Site-directed mutagenesis of the hinge peptide from the hemagglutinin protein: enhancement of the pH-responsive conformational change

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Environmentally responsive proteins and peptides are increasingly finding utility in various engineered systems due to their ability to respond to the presentation of external stimuli. A classic example of this behavior is the influenza hemagglutinin (HA) fusion protein. At neutral pH, HA exists in a non-fusogenic state, but upon exposure to low pH, the conformation of the structure changes to expose a fusogenic peptide. During this structural change, massive rearrangements occur in a subunit of HA (HA2). Crystallography data has shown that a loop of 28 amino acids (residues 54–81) undergoes a dramatic transition from a random coil to an alpha-helix. This segment connects to two flanking helical regions (short and long) to form a long, continuous helix. Here, we report the results of site-directed mutagenesis study on LOOP-36 to further understand the mechanism of this important stimulus-responsive peptide. The conformational transition of a bacterially expressed LOOP-36 was found to be less dramatic than has been previously reported. The systematic mutation of glutamate and histidine residues in the peptide to glutamines (glutamine scanning) did not impact the conformational behavior of the peptide, but the substitution of the glycine residue at position 22 with alanine resulted in significant pH-responsive behavior. Therefore this mutant stimulus-responsive peptide may be more valuable for future protein engineering and bionanotechnology efforts.

Keywords: circular dichroism/conformational change/hemagglutinin/site-directed mutagenesis/stimulus-responsive

Introduction
There is an increasing interest in the study of conformational changes in proteins and peptides as these features can be essential to the functionality of the native protein, and they can be exploited for the engineering of new proteins for important bioengineering applications (Banta et al., 2007; Chockalingam et al., 2007). One of the most famous examples of dramatic conformational changes can be found in the proteins that mediate membrane fusion. In the case of enveloped viruses, fusion is mediated by cell surface glycoproteins that undergo large structural rearrangements in response to cues from the targeted cells (Kielland and Rey, 2006). The hemagglutinin (HA) protein from the X-31 strain of the influenza virus has served as the standard model for this process, as structural information for this protein has been available for over 25 years (Wilson et al., 1981; Wiley and Skehel, 1987; Bullough et al., 1994). The protein dramatically refolds from a mesostable or ‘spring-loaded’ conformation at neutral pH to an extended fusogenic structure following the decrease in pH that occurs during endocytosis (Carr and Kim, 1993; Carr et al., 1997). However, the precise mechanism and sequence of events that lead to fusion of the viral and host membranes following this conformational change are still an area of active research (Tamm, 2003).

The mature HA protein is a homotrimer, and each monomer is originally synthesized as an HA0 precursor. This precursor is proteolytically cleaved into the functional HA1 and HA2 subdomains (Fig. 1A). The first 20 amino acids of the N-terminus of the HA2 domain constitute the hydrophobic fusion peptide, which inserts into the endosomal membrane. In the metastable conformation, the fusion peptide is folded adjacent to helices C and D. The helical segments A and C of each monomer are connected by a flexible hinge region B, which results in a helix-turn-helix hairpin structure. At the acidic pH of the endosome, the HA protein refolds into the energetically favorable fusogenic conformation, producing a massive rearrangement in HA2, including a dramatic extension of the helical region, and propelling the fusion peptide towards the targeted membrane. During this process the flexible hairpin hinge region adopts an α-helical conformation, resulting in the formation of a continuous 13.5 nm helical segment comprised of sections A–C (Fig. 1A, right panel).

The loop region was previously targeted for biophysical characterization, as this segment was predicted to have a high propensity for forming a coiled-coil motif (Carr and Kim, 1993). Two synthetic peptides containing the hinge sequence were created and the shorter 36-amino acid peptide (known as LOOP-36) was shown to be unstructured at neutral pH, but α-helical at pH 4.8. The acidic structure could be reversibly unfolded by increasing the temperature, and it was observed that the acidic structure formed trimers, while at neutral pH the peptide existed as a monomer. Therefore, the conclusion was that the LOOP-36 subunit of HA exists in a metastable state at neutral pH, and undergoes a conformational change at endosomal pH. The peptide is amphipathic, with predominantly hydrophobic amino acids at a and d positions, and a predominance of hydrophilic residues at the other positions (Fig. 1B). A projection of the peptide onto a helical wheel starting at the N-terminus shows an alignment of the hydrophobic residues forming a

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hydrophobic cluster responsible for the trimerization of the peptide and five glutamic acid residues at positions e which are responsible for the electrostatic repulsion at pH 7.2 that, according to Carr and Kim (1993), destabilizes the folded helical conformation. Despite the fact that LOOP-36 undergoes dramatic structural changes, the structural determinants of this event have not been systematically studied.

Here, we report the results of a site-directed mutagenesis study on the stimulus-responsive LOOP-36 peptide (residues 54–89) of the HA protein (Carr and Kim, 1993). We expressed LOOP-36 and mutant peptides in Escherichia coli (E. coli) as part of a self-cleaving fusion protein. The conformational transition of the purified LOOP-36 peptide was found to be less dramatic than has been previously reported. Substitution of a glycine residue at position 22 with alanine significantly affected the conformational behavior of the peptide, but the systematic mutation of glutamate and histidine residues to glutamines (glutamine scanning) did not affect the pH-induced conformational change. There has been recent interest in using the LOOP-36 peptide in future protein engineering and bio-nanotechnology projects (Dubey et al., 2004) and these results suggest that the G22A mutant peptide will be a better choice for these applications.

Materials and methods

Materials

Oligonucleotide primers were from Sigma Genosys. Restriction enzymes, the pTYB11 expression plasmid, and the IMPACT protein expression system were from New England Biolabs. Site directed mutations were made with the QuickChange mutagenesis kit from Stratagene. Super competent BL-21 cells were from Invitrogen. Centricon ultrafiltration units were from Millipore. All other chemicals were reagent grade from Sigma Chemical.

LOOP-36 cloning

Synthetic oligonucleotides (5’-AACCGTGTATATCGAAAAA ACACAACAAAAATTTCCACGAGTCTGAAAAGAAATTTC TCTGAAATGAGGTGATTCACGAAATTGCAT CTACGTTGAAGACCCAAAATCTAAC-3’ and 5’-TGAGAT TAGATTTTGGTGTCCTCAACGATTTTTCTCCAGGCTCC GGAATCGACCTCAACTTACGAGAATTTC TCGTGGAAATTTTCGTTGGTTTTTTGAT CTGGTGGAAATTTTCGTTGGTTTTTTGCTGAAACGCGC-3’) containing the LOOP-36 sequence with 5’ and 3’ overhangs compatible with the SapI and XhoI restriction sites, were annealed by heating at 95°C followed by slow cooling to room temperature. The synthetic DNA cassette was ligated overnight at 16°C with T4 ligase into the expression plasmid pTYB11, which was previously double digested with SapI and XhoI. SapI insertion of LOOP-36 into these pTYB11 multi-cloning site allowed us to obtain the peptide without the addition of extra amino acids flanking the LOOP-36 sequence.

LOOP-36 mutagenesis

The Quickchange system was used for all mutagenesis experiments. The primers used were as follows: G22A forward: 5’-CT CAAGGTGAAAGCTCTGATACG-3’, G22A reverse: 5’-CC TTGATACGACGTTTCAACTTCAGG-3’, H11Q forward: 5’-GA AAAACACAAACAAAAATTCACGAGATCGAAAAAAGA ATTCCTG-3’ H11Q reverse: 5’-CAGAGAAATTCCTTTTTCC AGATCCTGGAATTTTCTGTTGTTTTTTC-3’ E4Q forward: 5’-GTGTGACAGACGGTGTACATCGATATCCAAAAAAAACCGA AA-3’; E4Q reverse: 5’-TGGAAATTTTTCTGTTGGTTTTTTG AIAACAGCGTCTT-3’; E8Q forward: 5’-CGTGTATACGAAACTCAG-3’; E8Q reverse: 5’-CTGGTATACGAAACTCAG-3’; E19Q forward: 5’-CAAGGTGAAAGCTCTGATACG-3’, E19Q reverse: 5’-CAAGGTGAAAGCTCTGATACG-3’. Site-directed mutations were made with the QuickChange mutagenesis kit from Stratagene. Super competent BL-21 cells were from Invitrogen. Centricon ultrafiltration units were from Millipore. All other chemicals were reagent grade from Sigma Chemical.
Expression and purification of peptides
The pTYB11 plasmids containing the LOOP-36 peptide and its mutants were transformed into super-competent BL21 cells. The protein expression was induced by Isopropyl β-D-1-thiogalactopyranoside at 25°C and bacteria were collected 6 h after induction by centrifugation. Peptides were purified by affinity chromatography, using a chitin resin and chitin-binding domain tag, following the manufacturer’s instructions. The elute from the chitin affinity column was dialyzed (2000 Da membrane cut-off) three times against 10 mM phosphate buffer, pH 7.2. A fourth round of dialysis was performed against 10 mM triethyl ammonium bicarbonate buffer, pH 8.5. The peptide was then concentrated by ultra-filtration (Centricon, 3000 Da cut-off) and lyophilized to complete elimination of the volatile triethyl ammonium bicarbonate buffer. Purity was checked by SDS–PAGE and mass spectrometry.

Circular dichroism spectroscopy
Circular dichroism (CD) analysis of peptides was carried out with AVIV 202 CD instrument using a 250 μL quartz cuvette (0.1 cm path length). Spectra were collected from solutions at concentrations no <1 mg/ml, at pH 7.2 and pH 4.8 in 50 mM phosphate buffer. The correct pH value of every sample was verified immediately before the start of each CD experiment. Melting temperature experiments were carried out between 0 and 90°C with a temperature ramp of 2°C/min, and spectra were collected at 222 nm. Each experiment was repeated at least two times and CD spectra in each experiment are the average of three scans. CD spectra deconvolution analysis was performed using the CDNN (Bohm et al., 1992) and K2d (Andrade et al., 1993) programs.

Results
Mutant peptide selection
Sites for mutagenesis were selected based on the characteristics of the individual amino acids in the primary sequence. The LOOP-36 peptide has been shown to undergo a reversible conformational transition upon changing pH from 7.2 to 4.8. This suggests that the protonation of an amino acid sidechain could be involved in the structural rearrangement. There is a single histidine residue at position 11, which has a pKa of 5.2. Therefore, this side chain was substituted with a glutamine, which should maintain a similar degree of steric hindrance while preventing protonation upon a decrease in pH.

Since only one amino acid has a pKa within the pH range at which the structural transition was reported to occur, other amino acids that could be affected by the change in pH were also explored. The LOOP-36 sequence contains eight glutamic acid side chains, which have the next closest pKa to the pH at which the transition occurs. These residues were mutated to glutamines, which results in a conservative alteration in side-chain size, while eliminating a possible protonation effect.

Finally, the peptide also contains a single glycine residue at position 22, located at the end of loop B, before helix A (Fig. 1A). The conformational flexibility of glycine side chains generally destabilizes α-helices. Therefore, this residue was mutated to alanine, which is known to be an α-helix stabilizer (Matthews et al., 1987; Serrano et al., 1992).

Peptide production
The wild type peptide and the 10 mutant peptides (Table I) were expressed in E. coli as fusion proteins using the IMPACT expression system following the instructions of the manufacturer. One liter of shaker flask culture produced 300 to 500 μg of peptide. The final peptide concentration was determined by absorbance at 280 nm (extinction coefficient = 1290 M⁻¹·cm⁻¹). A purity of 95% was confirmed by SDS–PAGE and mass spectrometry, and the correct molecular weights indicated that no amino acids were added to the peptide sequences. The extinction coefficients were calculated using ProtParam program (Gasteiger et al., 2005).

Circular dichroism spectroscopy
The secondary structure content of all peptides was measured using CD spectroscopy at pH 7.2 and pH 4.8 (Table II). The LOOP-36 peptide exhibited mostly random coil structure at both pHs, with a minimum of absorbance at 205 nm and a
small fraction of α-helical content (222 nm peak) at pH 7.2 (12.6%) that did not increase upon acidification to pH 4.8 (11.5%) (Fig. 2A). The α-helical content of the H11Q mutant increased slightly upon acidification from 9.5% at pH 7.2 to 17.0% at pH 4.8 (Fig. 2B). The G22A mutant underwent a more significant conformational change with α-helical content increasing from 18.0% at pH 7.2 to 38.0% at pH 4.8 (Fig. 2C). The glutamate to glutamine mutants exhibited varying alterations in secondary structure (Table II, CD spectra not shown) with an increase in α-helical content at pH 7.2 for the mutations at position 16, 19 and 21 as compared with wild type. The glutamate to glutamine mutations at positions 4, 19, and 28 showed small increases in α-helical content upon acidification, but the magnitudes of the structural changes were less than what was observed with the G22A mutant.

**Sodium chloride titration**
Depending on the nature and concentration, salt ions can stabilize or destabilize protein secondary structure. To explore how near physiological salt concentrations may influence the observed structural transition, the CD spectra of wild type LOOP-36, H11Q and G22A were obtained at five NaCl concentrations in the range 0–150 mM (Fig. 3A–C). The addition of NaCl had only a minimal effect on helical content of the LOOP-36 peptide and the H11Q mutant peptide at both pH values. At pH 7.2 the wild type LOOP-36 peptide exhibited a decrease in intensity and a shift of a negative peak at 205 nm towards 207 nm as the NaCl concentration increased from 0 to 150 mM. No major changes were observed in the 220–230 nm region. At pH 4.8 a decrease in intensity of the 205 nm peak as well as a change of spectra shape were observed only at 100 mM NaCl.
At pH 7.2, the spectra of the H11Q mutant underwent a change in spectrum shape with a shift of the peak from 205 to 209 nm, and a decrease in its intensity, with the addition of 10 mM NaCl (Fig. 3B). No significant differences were observed with the addition of NaCl at pH 4.8. At pH 7.2, the G22A mutant exhibited no change in secondary structure. At pH 4.8, a spectral shift was observed at 100 and 150 mM NaCl, with a shift of the peak from 208 to 212 nm, as well as an increase in its intensity.
Figure 4 shows the α-helical content for wild type, H11Q and G22A at pH 7.2 and pH 4.8, with 150 mM NaCl. The wild type and H11Q peptides exhibited similar α-helicity at both pHs, corresponding to 10.7 ± 4.7% at pH 7.2 and 11.7 ± 3.2% at pH 4.8 for wild type and 12.3 ± 3.2% at pH 7.2 and 19.3 ± 6.0% at pH 4.8 for H11Q. However, the G22A mutant exhibited a dramatic increase in α-helical structure, from 22.5 ± 7.4% at pH 7.2 to 82.4 ± 6.8% at pH 4.8.

**Fig. 4.** α-helical content of LOOP-36, H11Q and G22A at pH 7.2 and pH 4.8 at 0°C and with 150 mM NaCl. The secondary structure content was calculated using K2d and CDNN programs.

In order to assess the thermal stabilities of the helices formed by LOOP-36, H11Q and G22A, we measured the ellipticity at 222 nm, by CD spectroscopy, between 0 and 90°C, at pH 7.2 and pH 4.8 (Fig. 5). At pH 7.2, LOOP-36, H11Q and G22A all exhibited a gradual decrease in the intensity of the peak at 222 nm with increased temperature, indicating an increase in α-helical content. LOOP-36 and H11Q exhibited similar behavior at pH 4.8, while the G22A mutant exhibited more changes in the helical structure.

**Enhancement of pH-response of hinge peptide from hemagglutinin protein**

Fig. 3. CD spectra of (A) LOOP-36, (B) H11Q and (C) G22A at pH 7.2 and pH 4.8, with different concentrations of NaCl. Spectra were collected at 0°C between 200 nm and 260 nm.
complex behavior, with an initial decrease in the ellipticity at 222 nm, followed by an increase starting at 5°C and reaching a maximum value around 25°C. The signal then decreased gradually, at a rate similar to LOOP-36.

To confirm the presence of α-helical structure at higher temperatures, full spectra were recorded every 10°C during a melting experiment (Fig. 6) and α-helical content was determined (Fig. 7). At pH 7.2 the LOOP-36, H11Q and G22A mutants gained secondary structure eventually reaching 71%, 20% and 64% α-helicity, respectively, with an isodichroic point at 208 nm for wt and G22A (Figs 6 and 7).

At pH 4.8 the LOOP-36 peptide gained less α-helical structure, reaching 44%, at 90°C, with an isodichroic point at 208 nm (Figs 6A and 7B). The thermal responsiveness of the H11Q mutant appeared to be unaffected by pH, with a similar α-helical content as observed at pH 7.2 (Figs 6B, 7). At pH 4.8, the G22A mutant initially contained 82% α-helical content at 0°C. Upon heating, it reached a minimum content of 37% at 20°C, and then α-helical content gradually increased to 62% at 90°C. No clear isodichroic point was observed before 20°C.

Discussion

We cloned, mutated and characterized the 36 amino acid region (LOOP-36) containing the sequence reported to be responsible for the structural change of the Influenza virus fusion protein during viral escape from the endosome. The LOOP-36 peptide contains a 28-amino acid region that undergoes a structural change from random coil to α-helix, during rearrangement of the HA fusion protein to an active, fusogenic conformation (Skehel and Wiley, 2000; Eckert and Kim, 2001). Crystallographic data of the full HA protein shows that LOOP-36 has 40% α-helical structure (14 of 36 amino acids) at pH 7.2 and becomes 100% α helical at pH 4.8 (Chen et al., 1999). To investigate the role of individual amino acids in this structural transition, we used a rational site-directed mutagenesis approach on the isolated and purified peptide sequence. Our results indicate that replacing the glycine residue at position 22 with alanine produced a peptide with an enhanced conformational transition: alanine has a greater helix-forming propensity than glycine, because it buries more solvent-accessible hydrophobic surface area upon folding and has less conformational freedom in the unfolded state (Matthews et al., 1987; Serrano et al., 1992).

In contrast with a previous report (Carr and Kim, 1993) our CD data shows that the LOOP-36 peptide isolated from the fusion protein possesses much lower α-helical structure content at pH 7.2, and does not undergo a structural transition upon decreasing the pH to 4.8 (Fig. 2A). The H11Q mutant also exhibited a small pH-induced change in α-helical content, while the G22A α-helical structure content doubled upon changing pH from 7.2 to 4.8 (Fig. 2C). The substitution of glutamic acid residues with glutamine (Table II) produced varying alterations in secondary structure. An increase in α-helical content at pH 7.2 as compared with the LOOP-36 peptide was observed for the mutations at positions 16, 19 and 21 without a structural transition upon a change in pH. The only mutants from the glutamine-scanning experiments that exhibited structural transition upon a change in pH were E4Q and E28Q mutant, but these changes were on the same order of magnitude as was observed with the H11Q mutant (Table II). Except for the glutamic acid in position 16, all the other glutamic acid residues are part of an acidic cluster (Fig. 1B) and the disruption of the cluster can explain the increase of alpha-helix content at pH 7.2 for the mutation at position 19 and 21. The mutation at position 28 appears to affect the structural transition ability of the peptide without changing the helix content at neutral pH, while the mutation at position 4 significantly reduces the helical content at neutral pH while the low pH helical content is similar to the low pH content of the LOOP-36 peptide.

Evidence suggests that the native pre-fusogenic conformation of HA is metastable, and separated from the stable fusogenic state by a kinetic barrier (Carr et al., 1997). The pH change is thought to destabilize the metastable state, making it possible to overcome the kinetic barrier. In the isolated LOOP-36 peptide, a change in pH may not be enough to drive the structural transition of the peptide from random-coil to α-helix. In the whole HA fusion protein, LOOP-36 is only partially accessible to water molecules. In the fusogenic state, its α-helical structure may be stabilized by intramolecular hydrogen bonds and the reduction of negative charges at low pH. In contrast, as an isolated peptide in aqueous solution, hydrogen bonds with water molecules may destabilize the α-helical structure making the random coil to α-helix transition less thermodynamically favorable. Disrupting the water shell around the peptide leads to a situation in which intramolecular hydrogen bonds are more likely to form (Garcia and Sanbonmatsu, 2001).
The glycine at position 22 is at the end of loop B, and its conformational freedom makes a change in pH insufficient to induce a transition in such a small peptide. Upon substituting glycine with alanine, the conformational freedom is reduced (Chakrabartty et al., 1991) and an increased helix content is observed at pH 7.2. The G22A mutant is also more responsive to pH changes: at pH 4.8 the helix content was double than that observed at pH 7.2. Our data suggests that the small increase of $\alpha$-helical content produced by alanine at pH 7.2 was sufficient to create a helix-nucleating domain (Fersht, 1995; Hummer 2001).

The effect of ionic strength was also investigated, by adding NaCl up to 150 mM. Salt concentrations above this did not produce any further spectral transition for any of the peptides. For the LOOP-36 and H11Q peptides, increasing the NaCl concentration up to 150 mM did not affect the $\alpha$-helical content at either pH (Fig. 3A and B), while the pH-dependent coil to helix transition was enhanced in the case of the G22A mutant in the presence of NaCl (Fig. 3C).

The effect of NaCl on the G22A transition can be explained by two mechanisms: NaCl may favor the folding process by neutralizing the negative charges of glutamic and aspartic acid that form clusters on the side of the helix; or NaCl may subtract water molecules from interaction with the peptide, therefore favoring the formation of intramolecular hydrogen bonds (Arakawa and Timasheff, 1982; Parsegian et al., 2000; Harries et al., 2005; Raman et al., 2005).

Figure 3 shows that the neutralization effect of NaCl alone is not sufficient to increase the helical content. Figure 3C shows that for the G22A mutant at pH 4.8 the $\alpha$-helical content increases at 100 and 150 mM NaCl, while at pH 7.2 this increase is not observed until 150 mM NaCl is reached. The fact that NaCl is necessary for the structural transition to occur supports the hypothesis of a combination of the two

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mechanisms mentioned above. At 50 mM salt, all the negative charges on the peptides are likely neutralized but no transition was observed at any pH for either LOOP-36 or the mutants. Our data indicates that an excess of ions are required not only to neutralize the negative charges on the peptide, but also to reorganize the water shell surrounding it, favoring the formation of intramolecular hydrogen bonds.

Thermal analysis of the peptides demonstrated complicated behavior of the G22A mutant. Increasing the temperature to 90°C induced an increase in negative signal intensity at 222 nm, corresponding to increased α-helicity, at both pHs (Figs 6C and 7). Interestingly, at pH 7.2 the α-helical content is similar for LOOP-36 and G22A throughout the 0–90°C temperature range. However, at pH 4.8, large differences were observed. The α-helical content of the G22A mutant was 80% at 0°C and this decreased to 40% at 20°C followed by an increase again to 60% at 90°C. In contrast, the LOOP-36 peptide exhibited a gradual increase in α-helicity with an increase in temperature, up to 40% at 90°C (Fig. 7B). The H11Q mutant also gained structure with temperature, though the change was of a much smaller magnitude than observed for LOOP-36 or the G22A mutant and the transition was unaffected by pH. These results cannot be due to the change in buffer pH during heating since the temperature coefficient (dPH/dt) for phosphate buffer is 0.0028 units/°C considering 25°C as starting point (Dawson et al., 1986), and therefore the change in pH between 0 and 90°C is 0.252 (absolute number) units of pH.

Previous studies have shown that the metastable conformation of the entire fusion protein at pH 7.2 can be transformed to the fusogenic form upon an increase in temperature (Carr et al. 1997). The authors suggest that heat induced HA1 rearrangement is necessary to release any constraints from the HA2 subunits and that the structural change of HA2 is due to the release from the metastable structure of the entire HA protein. Our data also suggests that LOOP-36 may contribute to the heat-dependant structural transition of the HA2 subunit due to its ability to gain structure at higher temperature (65% α-helical at 65°C).

Figure 8 shows a van’t Hoff plot for LOOP-36 and the G22A and H11Q mutants where K is the percentage of helical structure divided by the percentage of random structure. Analysis of the thermodynamic parameters extracted from the temperature-scan experiments (Table III) shows that the random coil to helix transition is purely an enthalpic process with TΔS<<ΔH and ΔS > 0 (Fig. 9). The enthalpy of the G22A mutant was calculated without considering the values at 0 and 10°C due to the non-linearity of these two values with the rest of the data. The calculated entropy values do not appear to be dependent on temperature (Fig. 9, open symbol) and this is likely due to the concomitant entropy decrease that occurs as helical structure is gained with increasing temperature.
A somewhat similar type of thermal behavior is observed in the well-studied elastin-like polypeptides, where increasing the temperature induces an increase in structure, with precipitation of high molecular weight peptides (Urry, 1988). In the case of elastin, it was shown that the hydration shell is disrupted, dehydrating valine side-chains, and resulting in intramolecular hydrogen bond formation and the formation of a beta-spiral secondary structure (Reiersen et al., 1998; Rousseau et al., 2004; Brovchenko et al., 2005). Similarly, it is possible that elevated temperatures disrupt the water molecule shell around LOOP-36, facilitating intramolecular hydrogen bond formation and a transition to alpha-helical structure. The NaCl titration and the temperature CD scan demonstrate that intramolecular hydrogen bond formation likely influences the folding process in LOOP-36. This possibility is supported by the fact that all peptides exhibited an increase in structure with temperature, and wild type and G22A reach a final structure with same alpha-helical content at pH 7.2 and 90°C. The isodichroic point at 208 nm indicates the presence of two states in equilibrium (Holtzer and Holtzer, 1992; Snow et al., 2002; Percec et al., 2004). Spectral analysis for secondary structure content showed that the alpha-helical content of the peptide increased, while the random coil content decreases with temperature (Fig. 7).

Our data are in contrast to what has been observed in previous studies (Carr and Kim, 1993) where it has been shown that the LOOP-36 peptide undergoes a pH-dependent transition from random coil to alpha-helix. We did not observe such a transition. One significant difference between our work and the previous experiments is that we used biologically derived peptides while the previous authors used N- and C-capped synthetic peptides. It is well documented that N-terminal acetylation increases the helical content of peptides (Chakrabarty et al., 1993). Second, synthetic peptides may be contaminated by smaller peptide byproducts from the synthesis process. We ruled out the possibility of an effect from the buffer: CD spectra of the cloned LOOP-36 were also acquired under the identical buffering conditions as reported previously and structural changes were also not observed (data not shown).

There is an increasing interest in stimulus-responsive peptides for a variety of applications, including biomaterials, drug delivery, tissue engineering, biosensors and nanodevices (Banta et al., 2007; Chockalingam et al., 2007). Chemo-physical properties of peptides are influenced by their protein environment: the behavior of an isolated peptide is often different from what can be observed in the native structure. Contrary to previous reports, we showed that indeed the pH responsiveness of isolated LOOP-36 is substantially different from what was described for the same sequence in the intact HA protein. Thorough analysis of individual amino acid contributions to the stabilization of the peptide structure allowed us to produce mutant peptides with enhanced pH-responsive properties. This is a first step towards the development of new peptide-based stimulus-responsive biomaterials.

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