A single amino acid can switch the oligomerization state of the α -helical coiled-coil domain of cartilage matrix protein

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We have studied the oligomerization of an α -helical coiled-coil using as an example a peptide corresponding to the C-terminal domain of cartilage matrix protein. By replacing one arginine residue, which forms an interchain ionic interaction with a glutamic acid residue, with glutamine, we found that this peptide assembles into a homotetramer at neutral pH in contrast to the native molecule which forms homotrimers. At acidic and basic pH, however, we again observed the trimer conformation. Another arginine, which is probably involved in an intrachain salt bridge, has no effect on the assembly. Our data demonstrate that besides the specific distribution of hydrophobic residues, interchain ionic interactions can be crucial in modulating the association behavior of α -helical coiled-coil domains.

Keywords: analytical ultracentrifugation/circular dichroism spectroscopy/heptad repeats/ionic interactions/synthetic peptides

Introduction

Coiled-coil formation of α -helical polypeptide chains is a frequently observed means for the specific oligomerization of both intra- and extracellular proteins. Meanwhile, the structures of >20 proteins containing coiled-coil domains have been solved at high resolution, including parallel and antiparallel aligned amphipathic helices consisting of two to five identical or different strands (Lupas, 1996; Malashkevich *et al.*, 1996; Momany *et al.*, 1996). On the primary structure level, the probability for forming a coiled-coil can be readily predicted on the basis of a repeating sequence motif which contains hydrophobic residues in a 3-4-3-4 spacing (Crick, 1953; Sodek *et al.*, 1972; Parry, 1982). When written as a heptad of the form $(abcdefg)_n$, positions *a* and *d* are chiefly occupied by apolar residues, whereas the other residues are in general of polar character. Positions *e* and *g* are frequently filled with charged residues which might contribute to stability, the specificity of helix association and their relative stagger

(for reviews see Cohen and Parry, 1990; Hodges, 1996). Such interacting right-handed helices wind around one another in a left-handed supercoil in which the hydrophobic residues become shielded from the aqueous environment and a residue from one helix (knob) packs into the space surrounded by four sidechains of the facing helix (hole) (for details see Lupas, 1996). Although the detection of a putative coiled-coil forming sequence region has become rather straightforward due to the availability of different computer programs (Lupas *et al.*, 1991; Berger *et al.*, 1995), it is still not clear what determines the oligomerization state of these helices. A systematic study of mutants of the yeast transcription factor GCN4 revealed the crucial role of the geometry of the residues in heptad positions *a* and *d* which in this case determine the assembly to two-, three- or four-stranded coiled-coils, but also allow for the co-existence of several oligomers (Harbury *et al.*, 1993; Gonzales *et al.*, 1996a,b). On the basis of several *de novo* designed peptides, it has been shown that interchain ionic attractions and repulsions can control the parallel and antiparallel alignment of α -helices within the coiled-coil (Monera *et al.*, 1994a). Furthermore, it was found that interhelical ionic interactions influence the folding and stability of such peptides (Zhou *et al.*, 1994a). Considering the different occurrence of specific apolar and charged residues in heptad positions *a/d* and *e/g* respectively, a profile method has been recently developed to distinguish between dimer and trimer formation with reasonable confidence (Woolfson and Alber, 1995).

By applying a synthetic peptide approach, we have shown that the 36 C-terminal residues of cartilage matrix protein (CMP) determine the assembly of three identical polypeptide chains by forming a three-stranded α -helical coiled-coil (Beck *et al.*, 1996). In the native protein, the homotrimer is further stabilized by disulfide-bridges formed between two cysteine residues directly preceding this domain (Hauser and Paulsson, 1994; Haudenschild *et al.*, 1995). CMP is a 148 kDa extracellular matrix glycoprotein specifically localized to cartilage (Paulsson and Heinegård, 1981). It seems tightly associated with aggrecan and is found co-localized with collagen type II fibrils (Paulsson and Heinegård, 1979; Winterbottom *et al.*, 1992). Sequences have been determined for chicken, mouse and human CMP, and they are highly similar (Kiss *et al.*, 1989; Jenkins *et al.*, 1990; Aszódi *et al.*, 1996). At the aminoterminal end and directly adjacent to the coiled-coil domain, CMP contains von Willebrand factor A domains (each ~200 residues in length) which are separated by an epidermal growth factor-like domain (~40 residues) resulting in a total length of ~470 residues per monomeric chain.

The coiled-coil domain of human and mouse CMP contains two arginine residues in corresponding positions where the first one (Arg479 in human, Arg483 in mouse)

Fig. 1. Sequences of synthetic peptides arranged in heptads. RR-CMP-C36 corresponds to the C-terminal 36 residues of human CMP. In QR-, RQ- and QQ-CMP-C36, arginine residues are replaced by glutamine (arrows). Residues in heptad positions *a* and *d* are printed in inverse font. Putative ionic interactions between charged residues either within a peptide chain (spacing $i \rightarrow i + 3$; $i \rightarrow i + 4$) or between adjacent chains in a coiled-coil conformation ($g-e'$) are indicated at top. Residues which are identical in all peptides are marked by dots. For comparison, the corresponding sequence parts of mouse and chicken CMP are included at the bottom. Numbers in the top rows refer to the positions within the complete human CMP sequence and the peptides respectively.

is followed three residues apart by glutamic acid. Based on the geometry of the α -helix and experiments performed on proteins and model peptides (Sundaralingam *et al.*, 1985; Marqusee and Baldwin, 1987), the spacing of oppositely charged residues by $i \rightarrow i + 4$ suggests that these amino acids could form an intrachain salt bridge. When written as heptads, the second arginine residue occupies position *g* and thus might be able to form an $i \rightarrow i' + 5$ interchain salt bridge with the glutamic acid in position *e* of the following heptad (i' prime indicates a residue from an adjacent chain; for the staggering and interaction of residues within the coiled-coil see McLachlan and Stewart, 1975). Interestingly, these latter arginine and glutamic acid residues are conserved in all three known sequences. To address the function of the arginine residues for the assembly and the stability of the CMP coiled-coil domain, we studied peptides in which they are replaced by glutamine. Glutamine was chosen to preserve the polar character of the side chain and as in the chicken CMP sequence, the first arginine of the human and mouse sequence is indeed replaced by Gln. We found that the second arginine is crucial for the trimeric structure of the coiled-coil at physiological pH. When replaced by glutamine, this domain forms a tetramer at pH 7, but trimers at acidic and basic pH. This suggests that interchain ionic interactions can modulate the oligomerization state of α -helical coiled-coil domains independently of the hydrophobic nature of the core residues.

Results

CMP-C36 related peptides designed to study ionic interactions

We have recently studied in detail a synthetic peptide with a sequence corresponding to the 36 C-terminal residues of human CMP by means of CD spectroscopy, gel filtration, chemical crosslinking and analytical ultracentrifugation (Beck *et al.*, 1996). The sequence of this peptide, which we will refer to as RR-CMP-C36, and its arrangement into heptad repeats is shown in Figure 1. Heptad positions *a* are mainly occupied by leucine residues, whereas position *d* shows an alternating pattern of leucine and valine residues. According to the criteria of Harbury *et al.* (1993), this configuration of apolar residues should favor a trimeric assembly which we indeed observed. Several

oppositely charged residues depict a spacing of $i \rightarrow i + 3$ and $i \rightarrow i + 4$ which might stabilize the helical structure by intrachain ionic interactions (Sundaralingam *et al.*, 1985; Marqusee and Baldwin, 1987). A single pair of charged residues, Arg27–Glu32, is located in heptad positions *g-e'* compatible with the formation of an interchain ionic interaction. We have now included in our study a set of three peptides, in which Arg19 (QR-CMP-C36), Arg27 (RQ-CMP-C36) or both arginines (QQ-CMP-C36) are replaced by glutamine and thus should be devoid of the corresponding ionic interactions. When the human and chicken CMP sequences are compared, they show an identity of only 56%, but four of the five residues in heptad position *d* as well as the second arginine (not the first one, however) are conserved (see arrows in Figure 1).

Purity and identity of the synthesized peptides were confirmed by amino acid analysis and Edman sequencing. Mass spectroscopy revealed single peaks at m/z ratios of 3997.7, 3967.3, 3968.9 and 3939.8 Da for RR-, QR-, RQ- and QQ-CMP-C36 respectively, which deviate <2 Da from the calculated masses.

The secondary structure of CMP-C36 related peptides is mainly α -helical

Far ultraviolet CD spectra show a high degree of α -helical structure for all four peptides at 5°C (at all benign solution conditions tested) as is qualitatively evident from the amplitudes observed at 192, 208 and 221 nm (Figure 2). The very high molar ellipticities observed under benign conditions around 220 nm ($\sim 30\,000$ deg cm²/dmol) did not increase significantly in the presence of the helix inducing solvent 1,1,1-trifluoroethanol (TFE). The observed ratios of ellipticities $[\Theta]_{220}/[\Theta]_{208}$ are 0.98–1.02 which decrease to 0.85–0.92 in the presence of 50% TFE. Values close to 1.0 were previously proposed as diagnostic of a coiled-coil structure (Lau *et al.*, 1984). The decrease upon addition of TFE which is known to disrupt tertiary and quaternary structures is indicative for extended or non-interacting α -helices (Lau *et al.*, 1984; Cooper and Woody, 1990).

Hydrophobic packing and one salt bridge contribute to the stability of RR-CMP-C36

To investigate the influence of hydrophobic packing on the integrity of the coiled-coil, we measured the stability

Fig. 2. CD spectra of CMP-C36 related peptides. Spectra were recorded in the presence (.....) and absence (—) of 50% TFE for RR- (A), QR- (B), RQ- (C) and QQ-CMP-C36 (D) (peptide concentrations: 50 μ M; 250 mM NaF, 20 mM $\text{KH}_2\text{PO}_4/\text{NaOH}$, pH 7.2, 5°C). The change of the ellipticity ratios at 208 and 220 nm reflects a TFE induced transition from interacting to single α -helices.

of our peptides in the presence of urea and guanidine-HCl by following the CD signal change at 221 nm. Assuming complete denaturation at 7 and 5 M urea and guanidine-HCl respectively, we calculated the fraction of folded peptides (Figure 3). In the case of urea, RR- and QR-CMP-C36 show very similar high stabilities with a transition midpoint $F = 0.5$, at which half of the molecules are folded and the other half are in a random coil conformation, of ~ 4 M urea. RQ- and QQ-CMP-C36 also have stabilities similar to each other, but considerably lower than the other two peptides (see Table I). If the salt concentration is increased from 0.1 to 0.25 M NaF, the urea concentration needed to achieve the transition midpoint increases by 0.4–0.5 M urea for all four peptides (data not shown). The similar increase in $[\text{urea}]_{1/2}$ values, upon increasing the ionic strength of the solvent, confirms that hydrophobic packing is the major force contributing to the stability of the coiled-coil. The relative change of the $[\text{denaturant}]_{1/2}$ values for the different peptides corresponds well with their thermal stability. Under corresponding conditions in the absence of denaturant, RR- and QR-CMP-C36 as one pair, and RQ- and QQ-CMP-C36 as another pair, show nearly identical melting temperatures of $\sim 62^\circ\text{C}$ and 58°C respectively.

If the more chaotropic guanidine-HCl is used as a denaturant, lower concentrations are sufficient to reach a value of $F = 0.5$ for RR- and QR-CMP-C36 (Figure 3B). For RQ- and QQ-CMP-C36, however, the necessary guanidine-HCl concentrations are indeed very similar to

Fig. 3. Stability of CMP-C36 related peptides against denaturants. Unfolding of peptides was monitored by the CD signal change at 221 nm at different concentrations of urea (A) and guanidine-HCl (B) and the fraction folded was calculated as described in Materials and methods (peptide concentrations: 50 μ M in 100 mM NaF, 20 mM $\text{KH}_2\text{PO}_4/\text{NaOH}$, pH 7.2, $T = 5^\circ\text{C}$). Symbols denote RR- (●), QR- (○), RQ- (■) and QQ-CMP-C36 (□). The free energy of unfolding ΔG was calculated assuming a two state transition with $K_{app} = F/(1-F)$ (insets; compare Table I).

those of urea (Table I). In contrast to urea, guanidine-HCl is a charged molecule and thus contributes to the ionic strength of the solvent. The high guanidine-HCl concentrations necessary to achieve the effect of urea therefore point to the stabilization due to hydrophobic interaction. For a series of α -helical coiled-coil model peptides it has been found that guanidine-HCl masks electrostatic interactions and the differences between $[\text{urea}]_{1/2}$ and $[\text{guanidine-HCl}]_{1/2}$ values vary depending on the contributions of hydrophobic and ionic interactions to protein stability (Monera *et al.*, 1994a,b; Smith and Scholtz, 1996).

The monophasic transition observed for all peptides both in the case of urea and guanidine-HCl, and in addition our observation that all peptides elute as single symmetric peaks upon size exclusion chromatography both under native and denaturing conditions (data not shown),

Table I. Stability of CMP-C36 peptides against denaturants and melting temperature at pH 7.2

	RR-CMP-C36	QR-CMP-C36	RQ-CMP-C36	QQ-CMP-C36
Urea:				
[Urea] _{1/2} (M)	4.00 ± 0.02	3.94 ± 0.02	1.94 ± 0.02	1.76 ± 0.02
Δ[Urea] _{1/2} (M)	–	0.06	2.06	2.24
ΔG _u ^{H₂O} (kJ/mol)	20.6 ± 0.4	18.2 ± 0.2	13.1 ± 0.5	11.8 ± 0.2
ΔΔG _u (kJ/mol)	–	0.3	13.1	13.4
Guanidine-HCl:				
[GuHCl] _{1/2} (M)	2.98 ± 0.02	2.81 ± 0.02	1.97 ± 0.02	1.66 ± 0.02
Δ[GuHCl] _{1/2} (M)	–	0.17	1.01	1.32
ΔG _u ^{H₂O} (kJ/mol)	25.1 ± 0.7	19.2 ± 0.5	18.6 ± 0.3	14.2 ± 0.2
ΔΔG _u (kJ/mol)	–	1.3	9.0	11.2
T _m (0.1 M NaF, pH 7.2) (°C)	62.3	61.2	58.8	57.0

[denaturant]_{1/2} denotes denaturant concentration at a folded fraction of $F = 0.5$; Δ[denaturant]_{1/2} is the difference in [denaturant]_{1/2} to RR-CMP-C36; ΔG_u^{H₂O} is an estimate of the (minimum) conformational stability at zero concentration of denaturants resulting from linear extrapolation (Pace, 1986), and ΔΔG_u the free energy difference to RR-CMP-C36 calculated as described in Materials and methods. Experimental conditions are as noted in the legend to Figure 3. For comparison, melting temperatures T_m (see Figure 4) are included.

allows us to assume a two state mechanism of unfolding. Thus, we can calculate the change of the conformational free energy of unfolding ΔG_u as a function of the concentration of denaturant (insets in Figure 3; see equation 4 in Materials and methods). Linear extrapolation to 0 M denaturant concentration reveals minimum ΔG_u^{H₂O} values of ~18–25 kJ/mol for RR- and QR-CMP-C36, and 12–19 kJ/mol for RQ- and QQ-CMP-C36 (Table I). The considerably higher values observed upon denaturation with guanidine-HCl further indicate the stabilizing effect of salt, but in general these values are found to be higher than when derived from urea denaturation curves (Pace, 1975). To avoid the uncertainties of ΔG_u^{H₂O} values due to the relative long extrapolation to 0 M denaturant, we calculated the free energy difference ΔΔG_u between the peptides relative to RR-CMP-C36 (equation 5; Table I). RR- and QR-CMP-C36 as one pair, and RQ- and QQ-CMP-C36 as another pair, which differ only with respect to a putative intrachain salt bridge between Arg19 and Glu22, show very similar values of [urea]_{1/2}, ΔG_u^{H₂O} and ΔΔG_u. Our data demonstrate that the loss of this interaction has only a small effect on the stability of the coiled-coil structure of CMP. In contrast, replacing Arg27 with an uncharged residue, and thus abolishing any ionic interaction with Glu32 of an adjacent chain, results in a drastic decrease in stability.

Depending on pH, ionic interactions stabilize and destabilize RR-CMP-C36

We have determined the isoelectric points of the CMP-C36 related peptides by isoelectric focusing gel electrophoresis. RR-CMP-C36 just enters the top of the gel (Figure 4A) which corresponds to a pI >9.6. Calculations based on the amino acid composition predicts a pI of 10.7. QR- and RQ-CMP-C36 run in an approximately identical position with a pI ~8.4 which is considerably smaller than expected (pI_{calc} = 9.6), whereas the pI of QQ-CMP-C36 corresponds to its calculated value of 7.2. As isoelectric focusing is a highly sensitive technique, the migration of the peptides as single bands further confirms the purity of our material.

To determine whether ionic interactions are involved in the stability of the coiled-coil structure, we measured the

thermal stability at various pH conditions by following the CD signal change at 221 nm upon heating. To diminish the masking of such effects by hydrophobic interactions (Yu *et al.*, 1996), but prevent aggregation effects observed at low ionic strength (Beck *et al.*, 1996), as a compromise we have chosen a salt concentration of 100 mM NaF. For RR-CMP-C36, the melting temperature T_m increases steeply by ~15°C when raising the pH from 8 to 9.5 and slightly decreases when raising the pH above 12 (Figure 4B). When compared with the other peptides at pH 7, RR-CMP-C36 shows the highest stability (T_m = 62°C) which slightly decreases with decreasing pH. The stability of the other peptides shows a trough-shaped pH dependence with minima between pH 6–8 (Figure 4B and C). Maximal stabilities are observed at pH <5 and pH >10. The Arg19→Gln change in QR- and QQ-CMP-C36 results in a small decrease of stability within the neutral pH range. The increased stabilities for all the peptides other than RR-CMP-C36 at acidic conditions can only be understood when assuming repulsive ionic interactions within this peptide (see Discussion).

Arg₂₇ modulates the oligomerization state of CMP-C36 peptides

To determine the oligomerization state of our peptides, we performed crosslinking experiments with bis(sulfosuccinimidyl)-suberate (BS³). BS³ preferentially reacts with primary amines including terminal α-amino groups and the ε-amino group of lysine (Partis *et al.*, 1983). Peptides were crosslinked for 1 h at a relatively high ionic strength of 0.25 M NaCl at pH 7.2 at varying crosslinker concentrations and analyzed by gel electrophoresis. At the maximal BS³ concentration applied, corresponding to a 25-fold molar excess over the peptide concentration, we observed nearly exclusively bands indicative of a trimer in the case of RR- and QR-CMP-C36, and a tetramer for RQ- and QQ-CMP-C36 (Figure 5A). These results indicate that replacement of Arg27 with glutamine favors the formation of tetramers, and Arg19 does not influence the oligomerization state. When compared with the position of marker proteins, the crosslinked peptides show lower apparent molecular masses than can be expected from their aggregation state. This indicates that the non-reducible

Fig. 4. Influence of pH on thermal stability. **(A)** Isoelectric focusing of CMP-C36 peptides. Isoelectric points (pI) of peptides are estimated relative to standard proteins (lane Std.) to >9.6, 8.4, 8.3 and 7.2 for RR-, QR-, RQ- and QQ-CMP-C36. As additional marker proteins, equine (me, pI = 7.4/7.0 major/minor band) and recombinant sperm whale myoglobin (mw, pI = 8.3/7.7 major/minor) were used. **(B)** and **(C)** pH dependence of the thermal stability of CMP-C36 peptides. Melting temperatures T_m were determined from transition curves recorded at 221 nm upon rising the temperature for RR- (●), QR- (○), RQ- (■) and QQ-CMP-C36 (□) (peptide concentrations: 50 μ M in 100 mM NaF, 20 to 50 mM buffer salts). At the bottom of (B), pK_a values of the charged residues of the peptides are indicated.

crosslinker keeps the associated peptide chains in a more compact conformation even in the presence of SDS.

To verify this dramatic effect of the exchange of a single amino acid on the oligomerization state, we analyzed the peptides crosslinked at a 25-fold molar excess of BS³ by mass spectroscopy (Figure 5B). These data support our finding that RR- and QR-CMP-C36 form covalently linked trimers, whereas the other two peptides form tetramers. Rather weak signals for higher aggregation states could

be observed around 26 000 and 35 000 Da for RR/QR- and RQ/QR-CMP-C36 respectively, which most probably represent hexamers and octamers (data not shown). For the covalent linkage of trimers and tetramers, a minimum of 2/3rd and 3/4th crosslinker molecules per peptide chain are necessary. Assuming that only the terminal amino group and the four lysine residues are reactive, a maximum of 5 BS³ molecules could bind per monomer in which case no oligomerization should be observed. From the known peptide masses and the mass of BS³ after removal of the sulfosuccinimidyl-groups upon hydrolysis, the mass spectroscopic data indicate that 3.1 ± 0.2 crosslinker molecules are bound to each peptide chain of RR- and QR-CMP-C36, whereas the RQ- and QQ-CMP-C36 tetrameric complexes each contain 2.7 ± 0.3 alkyl chains. Although these differences are small, they might indicate that some of the lysine residues are less accessible for BS³ in the tetrameric than in the trimeric configuration.

To avoid any putative crosslinking-induced artifacts, we measured the molecular mass of the peptides at different pH conditions by equilibrium sedimentation centrifugation. As we observed only a single species in velocity sedimentation runs (see below), masses were derived from the slope of the straight lines of $\ln c$ versus r^2 plots. For RR- and QR-CMP-C36, molecular masses result to ~12 000 Da indicating a trimeric configuration at all tested pH values (Table II). Similar masses were obtained for RQ- and QQ-CMP-C36 at acidic and basic conditions. At neutral pH, however, the molecular mass of ~16 000 indicates a tetrameric state for these samples.

Sedimentation velocity runs performed at pH 3 and pH 7.2 show sedimentation coefficients of $s^{0}_{20,w} \sim 1.2S$ except for RQ- and QQ-CMP-C36 which at pH 7.2 sediment significantly faster (Table II). When compared with globular molecules of similar size, these values indicate an extended shape for all four peptides. Assuming a rigid rod structure, and a degree of hydration of 1 g H₂O per gram protein, analysis of the data according to Bloomfield *et al.* (1967) results in a length of 5.1 ± 0.2 nm and a diameter of 3.0 ± 0.1 nm for the hydrated peptides in the trimeric conformation. For RQ- and QQ-CMP-C36 at pH 7.2, the diameter increases to 3.5 nm. With a helix rise of 0.153 or 0.152 nm per residue, as determined for a three- and four-stranded α -helical coiled-coil (Harbury *et al.*, 1993, 1994), a 36-mer peptide would extend to a length of 5.51 nm indicating a good correlation with our data evaluation.

Folding of trimeric and tetrameric CMP-C36 peptides is an all-or-none process

We have analyzed the coiled-coil to random coil transition of the CMP-C36 related peptides. For non-covalently linked multichain proteins, which simultaneously unfold and dissociate, T_m is a function of protein concentration. The concentration dependence of T_m can be related to the mechanism of transition and allows the determination of the enthalpy change of this process (see Materials and methods, equation 3). T_m values for the four peptides determined at pH 7.2 as a function of concentration are shown in Figure 6A. The data fit to straight lines when plotted as $1/T_m$ versus $\ln [N (0.5 c_0)^{N-1}]$ where the number of peptide chains N is assumed as 3 for RR- and QR-CMP-C36 and 4 for the other two peptides (Figure 6B

Fig. 5. Crosslinking of CMP-C36 peptides. (A) Peptides were incubated with various concentrations of BS³ (top row, in mM) for 1 h and analyzed by SDS Tricine gel electrophoresis (a, RR-; b, QR-; c, RQ-; d, QQ-CMP-C36). Left lanes show molecular weight marker proteins. (B) Mass spectra of crosslinked CMP-C36 peptides. Peptides as indicated at the right of the curves were crosslinked at a 25-fold molar excess of BS³ for 1 h. Analysis was performed by electrospray mass spectrometry. Dotted lines indicate the position of the different oligomers (top), and numbers are the molecular masses of the peak signals.

Table II. Molecular masses and sedimentation coefficients of CMP-C36 peptides as determined by analytical ultracentrifugation

Peptide	pH 3.0		pH 7.2		pH 11.5
	M _r (Da)	<i>s</i> _{20,w} ⁰ (S)	M _r (Da)	<i>s</i> _{20,w} ⁰ (S)	M _r (Da)
RR-CMP-C36	12 900	1.25	13 300	1.20	12 100
QR-CMP-C36	12 400	1.22	12 000	1.16	11 800
RQ-CMP-C36	12 500	1.20	16 100	1.48	12 100
QQ-CMP-C36	12 200	1.18	15 500	1.44	12 300

All measurements were performed in the presence of 250 mM NaCl. As the available centerpieces were not base resistant, sedimentation coefficients could not be measured at pH 11.5.

and C). This linear relationship indicates that unfolding proceeds via a two-state transition which is also reflected by the apparently monophasic single denaturation curves (data not shown). Furthermore, when unfolding was monitored at one concentration for each peptide by recording consecutive spectra, only a single isodichroic point could be observed which has been suggested to be indicative of a two-state transition (Engel *et al.*, 1991). The transition enthalpies ΔH^0 derived from the slope of the curves and normalized to the number of residues per oligomer result to -4.2, -3.9, -3.3 and -3.0 kJ/mol/res for RR-, QR-, RQ- and QQ-CMP-C36 respectively. These

data suggest that the contribution of Arg19 to the transition enthalpy is ~-11 kJ/mol per peptide chain both within the trimeric as well as in the tetrameric configuration. Interestingly, the Arg27→Gln exchange, which is accompanied with the change in the oligomerization state, results in a slight increase of the absolute transition enthalpy (Figure 6; compare RR- to RQ- and QR- to QQ-CMP-C36). At the midpoint of transition, the free energy ΔG becomes zero, and the melting temperatures observed for the tetramers are lower than those of the trimers. From $\Delta G = \Delta H - T\Delta S$ it thus follows that the transition entropy ΔS must be higher in the tetrameric state.

Fig. 6. Concentration dependence of the thermal stability of CMP-C36 peptides. (A) CD thermal equilibrium transition profiles were recorded at 221 nm and melting temperatures T_m were determined for a peptide concentration range of 1 to ~80 μM in 250 mM NaF, 20 mM $\text{KH}_2\text{PO}_4/\text{NaOH}$, pH 7.2. Assuming a two-state transition from a 3-stranded (B) or a 4-stranded α -helical coiled-coil conformation (C) to single random coil chains, data were linearized as described in Materials and Methods. From the slope of linear least square fits, the free standard enthalpies ΔH^0 result to -458, -423, -471 and -427 kJ/mol for RR- (●), QR- (○), RQ- (■) and QQ-CMP-C36 (□) respectively.

Discussion

α -helical coiled-coil domains appear to be one of nature's favorite building blocks for the assembly and defined alignment of adjacent protein chains. Within the apparently simple grammar of arranging apolar amino acids in a repeating 3–4 spacing and filling the remaining positions within a heptad with polar residues, there are still a fascinating number of possibilities left to arrange such helices as homo- or heterodimers, -trimers, tetramers or pentamers either in a parallel or antiparallel fashion. Such domains can form either by the assembly of several polypeptide chains or by backfolding within single chains (for examples see Lupas, 1996). A few rules of this grammar are meanwhile understood to allow some predictions on the possible arrangements based on the knowledge of the primary structure only. One critical role can be

attributed to the specific nature of the hydrophobic residues in heptad positions *a* and *d* as these have different geometries with respect to the facing helices. Thus, by systematically replacing the original residues of the *a/d* positions within the yeast transcription factor GCN4 leucine zipper domain with either leucine, valine or isoleucine, Harbury *et al.* (1993) could generate dimers, trimers or tetramers. Very recently, high resolution structures of GCN4-related peptides have shown the critical involvement of a single polar residue which is buried in the hydrophobic core of the coiled-coil. In this case, the structural selectivity results from the side-chain stereochemistry with the surrounding structural environment (Gonzales *et al.*, 1996a,b). From a large number of *de novo* designed model peptides, a number of rules have become apparent (for a review see Hodges, 1996). Besides the residues in *a/d* positions, those in *e* and *g*, and especially their charged character, can further contribute to the coiled-coil assembly and chain selection (Vinson *et al.*, 1993; Woolfson and Alber, 1995). In the same way that simplistic grammar book examples are rarely used in literature, the major difficulty in applying such rules to 'real world' proteins is that they are much less regularly structured than model peptides.

As we found that a peptide covering the sequence region of the C-terminal 36 residues of human CMP was capable of forming a very stable homotrimeric coiled-coil structure at different solution conditions (Beck *et al.*, 1996), we endeavored to use this as a model system to study the contribution of ionic interactions to stability. We thus designed peptides QR-, RQ- and QQ-CMP-C36 in which the only two arginine residues are replaced by glutamine. The hydrophobic core residues, namely leucines in heptad position *a* and alternating leucine/valines in *d*, remain unchanged and are consistent with a trimeric assembly applying the principles derived from the work of Harbury *et al.* (1993).

To focus on the contribution of electrostatic interactions, we measured the thermal stability of the different peptides at different pH conditions (Figure 4). At a pH between 5 and 10, most arginine, lysine and glutamic acid residues are charged and can contribute to several salt bridges (Figure 1). At a pH <4 and pH >12, the charged residues become protonated and deprotonated respectively. Therefore, in these ranges no stabilization by ionic interactions can be expected. For RR-CMP-C36, the maximum T_m values are observed between pH 10–12, and at pH 12 most of the lysine residues are not charged. We thus conclude that either intrachain (Arg19–Glu22) and/or interchain (Arg27–Glu32) pairs of arginine-glutamic acid residues are responsible for the observed maximal thermal stability. This view is supported by the decrease of stability when the pH is raised above the $\text{pK}_a \pm 12$ of arginine. In the absence of Arg19 (QR- and QQ-CMP-C36), the thermal stability decreases in the basic pH range but substantially increases at acidic pH. This is clear evidence for the contribution of Arg19 to α -helix stability, although the absolute contribution might be overestimated due to relatively high electrolyte concentration (100 mM NaF) present during all of our measurements (Yu *et al.*, 1996). The lower T_m values of RR-CMP-C36 at acidic pH most probably indicate repulsion effects. It is tempting to speculate that Arg19, Lys20, Lys26 and Arg27 located in

positions *f* and *g* of consecutive heptads (Figure 1) are responsible for the low stability. If one of these arginines is replaced by glutamine (QR- and RQ-CMP-C36), higher T_m values are observed at a low pH, and the stability further increases for QQ-CMP-C36. When normalized to $\Delta G = 0$ for a pair of alanines in heptad positions *e* and *g*, the coupling energy for the Arg27–Glu32 pair (*g/e'*) in a coiled-coil can be estimated to $\Delta\Delta G = -4.5$ kJ/mol (determined for dimers, Krylov *et al.*, 1994). Intrastand ionic interactions between Glu–Lys and Arg–Glu pairs (Figure 1) can contribute to stability with about -1 to -2 kJ/mol per pair (Scholtz *et al.*, 1993; Zhou *et al.*, 1993). In our case, we found a difference of 0.8 kJ/mol per Arg–Glu pair for RR- and QR-CMP-C36 and 0.3 kJ/mol for RQ- and QQ-CMP-C36 assuming a trimeric and tetrameric configuration respectively (Table I). These lower values might be partially due to the less regular structure of the CMP coiled-coil when compared with the model peptides and the higher order of oligomerization. For globular proteins it has been shown that the contribution of intrachain salt bridges to stability depends significantly on their location within the tertiary structure (Hensch and Tidor, 1994; Waldburger *et al.*, 1995). For comparison, the contribution of a hydrophobic coupling energy for a leucine pair (*a/d'*) with respect to alanine in dimeric coiled-coils has been determined to be of the order of 10 kJ/mol (Zhou *et al.*, 1992).

It is interesting to compare the pH dependence of the thermal stability with that of a 35-mer peptide related to the oncoprotein Jun. Besides its similar size, it contains the same type and number of charged residues except that only three, instead of four lysines are present. Rather than a three-stranded coiled-coil, however, it forms dimers. At a pH of ~ 9 , the stability rises sharply from around $T_m = 40$ – 45°C (pH 2–8) to reach a maximum of 58°C at pH 11.5 (O'Shea *et al.*, 1992). This difference of 18°C is in the same range as that observed for RR-CMP-C36.

The tetramer assembly of RQ- and QQ-CMP-C36 at a physiological pH results in a comparably low thermal stability (Figure 4C), whereas, in their trimeric conformation at acidic and basic pH, the stability is similar to RR- and QR-CMP-C36. Yet we know neither in what pH range this conformational transition occurs nor what mechanism leads to this profound rearrangement of peptide chains. The rather sharp increase in T_m observed around pH 5.5 and 8.5 for QQ-, and pH 7.5 for RQ-CMP-C36 might be indicative of this process. For RQ-CMP-C36, we noticed some inflection around pH 5 which might suggest a broader co-existence range of different oligomers.

Our observation of a tetrameric rather than trimeric state at physiological pH upon the exchange of a single arginine with glutamine requires the consideration of responsible interactions (Figure 7). As hydrophobic packing is the driving force for coiled-coil formation, we assume that in the tetrameric state, the large apolar residues Val4, Phe6 and Leu13 located to heptad positions *g* and *e* form knob-into-holes packing with residues in position *a'* and *d'* respectively, of adjacent chains. In the trimeric state, however, these *a–g'* and *d–e'* interactions might not form due to their larger distance from each other. Indeed a lower helix separation has been observed for tetrameric than for trimeric GCN4-related coiled-coils, and residues

Fig. 7. Schematic 'helical wheel' representation of a tetrameric α -helical coiled-coil as seen from the NH_2 -terminus. The upper and left parts show the putative hydrophobic interactions within RQ- and QQ-CMP-C36, the right and lower parts show the putative interchain ionic interactions. As residues in heptad positions *g* and *e* become more buried in a tetrameric rather than trimeric conformation, the large apolar residues Val4, Phe6 and Leu13 most probably interact with the hydrophobic residues in positions *a* and *d* stabilizing this chain arrangement. The closer distances between heptad positions *b* and *c* observed for a tetramer should allow that charged residues in this spacing might come into close contact with those in positions *g'* and *e'* respectively. Thus salt-bridges between Lys9–Glu11' and Lys20–Glu22' should be possible in the 4-stranded conformation. In the case of RR- and QR-CMP-C36, however, which contain arginine rather than glutamine in position 27, at neutral pH this residue probably forms a salt bridge with Glu32 in heptad position *e'*.

in positions *g* and *e* are less exposed to the solvent (Harbury *et al.*, 1993, 1994). This allows for closer packing of these edges within the tetramer conformation. The same argument holds for a decrease in the distance between *b–g'* and *c–e'* residues and ionic interactions of this type are found in the GCN4 p-LI tetramer (Harbury *et al.*, 1993). For RQ- and QQ-CMP-C36, this should allow for salt bridge formation between Lys9–Glu11' (*c/e'*) and Lys20–Glu22' (*g/b'*). Such interactions might be responsible for the lower accessibility of lysine residues as observed by the lower degree of BS³ binding. It is noteworthy that all these residues are conserved in the three known CMP sequences (Figure 1). Upon protonation and deprotonation of the charged residues, the tetramer is converted to trimers. Although interchain Glu–Lys pairs contribute to stability only with relatively low coupling energies (-0.6 or -3.8 kJ/mol per pair when in *g–e'* or *e–g'* position of a dimer; Krylov *et al.*, 1994; -1.6 kJ/mol

per pair acc. Zhou *et al.*, 1994b), this seems sufficient to modulate the conversion of the oligomerization state. The fact that the Arg19→Gln exchange has no effect on oligomerization is compatible with a localization of this residue in heptad position *f* where, in a two- to four-stranded coiled-coil, it is unlikely to interact with adjacent peptide chains.

In summary, our data present the first experimental evidence that interchain ionic interactions can modulate the oligomerization state of α -helical coiled-coils. In the case of our CMP related peptides, the exchange of a single arginine residue to an uncharged, but polar residue results in a conversion from a trimeric to tetrameric coiled-coil. As this effect is only observed at neutral pH, the specific localization of apolar and/or other charged residues must be crucial for this process.

Materials and methods

Peptide synthesis and purification

Peptides were synthesized by solid-phase chemistry on a Milligen/Biosearch model 9050 synthesizer using Fmoc chemistry and cleavage was performed as described (Beck *et al.*, 1996). Crude peptides were purified by reversed phase HPLC on a Vydac C18 column (2.2/25 cm, 10 μ m; The Separations Group, Hesperia, CA) operated at a flow rate of 4 ml/min. Final purification was achieved by a second chromatography step on a Vydac C8 column (1.0/25 cm, 5 μ m; eluted at 2 ml/min). Both columns were eluted with a linear binary gradient of acetonitrile/H₂O from 0–60% containing 0.1% TFA (steepness of gradient: 0.25%/min around the pre-evaluated elution position, otherwise 1%/min). Peptides eluted between 51 and 58% acetonitrile on the C18 column, whereas on the C8 column, elution was observed between 43 and 50% acetonitrile in the order of RR-, QR-, RQ- and QQ-CMP-C36. Peptide stock solutions were prepared by dissolving the lyophilized material in H₂O (~2 mg/ml) and were stored at –130°C. Peptides were characterized by amino acid compositional analysis, sequencing and mass spectrometry.

Buffers

Peptide buffer conditions were adjusted by dilution with stock solutions at least 12 h before measurements were performed. To achieve maximum transparency in the far UV, for CD measurements NaF (>99.5%, Fluka) was used as a salt rather than NaCl. Guanidine-HCl was from Pierce (Rockford, IL). For pH variation, we used 20–50 mM final buffer concentrations of HCl/KCl (pH 1.5–3), acetic acid/Na-acetate (pH 3–6), KH₂PO₄/NaOH (pH 6–8), boric acid/NaOH/KCl (pH 8–12.6). pH values were measured with a micro-combination electrode (PHR-146, Lazar Research Lab., Los Angeles, CA) in the presence of peptides at 25°C. Urea and guanidine-HCl titration series were prepared from ~10 and 8 M stock solutions respectively, and final concentrations were determined by refractometry (Pace, 1986). All buffers were prepared with MilliQ water, filtered (0.22 μ m) and degassed.

Analytical ultracentrifugation

Sedimentation velocity and sedimentation equilibrium measurements were performed on a Beckman model E analytical ultracentrifuge equipped with a modified scanner (Flossdorf, 1980). Absorbance was measured at 237 nm. Runs were carried out at 5°C in an AnJ-Ti rotor using 12 mm Kel-F double-sector centerpieces for sedimentation equilibrium measurements and corresponding aluminum-filled Epon centerpieces for sedimentation velocity experiments. Running conditions were 24 000 r.p.m./72 h (2–3 mm column height) and 52 000 r.p.m. with 8 scans in 45 min intervals for equilibrium and velocity sedimentation respectively. Partial specific volumes v_2 were calculated to 0.772, 0.771 and 0.770 ml/g for RR-, QR/RQ- and QQ-CMP-C36 respectively, from the weight average of v_2 of the individual amino acids using the consensus scale of Perkins (1986). Minimum molecular weights were derived from the slope of $\ln c$ versus r^2 plots (r = distance from the rotor center) after linearity had been optimized by adjusting the baseline. Sedimentation coefficients were corrected to water and zero protein concentration by standard procedures (Van Holde, 1985). Hydrodynamic dimensions were calculated as described (Bloomfield *et al.*, 1967; Beck *et al.*, 1996).

Circular dichroism measurements

CD spectra were recorded on a JASCO J-500 A spectropolarimeter with thermostatted quartz cells (Hellma, Mülheim, F.R.G.) of 0.05–2 mm path-length (for details, see Beck *et al.*, 1996). Thermal transition curves were recorded at 221 nm. Temperature gradients were generated with a programmer-controlled circulating water bath at heating and cooling rates of 12 to 30°C/h. Temperature was monitored in the cell with a thermistor. Transition curves were normalized to the fraction of folded peptide *F* with $F = ([\Theta] - [\Theta]_u)/([\Theta]_n - [\Theta]_u)$ where $[\Theta]_n$ and $[\Theta]_u$ represent ellipticities of the fully folded and unfolded species respectively, and $[\Theta]$ is the observed ellipticity at 221 nm. Peptide concentrations were determined in triplicate by amino acid analysis with an accuracy of $\sim \pm 3\%$.

Other analytical methods

SDS-PAGE was performed according to Schagger and Jagow (1987) using precast 16% Tricine gels (Novex, San Diego, CA, USA). Isoelectric points of the peptides dissolved in H₂O were determined on precast pH 3–10 isoelectric focusing gels (Novex) calibrated with a standard protein mixture (Bio-Rad Laboratories). Gels were stained with Coomassie Blue R. Crosslinking was performed using BS³ (Pierce), a water soluble homobifunctional *N*-hydroxysuccinimide ester analog with a spacer arm length of 1.14 nm. For gel electrophoresis, peptides ($c_{\text{final}} \sim 100 \mu\text{M}$, in 250 mM NaCl, 20 mM KH₂PO₄/NaOH, pH 7.2) were incubated at various BS³ concentrations for 1 h at 25°C under gentle vortexing. For mass spectroscopic analysis, crosslinking was performed in the same manner with 2.5 mM BS³. Unbound BS³ was removed and buffer exchanged to 0.2 M ammonium acetate by passing the samples over a Fast Desalting column (Pharmacia Biotech, Uppsala, Sweden). Peptides were concentrated by evaporation in a Speed-Vac and analyzed on a triple quadrupole electrospray mass spectrometer (Perkin Elmer Sciex, Norwalk, CT) by the Mass Spectrometry Facility of the University of Washington (Seattle, WA).

Homogeneity of the peptides was tested by size exclusion chromatography on a Superose 12 column (1.0/30 cm, Pharmacia Biotech) equilibrated in 100 mM NaF, 20 mM KH₂PO₄/NaOH, pH 7.2, in the absence and presence of 7 M urea. Elution was performed at 20 ml/h.

Assembly mechanism and calculation of thermodynamic parameters

Equilibrium melting curves were interpreted assuming a two-state (all-or-none) mechanism, where *N* unfolded chains *u* combine to a native α -helical oligomer *n* in coiled-coil conformation (for details see Engel *et al.*, 1977; Marky and Breslauer, 1987):

The equilibrium constant *K* is

$$K = c_n/c_u^N = F/Nc_0^{N-1} (1 - F)^N \quad (1)$$

where $c_0 = c_u + Nc_n$ is the total concentration of peptide chains and $F = Nc_n/c_0$ the degree of conversion to the coiled-coil. From

$$\Delta G^0 = -RT \ln K = \Delta H^0 - T\Delta S^0 \quad (2)$$

and equation (1) it follows that at the midpoint of the transition ($F = 0.5$ and $T = T_m$),

$$1/T_m = \{\Delta S^0 + R \ln [N (0.5 c_0)^{N-1}]\}/\Delta H^0 \quad (3)$$

where ΔG^0 is the standard free energy (Gibbs free energy), ΔH^0 standard enthalpy, ΔS^0 standard entropy, *T* the absolute temperature and *R* the universal gas constant. Thus, for non-covalently linked peptide chains, $1/T_m$ is linearly correlated to $\ln [N (0.5 c_0)^{N-1}]$ and ΔH^0 can be calculated from the slope of the straight line.

The free energy change upon urea and guanidine-HCl induced unfolding (ΔG_u) was calculated from equation (2) assuming a two-state mechanism with an apparent equilibrium constant $K_{app} = F/(1 - F)$ and plotted versus denaturant concentration. A least squares analysis was used to fit the data to

$$\Delta G_u = \Delta G_u^{\text{H}_2\text{O}} - m [\text{denaturant}] \quad (4)$$

where *m* is a measure of the dependence of the free energy on denaturant concentration and $\Delta G_u^{\text{H}_2\text{O}}$ an estimate of the minimum conformational stability in the absence of denaturant (Pace, 1986). To avoid the errors resulting from the long extrapolation to 0 M denaturant, the differences of ΔG_u with respect to RR-CMP-C36 are derived from

$$\Delta\Delta G_u = \langle m \rangle \Delta [\text{denaturant}]_{1/2} \quad (5)$$

where $\langle m \rangle$ is the average value of m and $[\text{denaturant}]_{1/2}$ the concentration of denaturant at $F = 0.5$ (Serrano *et al.*, 1990).

Acknowledgements

We thank Ms Santosh Kumar (Department of Biochemistry, University of Washington, Seattle, WA, USA) for expert mass spectroscopic analysis. Furthermore, we thank Dr Nick Morris (Shriners Hospital, Portland) for critical reading of the manuscript and constructive criticism. This work was supported by the Shriners Hospital for Children.

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Received on February 5, 1997; revised on April 2, 1997

Note added in proof

While this paper was in press, a new protein with a sequence and domain structure very similar to CMP was described [Deák, F., Piecha, D., Bachrati, C., Paulsson, M. and Kiss, I. (1997) Primary structure and expression of matrilin-2, the closest relative of cartilage matrix protein within the von Willebrand factor type A-like module superfamily. *J. Biol. Chem.*, **272**, 9268–9274]. As this protein could not be detected in cartilage, it was named matrilin-2, and for CMP the name matrilin-1 has been suggested. Only 12 of the 36 residues covered by peptide RR-CMP-C36 are identical between human matrilin-2 and matrilin-1/CMP. Arg27 of peptide RR-CMP-C36 is also contained in matrilin-2, whereas Arg19 is replaced by glutamine as in peptide QR-CMP-C36. This might indicate that Arg27 (which could be replaced by a lysine residue as it is found in a corresponding position in thrombospondin-1) is of crucial importance for the structural integrity of this protein family.