resonances of the labelled leucine (0.7–1.1 ppm) via $^{13}$C edited proton spectra. The spectra revealed the presence of one further species, which is deshielded to higher ppm values at elevated temperatures (data not shown). At lower peptide concentration three distinct peaks were seen in the $^{1}$H,$^{15}$N HSQC and the $^{1}$H,$^{13}$C edited proton spectra (Fig. 5, 0% TFE and 6A upper spectrum), respectively.

Molecular dynamics simulation

The relative stability and preferred orientation of the APNMR peptide coiled coil was also investigated using molecular dynamic simulation techniques. Two series of simulations starting from idealized parallel and antiparallel configurations were performed consisting of 4 independent runs of 10 ns each for each orientation.

In 2 of the 4 simulations started from an antiparallel orientation the structure of the peptides converged to a classical coiled coil dimer configuration as illustrated in Fig. 7A. The peptides contain a high degree of $\alpha$-helix ($\sim$85%) and interact via a closely packed hydrophobic interface with the exception of the first and last heptad partner. The intermolecular distance between the leucine carbon atoms at the peptide interface is on average 3.5 Å with the width of the distributions at half weight being $\sim$1.0 Å. Once formed the dimeric structure was quite stable with no change being observed when the simulations were extended to 20 ns. In the other 2 simulations initiated from an antiparallel orientation the peptides did not form a classic coiled coil. There was some loss of helical structure in the individual peptides which then interacted with each other in an irregular way.

In none of the 4 simulations in which the two peptides were initially placed in a parallel orientation was the formation of a coiled coil observed. In all cases the individual helices partially unfolded and the peptides did not interact in a regular manner suggesting that a parallel orientation is unstable (see Fig. 7B). To confirm this two additional simulations were performed in...
which the α-helical structure of the peptides was restrained to prevent any unfolding of the individual peptides. Even with the peptide restrained to be α-helical we were unable to generate a stable parallel coiled-coil with the peptides moving away from each other during the 10 ns simulation.

The simulations also provide an opportunity for comparison with the results obtained using NMR. In the analysis of the NMR results it is assumed that in an antiparallel orientation the phenylalanine in position 9 packs against the leucines of the other peptide. In one of the two simulations where an antiparallel coiled coil was stable the phenylalanine at position a did interact closely with the leucines at positions a’ and d’ in the opposing chain. In the other case the phenylalanine at position 9 remained at some distance, >7 Å, from any carbons of the opposing leucines. Furthermore, in the simulations the two peptides readily packed in a variety of different ways suggesting the different chemical shifts observed in the absence of TFE could correspond to different packing arrangements as well as different oligomerization states.

Experimental

Peptide synthesis and purification

All peptides were synthesized by standard Fmoc chemistry on Fmoc-Gly-OWang (0.71 mmol g\(^{-1}\)) and Fmoc-Glu(OtBu)-OWang (0.55 mmol g\(^{-1}\)) resins using a ACT 90 peptide synthesizer (Advanced Chem Tech, Louisville, KY, USA). Purification was carried out by preparative reversed phase high-performance liquid chromatography (HPLC) on a Vydac\(^{\circledR}\) C4 column (10 μM, 300 Å, 250 × 22 mm, Grace Vydac, Hesperia, CA, USA). The molecular weight of the products was determined by MALDI-TOF mass spectrometry using a Voyager-DE\textsuperscript{TM} RP MALDI-TOF Mass spectrometer (Applied Biosystems, Foster City, CA, USA) and its purity was determined by analytical HPLC (Vydac\(^{\circledR}\) C4, 10 μM, 300 Å, 250 × 4.6 mm, Grace Vydac, Hesperia, CA, USA).

CD-spectroscopy

CD measurements were carried out on a J-715 spectrometer equipped with a temperature controller (Jasco inc., Easton, MD, USA) using a quartz cell of 1 mm path length. Denaturation curves were recorded at 222 nm from 20 to 90 °C at a resolution of 0.5 °C with a temperature slope of 5 °C min\(^{-1}\). The acquired spectra were analyzed using Microcal Origin 6.0 (OriginLab Corporation, Northampton, MA, USA). Measurements were carried out in Phosphate buffer (10 mM, 250 mM NaCl) at pH 7.0 with an overall peptide concentration of 50 μM. GdnHCl was taken from a 8 M stock solution in the corresponding phosphate buffer.

Fluorescence spectroscopy

Fluorescence spectra were recorded on a Luminiscence Spectrometer LS 50B (The Perkin Elmer Cooperation, Boston, MA, USA) equipped with a Julabo temperature controller F12 (Julabo GmbH, Germany) using 4.5 ml PMMA cuvettes (Dispolab-Kartell, Milano, Italy). Spectra were recorded from 350 to 600 nm with an excitation wavelength of 320 nm and a scan speed of 250 nm min\(^{-1}\). The acquired spectra were analyzed using FLWinLab 2.0 (The Perkin Elmer Cooperation, Boston, MA, USA) and Microcal Origin 6.0 (OriginLab Cooperation, Northampton, MA, USA). Measurements were carried out in Phosphate buffer (10 mM, 250 mM NaCl) at pH 7.0 with an overall peptide concentration of 10 μM. GdnHCl was taken from a 8 M stock solution in the corresponding phosphate buffer.

ESI-FTICR-MS

ESI-FTICRMS measurements were performed on an Apex II FTICR mass spectrometer with a 7T superconducting magnet (Bruker Daltonics, Billerica, MA, USA) equipped with an ESI source (Agilent Technologies, Waldbronn, Germany). A Cole-Parmer syringe pump (Cole-Parmer, Niles, IL, USA) was used to infuse the solution. The dry gas temperature was set to 150 °C to prevent dissociation of the non-covalent complexes. The analysis was performed in the positive ionization mode with a capillary-exit-skimmer voltage (DCS) of 60 V. For the ESI-FTICR measurements the peptide was dissolved in 10 mM NH\text{Ac}-solution (pH = 5) forming a 50 μM solution.

NMR spectroscopy

The NMR experiments were performed with a Bruker Avance 700 spectrometer equipped with a 5 mm inverse triple-resonance probehead or a Bruker Avance DRX 400 with a 5 mm TBI or BBO probehead. All experiments were performed at 298 K. 1D\(-\text{13C}\) edited proton spectra were recorded as follows: after a 90° pulse on protons and 13C a gradient (1 ms, 27.5 G cm\(^{-1}\)) was applied. A 180° pulse on protons was followed by a gradient (1 ms, 22 G cm\(^{-1}\)), a 90° pulse on carbons and a final gradient (1 ms, 16.56 G cm\(^{-1}\)) before acquisition under GARP decoupling.

The pulse sequence for the NOEs spectra began with developing of the NOE effect by applying three 90° pulses and carbon decoupling (180° pulse) during the preparation period. In the second part only the 13C bound protons were selected by an initial INEPT step (with a 1 ms trim-pulse before the second 90° pulse). After a 180° pulse on protons and a 1 ms gradient (44 G cm\(^{-1}\)) a 180° pulse on carbon was applied. Between the back-INEPT step and acquisition a further gradient was applied (1 ms, 11.06 G cm\(^{-1}\)). Acquisition was done under GARP decoupling.

The samples contained typically 1–3 mM peptide and 100–300 mM sodium chloride (the peptide to NaCl ratio was 1 : 100) and DSS in 50 mM sodium phosphate buffer, pH approx. 7. The samples were prepared in water with 10% D\text{2}O or in pure D\text{2}O.

Molecular dynamics simulations

Two series of simulations of the APNMR peptide starting from idealized parallel and antiparallel coiled coil dimers in which the leucines of both peptides are oriented to the core of the structure were performed. The secondary structure of the individual peptides was modelled as an ideal α-helix, generated using the program WHATIF.\textsuperscript{38} The two peptides were placed in a periodic rhombic dodecahedron box separated by a small distance (minimal distance = 4 Å) to which around 11000 water molecules were added. The simple point charge (SPC) water model\textsuperscript{37} was used to describe the water. The GROMOS96 (43a2) force field\textsuperscript{39} was used to describe the peptide. The protonation state of the residues was chosen appropriate for approximately pH 7. The N termini, arginines and lysines were protonated while the C termini and glutamates were deprotonated.

All simulations were performed using the GROMACS package, version 3.0b5a1 (http://www.gromacs.org) at constant temperature and pressure. The water and peptides were coupled separately to a temperature bath\textsuperscript{42} at 25 °C using a coupling constant of 0.1 ps. The pressure was maintained by weak coupling\textsuperscript{42} to a reference pressure of 1 bar, with a coupling time of 0.5 ps and an isothermal compressibility of 4.6 × 10\(^{-5}\) bar\(^{-1}\). Nonbonded interactions were evaluated using a twin range cutoff of 9 and 14 Å. Interactions within the shorter and longer cutoffs being updated every step and every five steps respectively. Beyond the 14 Å cutoff a reaction field correction with a dielectric constant ε of 78.0 was used. The bond lengths and angles in water were constrained using the SETTLE algorithm\textsuperscript{40} while the LINCS algorithm\textsuperscript{40} was used to constrain bond lengths within the peptide. The equations of motion were integrated using the leap-frog method with a timestep of 2 fs.
The initial velocities were taken from a Maxwell distribution at 25 °C with different random distributions used for each simulation. The minimum distance between periodic images of the peptides during the simulations was always > 14 Å.

Conclusion
The folding characteristics of a de novo designed 41 amino acid antiparallel coiled coil peptide was studied by a combination of several methods including FRET, NMR, ESI-FTICR-MS, and molecular dynamics simulation. Antiparallel peptide folding was forced by the amino acid design in the charged region of the coiled coil peptide.

FRET experiments showed antiparallel folding of the coiled coil peptide. Appropriate labelling enabled the application of high-resolution NMR methods for the detection of interhelical amino acid interactions in a coiled coil peptide and, based on that, the exact correlation of the helix alignment. Furthermore, NMR experiments in combination with ESI-FTICR-MS were used to investigate the oligomerization equilibria of the peptide. Both methods reveal the existence of a coiled coil dimer. Molecular dynamics simulation showed that the preferred orientation of the peptide in the dimer is antiparallel as only this alignment leads to a system which is stable over a period of 20 ns.

Thus, the proof of the design concept could be achieved by all of the applied methods.

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