The effects of individual amino acids on the fast folding dynamics of α-helical peptides

Edward A. Gooding,*[a] Angela Pozo Ramajo,[b] JunWei Wang,[b] Colin Palmer,[a] Elizabeth Fouts[a] and Martin Volk*[b]

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Nanosecond temperature jump experiments coupled to time-resolved infrared spectroscopy were carried out on a series of alanine-based peptides containing different guest amino acids to study the effects of residues with different helix propensities on the helix–coil dynamics.

The question of how proteins fold to their unique and highly organised functional three-dimensional structure is one of the most challenging questions currently investigated in biological research. The first steps during the folding of a protein may involve the formation of secondary structures, which occurs on the nano- to microsecond time scale; only in recent years has it become possible to directly observe this process.[1,2] In particular, the stability and dynamics of the α-helix, the most abundant secondary structural motif, have been at the focus of experimental investigations, which mostly made use of simple alanine-based model peptides, thus avoiding the heterogeneity and complexity of natural proteins.

Alanine-based peptides are known to form helical structures in aqueous solution because of the high helix propensity of alanine. Introducing a guest amino acid into an alanine-based peptide modifies its helical stability, which allows one to investigate the intrinsic stabilising or destabilising effects of particular residues and their interactions with the polypeptide backbone. Similarly, it is possible to study the effects of particular residue–residue interactions on helix stability by incorporating the relevant amino acids at well-defined positions. Numerous studies of this kind have resulted in a detailed picture of the various factors governing thermodynamic stability of α-helices. Using increasingly sophisticated modifications of helix–coil theory, which analyses helical stability in the framework of statistical mechanics concepts, it is now possible to predict the (average) helicity of a peptide with a given primary sequence under particular conditions (temperature, pH and ionic strength) with high accuracy.[3–6]

The investigation of *dynamic* aspects of the helix–coil transition, which are of particular importance for understanding the process of protein folding, has started only recently. Helix–coil relaxation has been shown to proceed on the 10 to 100 ns-time scale;[1,7] and peptide length,[8] temperature,[8–10] solvent viscosity[10] and ionic strength[11] have been shown to affect the relaxation time. However, so far no systematic study has been carried out to investigate the effect which the substitution of individual amino acid residues may have on the dynamics of the helix–coil transition.

Here, we show that it is now possible to investigate the effects of individual guest amino acids on the folding *dynamics* of alanine-based host peptides, in analogy to the extensive work that has been carried out on helix *stability*.

We have studied the folding dynamics of a series of alanine-based α-helical peptides of the structure Ac–$(AAXAA)_4$GY–NH_2, where X stands for R, K, E or Q, respectively;† in the following, these peptides are denoted as AR, AK, AE and AQ. All guest amino acids used here have polar side chains, but they include positively charged (R and K), negatively charged (E) and neutral (Q) residues, with significantly different helix propensities.[3] Consequently, the helicity (fraction of residues in helical conformation), which was determined by CD spectroscopy,‡ was found to vary significantly for the different peptides, between 44% and 66% at 28 °C, see Table 1.§ FTIR spectra show an amide I′ band¶ with a maximum near 1635 cm^{-1} at low temperatures, which gradually broadens and shifts to higher frequencies upon increasing the temperature, see Fig. 1 for representative spectra. The amide I′ band can be decomposed into subbands corresponding to α-helical and random coil conformers, and it is the changing population of conformers that results in the apparent broadening and blue shift upon heating.[1,12] In agreement with CD spectra, the FTIR spectra confirm that the peptides undergo the well-characterised broad temperature-induced transition from helical to random coil conformation observed for α-helical model peptides, with the amide I′ band of solvated α-helices in D_2O found around 1630 cm^{-1}, and that of random coil structures closer to 1645 cm^{-1}.[1,12]

Thus, a sudden increase in the temperature shifts the equilibrium towards less folded conformations, and the relaxation towards the new equilibrium can be monitored by a suitable technique, such as nanosecond time-resolved IR spectroscopy of the amide I′ band. Temperature jumps of 5 °C on the nanosecond time scale were induced by short laser pulses and the ensuing IR absorbance changes were monitored with a time resolution of 14 ns.‖

Table 1 Helicity, h_{23} and h_{28} (±1.5%), at 23 and 28 °C and helix–coil relaxation time, τ_{rel}, after temperature jumps from 23 to 28 °C for peptides AR, AK, AE and AQ

	h_{23} (%)	h_{28} (%)	τ_{rel} (ns)
AR	73	66	184 (±2)
AK	67	60	194 (±4)
AE	58	51	147 (±2)
AQ	50	44	117 (±3)

[a]*Department of Chemistry and Biochemistry, Swarthmore College, Swarthmore, PA 19081, USA. E-mail: egoodin1@swarthmore.edu; Fax: +1-610-328-7355; Tel: +1-610-328-8558*
[b]*Department of Chemistry and Surface Science Research Centre, University of Liverpool, Liverpool, UK L69 3BX. E-mail: m.volk@liv.ac.uk; Fax: +44-151-794-3870; Tel: +44-151-794-3317*

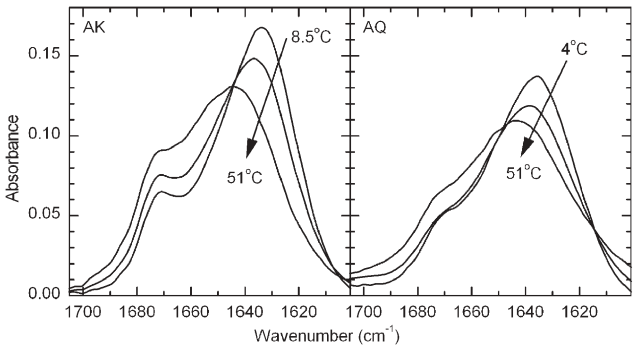

Fig. 1 Temperature-dependent FTIR spectra in the amide I′ band of peptides AK (8.5, 24 and 51 °C) and AQ (4, 26 and 51 °C). The shoulder at 1672 cm^{-1} is due to the TFA counterion.

The inset of Fig. 2 shows the results for peptide AK at 1582 cm^{-1}, where only D_2O has significant absorbance, and at 1632 cm^{-1}, where D_2O and the peptide contribute. The D_2O absorbance decrease, arising from a shift of the D_2O absorbance band with temperature, appears instantaneously within the time resolution of our setup and remains constant up to the millisecond time scale, when the sample cools due to thermal diffusion (data not shown). In addition to this instantaneous bleach of the D_2O absorbance, and a similar instantaneous bleach of the peptide absorbance due to a temperature-induced shift of the amide I′ band,[12] the absorbance at 1632 cm^{-1} shows a significant dynamic component on the 100 ns-time scale, which is due to the helix–coil relaxation towards the new equilibrium at the higher temperature. The temperature jump-induced absorbance changes at 1632 cm^{-1} for all four peptides are shown in Fig. 2. For better comparability, these curves have been normalised to the amplitude of the dynamic component on the 100 ns-time scale. All data can be well fitted using a single exponential function;** the resulting helix–coil relaxation times are summarised in Table 1.

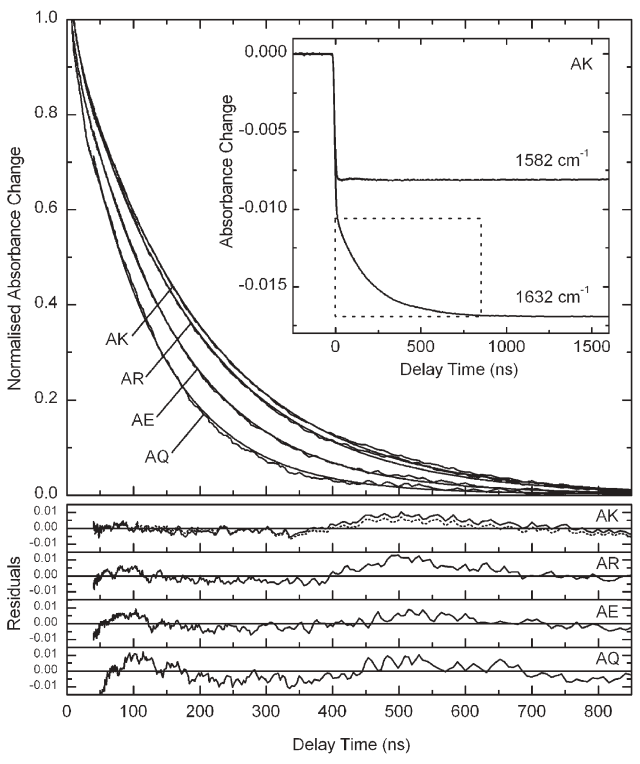

Fig. 2 Absorbance changes at 1632 cm^{-1} observed after temperature jumps from 23 to 28 °C for peptides AK, AR, AE and AQ in 0.2 M NaCl–0.05 M phosphate buffer (pH 7)–D_2O, normalised to the amplitude of the dynamic component on the 100 ns-time scale. Also shown are the results of single exponential fits of the data and the resulting residuals (solid lines). The dotted line shown in the residual plot for peptide AK are the residuals of a biexponential fit. Inset: Absorbance changes at 1582 and 1632 cm^{-1} for peptide AK, not normalised; the dotted rectangle indicates the data range selected for the main figure.

Short α-helical alanine-based peptides exist in an equilibrium between many (folded, partially folded and unfolded) states.[7,13] Previous experimental results have shown that the relaxation dynamics observed on the 100-ns time scale encompass both helix propagation (elongation of existing helical segments) and nucleation (formation of the first helical turn) and their reverse processes, with very different activation energies and entropies.[7,14] Thus, helix–coil relaxation is a multi-step process and cannot be described by a two-state kinetic model, although it may show close to single exponential behaviour;[15] any detailed analysis (including the attempt to obtain information on transition states and activation energies) requires the use of more advanced modelling, such as the kinetic zipper model.[7,13]

It is obvious from Fig. 2 and the fit results in Table 1 that the four peptides have significantly different relaxation dynamics. Thus, individual amino acid residues have a considerable effect on helix–coil dynamics, similar to their effect on helix stability. Our results indicate a correlation between the helix–coil relaxation time and the helicity of the peptide, and thus the helical propensity of the guest amino acid. With increasing helicity, the helix–coil relaxation becomes slower up to an apparent maximum time constant at around 60% helicity (at the final temperature, at which the relaxation is taking place). This is in qualitative agreement with a simplifying theory of the helix–coil transition kinetics, which predicts the maximum relaxation time to occur at the midpoint of the transition, *i.e.* 50% helicity, when changing the (average) helix propensity.[16] However, this model applies strictly only to very long polypeptides and is based on a number of other simplifying assumptions (homogeneous peptide with identical residues, infinitesimally small perturbation, narrow relaxation spectrum), so that it is not surprising that the agreement is not quantitative. More detailed kinetic simulations, based on the kinetic zipper model for short peptides,[7,13] which are beyond the scope of this paper, will be needed to model and investigate in detail this apparent correlation between helix–coil dynamics and helical propensity.

On the other hand, there may be additional factors relating to the replacement of individual amino acid residues which modify the folding *dynamics* of α-helices, but do not, or not greatly, affect helical *stability*, similar to the effect of solvent viscosity, which has been shown to affect helix–coil dynamics without having any effect on helical stability.[10] One such factor, which may be important for the peptides investigated here, is the size and type of the counterions balancing the charges on some of the residues. Screening of electrostatic interactions by ionic clouds has been shown to affect helical stability,[17] but the ions and their hydration layer, which might move with the peptide backbone during folding/unfolding and will certainly cause additional

hydrodynamic friction, may have a further, purely *dynamic*, effect. We note that for the peptides investigated here the fastest dynamics were found for the neutral peptide AQ, which does not have any major ionic cloud. The negatively charged peptide AE (counterion: Na^+) has slower dynamics and the positively charged peptides AR and AK, with the larger counterions Cl^-, TFA^- and/or phosphate, have the slowest helix–coil relaxation. However, the size of the counterionic cloud and its hydration layer depend in a complicated manner on ion size, charge and polarisability,[18–20] and it will require further detailed investigations (*e.g.* using buffer solutions based on different salts at varying ionic strength) to investigate this effect in more detail.

In conclusion, our experimental results confirm for the first time that the substitution of individual amino acid residues in an alanine-based peptide not only affects the helical stability of the peptide, but also the kinetics of its helix–coil relaxation. This finding confirms that it is possible to study the effects of individual guest amino acids on α-helical folding dynamics, in a manner similar to the detailed investigation of α-helical stability carried out over the last two decades. In the long term, such measurements are expected to result in a more complete picture of the process of α-helix folding and to contribute to the discussion of dynamic control of protein folding pathways.

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Notes and references

† Abbreviations: Ac, acetyl; A, alanine; G, glycine; Y, tyrosine; R, arginine; K, lysine; E, glutamic acid; Q, glutamine. Peptides were synthesised using a PerSeptive Biosystems Pioneer solid-phase peptide synthesiser and purified by HPLC. Peptide identity and purity were confirmed by electrospray mass spectrometry. Prior to CD- and IR-measurements, peptides were lyophilised from D_2O to effect H/D-exchange. All peptides showed high solubility in D_2O, with the exception of AQ, for which slight aggregation was observed at the concentrations used here, although no indication for the formation of β-sheet structures was found in FTIR or CD spectra. All samples contained trifluoroacetate (TFA) counterions from the purification process, as indicated by a TFA absorbance band at 1672 cm^{-1}; we estimate the concentration of TFA in a 5 mM peptide sample to be 20–30 mM for the positively charged peptides AR and AK, and 5–10 mM for peptides AE and AQ.

‡ Peptides were redissolved to a concentration of 2–5 mM (43–105 mM on a per-residue basis) in 0.2 M NaCl–0.05 M phosphate buffer (pH 7)–D_2O and placed in a fused silica cuvette with 110 μm pathlength. Peptide concentration was determined from the tyrosine absorbance at 269 and 277 nm. CD spectra at 17, 24 and 31 °C were recorded on an Aviv 62 DS spectrometer, calibrated using (+)-10-camphorsulfonic acid. Fractional helicities were calculated from the CD signal at 222 nm following standard procedures.[21] The helicities at 23 and 28 °C were determined from the measurements by linear interpolation. FTIR spectra were recorded on a Digilab Excalibur FTS 3000 spectrometer with 1 cm^{-1} nominal resolution, using a 56 μm pathlength cell. Water vapour and temperature-matched solvent spectra were subtracted to obtain a flat baseline.

§ The helicity of 65% at 24 °C found here for peptide AK is larger than the value of approximately 59% at 20 °C reported previously for the same peptide in H_2O;[22] however, the previous result was obtained at an ionic strength of 0.1 M, whereas our results refer to an ionic strength of 0.3 M, and the helicity of peptides of this type is known to increase significantly with increasing ionic strength.[5]

¶ The prime indicates deuterated amides.

‖ Samples were dissolved to a concentration of 5 mM in 0.2 M NaCl–0.05 M phosphate buffer (pH 7)–D_2O, lyophilised, redissolved in D_2O to a concentration of 5 mM and placed in an infrared cell with 53 μm pathlength. Sample temperature was monitored by a calibrated Pt100 resistor and was kept at 23.0 ± 0.3 °C for all measurements reported here. Temperature jumps were induced by direct excitation of the overtone band of D_2O at 1970 nm with 7 ns pulses with an energy of 3 mJ (Nd:YAG/dye laser system with difference frequency generation, CCLRC Central Laser Facility loan pool system NSL4). The temperature jump size was measured using the induced D_2O absorbance change at 1582 cm^{-1}, calibrated from temperature-dependent FTIR spectra of neat D_2O, and was kept at 5 ± 0.3 °C. Time-resolved absorbance changes at 1582 and 1632 cm^{-1} were monitored using a tunable IR-laser diode and a 50 MHz IR-detector (overall signal rise time 14 ns). More details on the temperature jump setup and data analysis have been given elsewhere.[11] FTIR spectra at room temperature were taken before and after the temperature jump experiments; in all cases no significant differences between these spectra were found, confirming that no permanent structural changes had occurred during the course of the experiment.

** The residuals of the single exponential fits to the relaxation of all four peptides show a similar structure, arising from an experimental artifact which is also observed after temperature jumps in the absence of peptide. We assign this artifact to pressure wave effects: The sudden increase of temperature results in high pressure, which subsequently is released on a time scale determined by the speed of sound, with a pressure relief time of approximately 150 ns for a pump focus of 300 μm diameter; due to the resulting inertial outward motion of solvent molecules, the pressure then decreases to values below the surrounding pressure before recovering on the time scale of a few hundred nanoseconds.[23] This outward and inward flow of solvent molecules leads to a modulation of the solvent absorbance with a magnitude which can be estimated from the pressure modulation calculated by finite element calculations[23] and which corresponds reasonably well to the observed modulation. In agreement with this assignment a similar modulation is seen in the residuals of a biexponential fit, see Fig. 2, or a stretched exponential fit (data not shown), which shows that it does not arise from multi-exponential relaxation.

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