Acid destabilization of a triple-helical peptide model of the macrophage scavenger receptor

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Abstract Electrostatic interactions were studied in a triple-helical peptide, $(POG)_3$PKGQKGEKG$(POG)_4$, which contains a lysine-rich 9 residue sequence from the collagen-like domain of the macrophage scavenger receptor (MSR). This peptide adopts a stable triple-helical conformation only when the pH is higher than 4.5, corresponding to ionization of the Glu side chain. Modeling shows Glu forms ion pairs with one of the Lys residues, stabilizing the structure. Previously studied collagen-like peptides show relatively small contributions of electrostatic interactions to stability. The large magnitude of the pH mediated structural changes seen for this peptide suggests that specific placement of charged residues in the triple-helix conformation can generate strong electrostatic interactions.

Key words: Collagen; Triple-helix; Macrophage scavenger receptor; Triple-helical peptides; Electrostatic interactions; pH dependence

1. Introduction

The triple-helix is the major repeating structural element of all collagens and constitutes one of the basic structural motifs in a number of host defense proteins, including pulmonary surfactant apoprotein A, the mannose binding protein, C1q, and the macrophage scavenger receptor [1–3]. The triple-helix is composed of three supercoiled polyproline II-like chains, and has steric requirements for glycine as every third residue and a high imino acid content [4,5]. This generates a $(X\text{-}Y\text{-}Gly)_n$ sequence where X and Y are frequently Pro and Hyp, respectively.

A high proportion of charged residues (15–20%) is generally found in collagen triple-helical domains. A net excess of positive charges is observed for collagens, with most basic residues in the Y position of X-Y-Gly triplets [6,7]. All residues in the X and Y positions of the triple-helix are partially exposed to solvent [8], giving charged residues the potential for intramolecular interactions between the three chains and for interactions with other molecules. The highly conserved charged residues of collagen have been implicated in electrostatic interactions stabilizing the triple helix [9,10], the molecular association to form fibrils [11,12] and association of collagen with other molecules [13].

The triple-helical domains of C1q and macrophage scavenger receptor have a very high content of charged residues (20–25%), with a large excess of basic residues, which are again preferentially found in the Y position of triplets. Electrostatic interactions appear to be critical to the function of these host defense proteins, as they are in collagen. For example, a charged region of the C1q triple-helix is implicated in binding to the C1q receptor [2], and the basic collagen-like domain of the macrophage scavenger receptor has been identified as the region which binds to polyanionic ligands [14,15,16]. To investigate the electrostatic interactions which occur in a triple-helix, a synthetic peptide was designed which contains a highly charged sequence found in the macrophage scavenger receptor (MSR) and has been implicated in ligand binding [14,15]. This peptide is seen to adopt a stable triple-helical conformation, with electrostatic interactions as the dominant determinant of its stability.

2. Materials and methods

2.1. Peptide synthesis

The peptide $(POG)_3$PKGQKG*EKG$(POG)_4$, designated MSR-1 was synthesized with ^{15}N-enriched glycine (G*) at the indicated position, on an Applied Biosystems 430A peptide synthesizer using a standard N-t-Boc protection strategy on t-Boc-L-Gly-PAM resin. Side chain protection was benzyl for Hyp, benzyl ester for Glu and 2-chlorobenzyloxycarbonyl for Lys. All amino acids after position 5 were double coupled, and a hydrogen fluoride cleavage was used. The peptides were purified to greater than 95% by reverse-phase HPLC on a Vydac C-18 column. The identities of these peptides were confirmed by mass spectroscopy using a VG Analytical ZAB-T instrument at the Center for Advanced Food Technology (Rutgers University, New Brunswick NJ).

2.2. Circular dichroism spectroscopy

Samples for circular dichroism spectroscopy (CD) measurements were prepared at a concentration of 1.0 mg/ml, with pH values ranging from 1 to 13. Previous studies have shown triple-helix formation to be stable over this entire pH range [10]. Spectra were collected for samples prepared in both phosphate and cacodylate buffer systems to ensure that any observed effects were not due to buffer composition. Ionic strength was adjusted by the addition of NaCl. All solutions prepared at room temperature were equilibrated at 4°C for at least 24 h prior to CD measurements. All CD spectra were recorded on an Aviv Model 62DS spectropolarimeter with a Hewlett Packard Peltier thermoelectric temperature controller using a 1 mm path length cell. For equilibrium melts, the ellipticity at 225 or 223 nm was monitored as the temperature in the cell was increased (step size of 0.3°C, 3 min equilibration at each temperature) and signal averaged for 30 s.

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Abbreviations: CD, circular dichroism; NMR, nuclear magnetic resonance; TPPI, time proportional phase incrementation; *, indicates ^{15}N-enriched residue for NMR detection; T_m, midpoint of thermal transition; MSR, macrophage scavenger receptor; oxLDL, oxidized low density lipoproteins; O (one-letter code) and Hyp (three-letter code), 4-hydroxyproline; thus $(POG)_{10}$ is used for $(Pro\text{-}Hyp\text{-}Gly)_{10}$. The EK containing peptide is Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Glu-Lys-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly; MSR-1 peptide is used to denote the peptide Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Lys-Gly-Gln-Lys-Gly-Glu-Lys-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly.

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pH 2 $^{+}H_2$NPOGPOGPOGP$\overset{+}{K}$GQ$\overset{+}{K}$GE$\overset{+}{K}$GPOGPOGPOGPOGCOOH

pH 6 $^{+}H_2$NPOGPOGPOGP$\overset{+}{K}$GQ$\overset{+}{K}$G$\overset{-}{E}\overset{+}{K}$GPOGPOGPOGPOGCOO^{-}

pH 9 HNPOGPOGPOGP$\overset{+}{K}$GQ$\overset{+}{K}$G$\overset{-}{E}\overset{+}{K}$GPOGPOGPOGPOGCOO^{-}

pH 13 HNPOGPOGPOGPKGQKG$\bar{E}$KGPOGPOGPOGPOGCOO^{-}

Fig. 1. The amino acid sequence of MSR-1 indicating the charges present at different pH values.

2.3. Calculation of thermodynamic parameters

Thermodynamic parameters were extracted by non-linear least squares fitting of the equilibrium constant for the transition between monomer and trimer as previously described [17]. After correcting for a linear temperature dependence in ellipticity for both monomer and trimer, the equilibrium melting transitions were fit to a two state trimer to monomer transition. Thermodynamic parameters were calculated following previously described methods [17,18,19], using a standard state of 1 mM. The T_m and the van't Hoff enthalpy ΔH^0 were determined by curve fitting to the following equation, where c_0 is the initial peptide concentration:

$$K = \exp\left[\Delta H^0/RT \times (T/T_m - 1) - \ln(0.75c_0^2)\right]$$

Then ΔS^0 and ΔG^0 values were calculated:

$$\Delta S^0 = [\Delta H^0 - RT_m \times \ln(0.75c_0^2)]/T_m$$
$$\Delta G^0 = \Delta H^0 - 298\Delta S^0$$

2.4. NMR Spectroscopy

All NMR spectra were collected on a 500 MHz Varian VXR-500 spectrometer and processed on Silicon Graphics INDIGO$_2$ workstations using FELIX (Biosym Inc.). Each ^{1}H-^{15}N HSQC (heteronuclear single quantum coherence) [20] data set was collected and 90° phase shifted sinebell window functions were applied in both dimensions prior to Fourier transformation. Each spectrum consisted of 96 $t1$ increments of 1K complex data points. Quadrature detection in the $t1$ dimension was obtained by TPPI [21]. The sample concentration was 10 mM for all NMR experiments.

2.5. Molecular modeling

Starting structures for MSR-1 were generated from $(POG)_{10}$ coordinates [22] using Insight II (Biosym Inc.). The charges on K and E were considered explicitly to mimic ionized states of E and K residues. The model peptides were energy minimized with IMPACT program [23]. A distance dependent dielectric constant ($\varepsilon = r$) was used.

3. Results

A 30-mer peptide denoted as MSR-1 was designed to include 9 residues of the human macrophage receptor, residues 333–341. It is known that peptides with Gly as every third residue and a high imino acid content will adopt a triple-helical conformation, and the triplet Pro-Hyp-Gly is the strongest promoter of triple-helix formation [18,24]. Thus the MSR 9-residue sequence was capped with POG triplet repeats to provide stability, giving the final sequence $(POG)_3$PKGQKGEKG$(POG)_4$ (Fig. 1). The circular dichroism (CD) spectrum of the MSR-1 peptide shows a maximum ellipticity at 223 nm, which is characteristic of the triple-helical conformation (Fig. 2, inset). With increasing temperature, the amplitude of this maximum undergoes a sharp decrease near 30°C (pH 7, 1 mg/ml). This sharp thermal transition, similar to that seen for trimer-to-monomer transitions of our other triple-helical peptides [10,17], suggests that MSR-1 adopts a triple-helical structure at temperatures

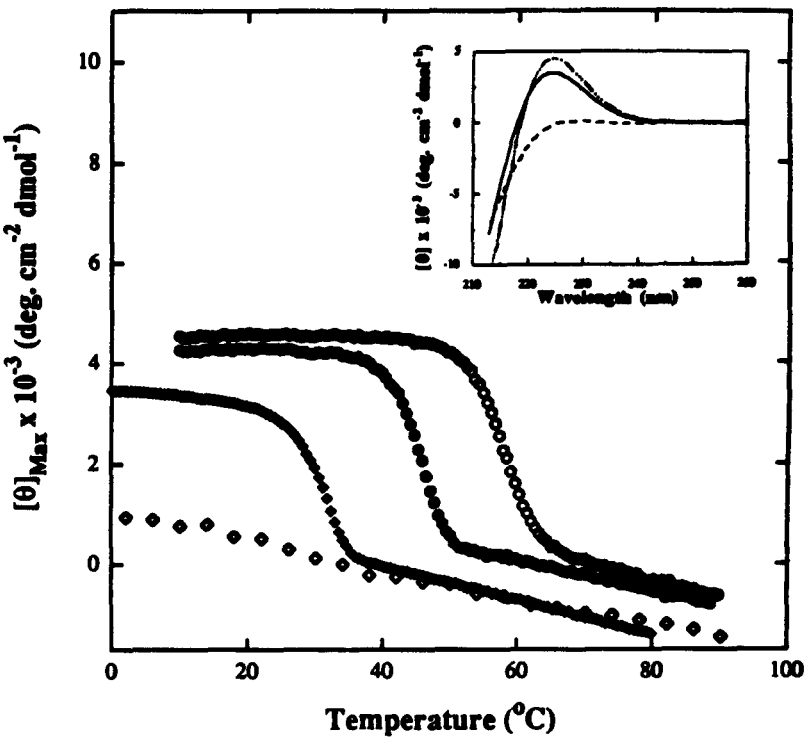

Fig. 2. Equilibrium melting profiles of $(POG)_{10}$ (○), EK-containing peptide (●), MSR-1 (◆) at pH 7; and MSR-1 at pH 3 (◇). The inset shows CD spectra of peptides $(POG)_{10}$ (···–···) at 2°C , MSR-1 at 2°C (—), and MSR-1 at 50°C (- - -) at 1 mg/ml, pH 7.

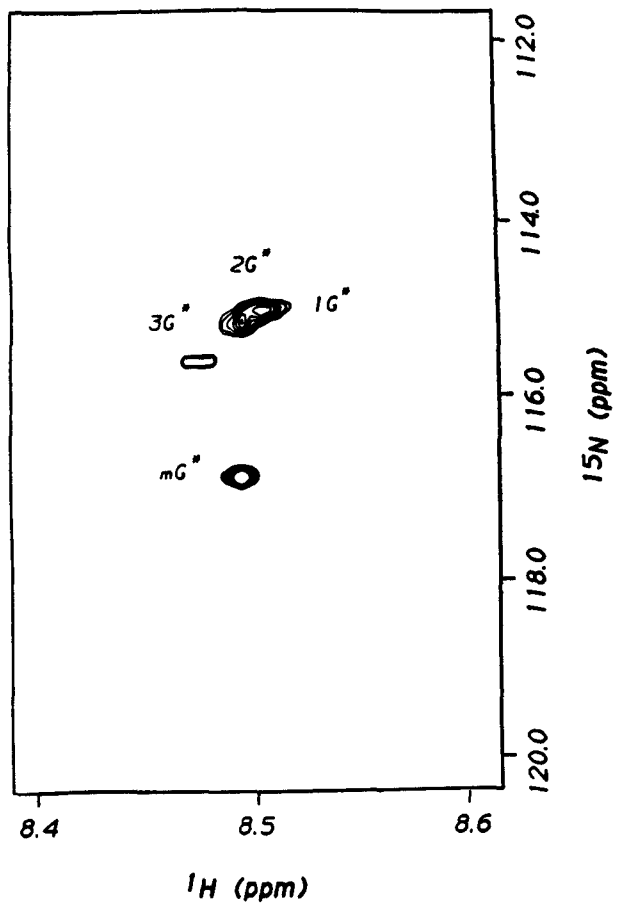

Fig. 3. ^{1}H-^{15}N HSQC (heteronuclear single quantum coherence) spectra in 90% H_2O/10% D_2O solution at pH 5.5, 10°C for MSR-1 (10 mM concentration). The resonances observed are for the ^{15}N-labeled Gly at position 15. The resonances from the three chains of the triple helix are designated with the chain number 1, 2, or 3 in the prefix, and the resonance from the monomer form is designated as 'm'.

below 30°C (Fig. 2). The trimeric nature of the MSR-1 peptide was confirmed by NMR spectroscopy, using the ^{15}N-enriched glycine incorporated into the residue at position 15. Heteronuclear ^{1}H-^{15}N correlation NMR spectra of MSR-1 at low temperature show four cross peaks for the ^{15}N Gly (Fig. 3). One cross peak is present at high as well as low temperature and is due to monomer form. The other three peaks are present only at low temperature and can be attributed to the three distinct chains in a triple-helical structure [25], consistent with a monomer–trimer equilibrium.

The 30-mer MSR-1 peptide (T_m = 30°C) is much less stable than the repeating polytripeptide $(POG)_{10}$ (T_m = 62°C, Fig. 2) [18,25], or the homologous EK-containing peptide, $(POG)_4EKG(POG)_5$ (T_m = 46°C, Fig. 2) [10]. The presence of triplets from the MSR sequence is destabilizing compared with POG triplets, as expected if the imino acid content is the dominant determinant of stability. Thermodynamic parameters were calculated from equilibrium melting curves to clarify the basis of this decreased stability [17,18]. The enthalpy values for MSR-1, the EK-containing peptide and $(POG)_{10}$ are −200, −175 and −160 kcal/mol, respectively, and the entropy values are −650, −520 and −470 cal/mol·K, respectively, at pH 7. The destabilization of MSR-1 peptide compared to the other peptides is due to its less favorable entropy term, consistent with its lower content of imino acids [26]. The MSR-1 peptide has the most favorable enthalpy term, which is expected on the basis of its high content of residues that can participate in hydrogen bonding and electrostatic interactions.

To examine the effect of ionizable side chains on this highly charged triple-helical peptide, the thermal stability of MSR-1 was determined as a function of pH. At pH values of 4.5 and above, a sharp thermal transition is observed. There is little variation in the Tm over the pH range of 4.5–13 (Fig. 4). In contrast, at pH values below 4.5, there is only a small linear decrease in ellipticity as the temperature is raised, with no sharp transition (Fig. 2). Such a linear decrease with no transition is found in peptides which do not associate as trimers [17]. The mean residue ellipticity of the maximum near 223 nm, which relates to triple-helix content, was found to be significantly lower at pH values below 4.5 than at higher pH values (Fig. 4). These data indicate that MSR-1 forms a stable triple-helix at the concentrations used for the CD studies (1 mg/ml) for pH values of 4.5 and above, but does not form a triple helix at more acidic pH values, even at low temperature.

Possible ionization events in MSR-1 that could take place with pH changes include the protonation of Glu and Lys side chains and the N- and C-termini (Fig. 1). The dramatic appearance of triple-helical structure at pH 4.5 or higher in MSR-1

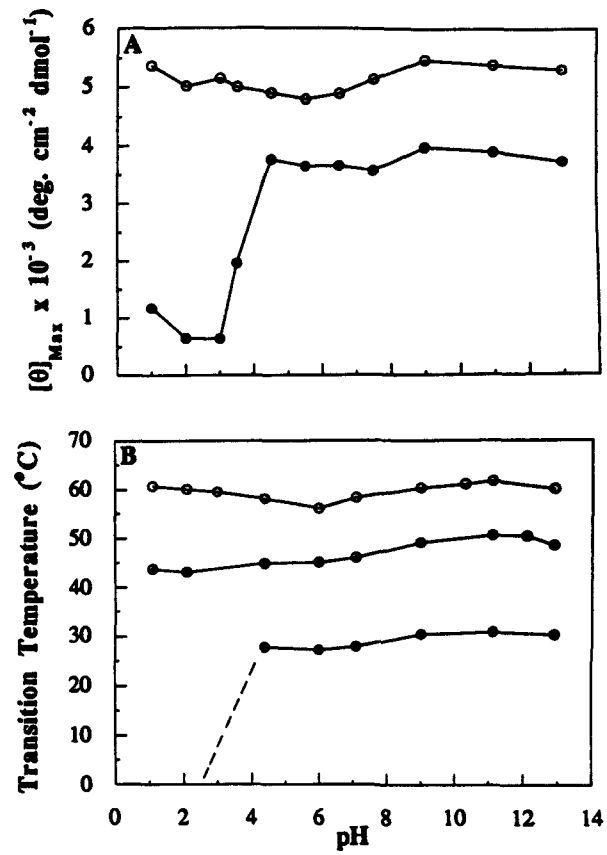

Fig. 4. (A) Mean residue ellipticity plotted as a function of pH for $(POG)_{10}$ (○) and MSR-1 (●) peptides, (1 mg/ml, 2°C). (B) Plot of melting temperature as a function of pH for $(POG)_{10}$ (○), EK-containing peptide (⊕) and MSR-1 (●), at 1 mg/ml. At all pH values below pH 4.5, no sharp transitions for MSR-1 are seen and therefore T_m values could not be obtained; a dashed line is used to indicated that the T_m is less than 2°C at all pH values.

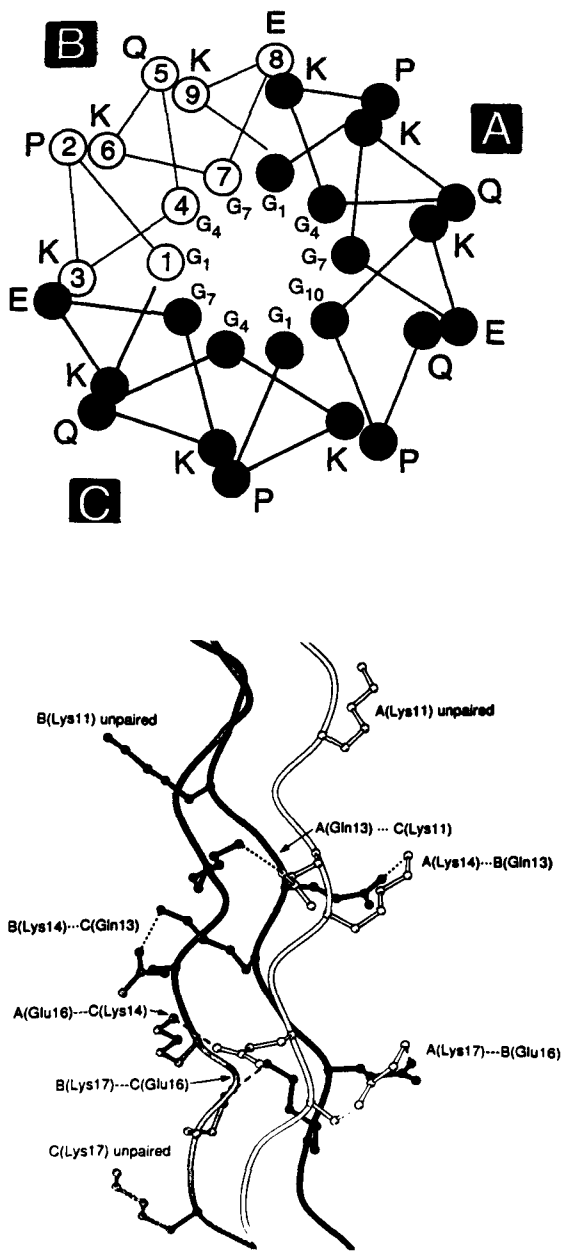

Fig. 5. (A). Helix wheel diagram showing a cross-section of the triple-helix in the central region of the 9 residue MSR sequence. (B). Axial view of the energy minimized structure of MSR-1. Prefixes A, B, and C refer to the three chains of the molecule. The residues that form interchain ion pairs are: A(Glu^{16})–C(Lys^{14}); A(Lys^{17})–B(Glu^{16}); B(Lys^{17})–C(Glu^{16}). The residues that participate in interchain hydrogen bonding are: A(Gln^{13})···C(Lys^{11}); A(Lys^{14})···B(Gln^{13}); B(Lys^{14})···C-(Gln^{13}). Unpaired ionizable side chains are: A(Lys^{11}); B(Lys^{11}); C(Lys^{17}).

occurs close to the expected pK value for the ionization of glutamic acid. Determination of Glu pKs by NMR have indicated values of 4.2 for a tetrapeptide [27] and 3.8 for Bovine Pancreatic Trypsin Inhibitor [28]. The midpoint of the transition of the mean residue ellipticity of MSR-1 is near pH 3.8 (Fig. 4A), and the somewhat low value may be caused by the interactions of Glu residues with Lys side chains. Very little variation is observed in the value of the T_m and the mean residue ellipticity over the pH range of 4.5–13 (Fig. 4), suggesting the loss of charge on the lysine residues and the deprotonation of the amino terminus have only small effects on stability. A small change in ellipticity is noted below pH 2, which could be related to deprotonation of the C-terminus.

4. Discussion

Charged residues in collagen-like domains play a critical role in ligand binding for MSR [14,15] and for C1q [2]. The extended nature of this rod-like domain makes charged residues available for interaction with other molecules. The absence of a net dipole moment and its unique geometry result in distinctive electrostatic interactions in this triple-helix motif [10]. The C1q triple-helix stability shows pH dependence, increasing 12°C in going from pH 4 (48°C) to pH 8 (60°C) [29], while collagen thermal stability increases by 7°C in going from pH 1 (32°C) to pH 7 (39°C) [30]. The striking pH dependence of MSR-1 stability shows it is possible to model electrostatic interactions in a triple-helical peptide.

The magnitude of the pH dependent change in stability seen for MSR-1 (>30°C) is much greater than seen for other triple-helical peptides containing Glu and Lys residues. For example, the EK-containing peptide shows a T_m of 43°C at pH 3 and T_m of 46°C at pH 7 [10], and a peptide with two Lys residues with two potential ion pairs GKOGEOGPKGDAGAOGAO-(POG)$_4$GV, shows a maximum variation of 9°C over the pH range 1–13 [10]. In all triple-helical peptides studied other than MSR-1, electrostatic interactions are a relatively minor factor in stability, small compared to the influence of imino acid content ([10]; unpublished observation). The anomalously large pH effect and predominance of electrostatic interactions in the stability of MSR-1 is dependent on the 9 residue amino acid sequence which has Lys in three consecutive Y positions with one adjacent to a Glu. The effect of side chain on triple-helix stability is consistent with conformational energy calculations [31].

At low pH, there are three positively charged lysine residues per MSR-1 chain, and the triple-helix, which would include 9 Lys residues evenly distributed around the helix in an axial length of 3 nm, is not stable. It appears likely that the charge repulsion which would result from the high density of positively charged side chains in a triple-helix is responsible for the destabilization of the trimer relative to the monomer. As the pH is raised above 4.5, the Glu side chain becomes negatively charged and the triple-helix is stabilized (Fig. 1). It is the context of Lys residues surrounding the EKG triplet in MSR-1 peptide which results in its dramatic pH dependence, since the ionization of Glu in the EK-containing peptide shows only a minor effect on stability (Fig. 4). To probe the molecular basis for triple-helix stabilization, molecular modeling studies were performed. Examination of the 9 Lys residues in the energy minimized triple-helical structure of MSR-1 shows that three Lys are involved in intramolecular ion pairs with Glu, three Lys participate in hydrogen bonding with Gln residues, and three Lys side chains are unpaired (Fig. 5B). Since all residues in X and Y positions of a triple-helix are substantially exposed to solvent [8], the unpaired lysines are potential candidates for intermolecular interactions, such as ligand binding.

A trimer peptide containing a similar sequence from bo-

vine macrophage scavenger receptor, preceded by N-terminal Pro-Hyp-Gly triplets, was synthesized previously, with a design that included covalent cross-linking at the N-terminus [32]. This peptide adopts a triple-helical conformation with a melting temperature of 20°C. The greater stability of MSR-1 relative to this cross-linked trimer is likely to be due to its having stabilizing Pro-Hyp-Gly sequences at both ends, while the cross-linked trimer contains stabilizing features only at the N-terminus. Cross-linked triple-helical peptides which include both a C-terminus cross-link and stabilizing Pro-Hyp-Gly triplets at the N-terminus show a high thermal stability [33].

The more than 30°C variation in MSR-1 peptide stability is the largest dependence on pH reported for any triple-helix. It is reminiscent of the strong effect of pH on the 3-stranded coiled coil structure found in influenza hemagglutinin [34,35], and both supercoiled helical structures have a high charged content as a result of their extended conformations [36]. A decrease in pH from 7 to pH 5 greatly stabilizes the 3-stranded coiled coil, as a result of the reduction in charged repulsion of the highly acidic region [34,35]. In contrast the stabilization of the triple-helix occurs at pH values greater than 4.5, when one of the charged Lys residues can participate in ion pairing with an ionized Glu.

The sequence studied is found in a region of MSR critical to ligand binding [14,15] and a similar region, including Lys residues in the Y positions of three adjacent Gly-X-Y triplets of which one is a Gly-Glu-Lys triplets, is found in type IV collagen (personal observation) and in a recently discovered macrophage receptor [37]. This highly charged sequence with its strong electrostatic interactions is likely to be important for ligand binding in all of these molecules, and its destabilization at acid pH values may play some role in dissociation of ligands from the macrophage receptors in the acidic endosomes.

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